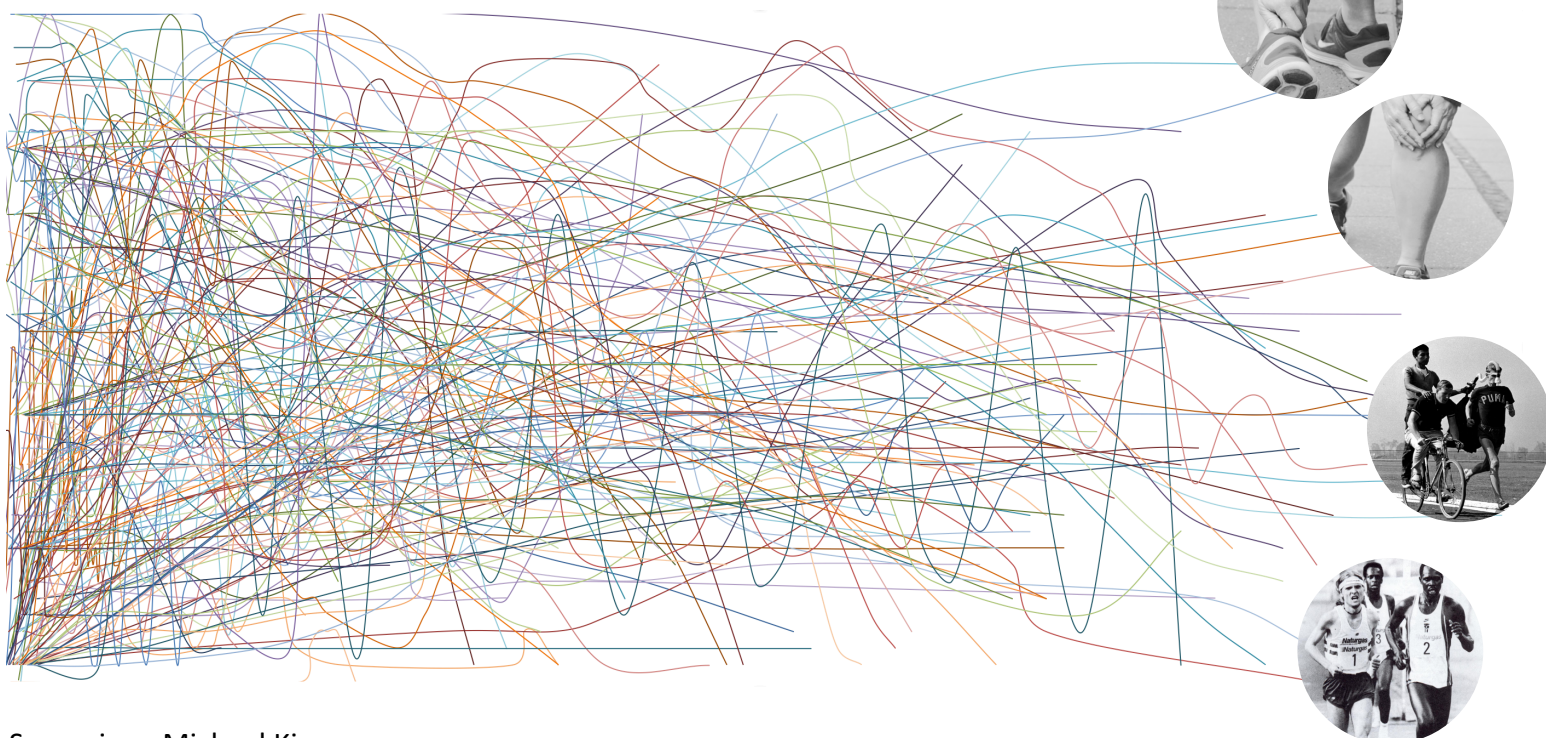# The mechanisms behind the development of tendinopathy: Early structural, inflammatory, nociceptive and clinical changes

PhD thesis

Peter Hung Thanh Tran

Supervisor: Michael Kjær

Principal co-supervisor: Stig Peter Magnusson

Submitted: October 28, 2019

Title

The mechanisms behind development of tendinopathy: Early structural, inflammatory, nociceptive and clinical changes

Principal supervisor

Michael Kjær

Institute of Sports Medicine, Department of Orthopedic Surgery M, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Principal co-supervisor

Stig Peter Magnusson, Institute of Sports Medicine, Department of Orthopedic Surgery M, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Assessment committee

Tine Alkjær

Associate Professor, PhD

Faculty of Health and Medical Sciences,

Department of Biomedical Sciences,

University of Copenhagen, DK

Martin Lind

Professor, overlæge, dr.med., PhD

Ortopædkirurgisk afd.,

Aarhus Universitetshospital

Palle Juul Jensens Boulevard 99

8200 Aarhus N

Pernilla Eliasson

Associate Professor, PhD

Department of Orthopaedic Surgery

Lindköping University

Sweden

Submission date

October 28, 2019

Cover and layout

All created by Peter Hung Thanh Tran

The front page illustrates a graph from all included patients and how their symptoms developed through time (x-axis) on a scale from 0 - 10 (y-axis, 10=most painful).

Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital, and Center for Healthy Aging, Faculty of Health Sciences, University of Copenhagen, 2400 Copenhagen NV, Denmark. This PhD thesis fulfills the requirements of achieving a PhD degree from the University of Copenhagen.

Content

Preface and acknowledgements

Back in autumn 2014 I wrote an email to Michael Kjær asking him if he had any projects available for an ambitious sports enthusiast and hard-working physician like me. Despite encouraging words about myself I received no response. I waited several weeks before attempting to call Michael but there was no response on the phone. On my second attempt, I reached a lovely female voice, his secretary, who replied that he was out of the office. This might have been a sign to stop bothering him right here. But sometimes my sturdiness and perseverance actually are an advantage, as something pulled me towards this place. I was certain that in this place my interests and curiosity could be satisfied in a setting with amazing people. My third call reached Michael Kjær himself, and so started my adventure at the Institute of Sports Medicine Copenhagen.

A special thank and gratitude to my supervisor, Michael Kjær, for taking me under his wing. Your knowledge, understanding and motivation helped me become a better person. You believed in me during times when I doubted myself, motivated me when I was facing the abyss and most importantly, you provided me guidance that extends far beyond research. I am forever grateful for all the things you have done.

Peter Magnusson, you possess a calm and focused personality that has always inspired me. During our meetings you made me feel relaxed and it helped me speak my mind. Thanks to you we were able to push things forward with our collaborators, and I will never forget how bravely you fought for this project. Thank you for having my back all the time.

Even though not my supervisor I would like to thank Peter Schjerling for some very interesting discussions and of course your expertise in biochemistry. With you analyzing my samples I was 100 % sure it would be made perfect and helped me sleep tight, knowing that your high standards are the best I could wish for.

My nano-friend, René Svensson, you are smart and hard-working. With you watching over my data-analysis I was confident we would do it the right way. If I ever had to assemble a dream-team, you would be my first pick. Your skills in troubleshooting and intelligent solutions will have us find a way through every hopeless situation. Thank you for helping me and being patient while doing it.

"I have thought often of what it all means. Life thrusts you into a competitive environment. How do you prepare for the realities and the unknown? Hopefully, you have a mentor, a Bowerman who pushes you at that critical time. A time when someone has a belief in your future more than you do. It's not about how long you live but how you contribute. It's about doing your best and doing the right thing. It's about recovering from your mistakes and not giving up. It's about the baton pass to a new generation. It's about the realization that you can not go it alone. It takes a team.

In the end, you are somewhere in the middle, part of a never-ending process. The future will never remember what was in your bank account or what kind of a car you drove. The future will remember that wild ride of life where you believed in others and left a gift behind for someone else to dream the impossible. That gift was your own life. It does not matter whether it was long or short. What did you leave behind?"

- Geoff Hollistor, Out of Nowhere,

The Inside Story of How Nike Marketed the Culture of Running.

Abbreviations

AT: Achilles Tendon

PT: Patella Tendon

CSA: Cross sectional area

Over-use injuries in tendons: Tendinopathy

HSR: Heavy slow resistance training

BMI: Body mass index

MRI: Magnetic resonance imaging

IFM: Inter-fascicular matrix

HC: Healthy control

C: Normal contra-lateral leg

T all: All tendinopathic

US: Ultrasound

PCR: Polymerase Chain Reaction

ROI: Region of interest

PD: Power Doppler

FDL: Flexor digitorum longus

English summary

Tendon overuse-injuries (tendinopathy) develop from overloading the tissue and occur in leisure activity as well as occupational activity, representing a significant socio-economic burden. Patients, often sports athletes, present with symptoms of pain, swelling and feeling of stiffness. Further examination of the injured tendon reveals hyper-vascularization, disorganized collagen fibrils and increased GAGs (glycosaminoglycan). It remains unknown how tendinopathy develops; thus, the early mechanisms that cause these clinical manifestations are not well-understood. Tendinopathy was previously thought known to be of degenerative nature with little to no inflammation. Whether inflammation is present at and/or precedes tendinopathy is still unclear.

The aim of the present work was to demonstrate the spatiotemporal distribution of pathological changes in early tendinopathy with focus on structure and tissue signaling. Active athletes with soreness in their Achilles or patella tendon within the last three months were included and examined by clinical assessment, questionnaires, ultrasound, blood samples, biomechanical tests, MRI (magnetic resonance imaging) and patella biopsies. They were divided into three groups based on the duration of their symptoms: 0-1 months, 1-2 months and 2-3 months, but all received same tests. Biopsies were analyzed by PCR for key regulators of inflammation, tissue remodeling and nociceptive substances.

Ultrasound demonstrated clinical changes with increased thickness and CSA (cross-sectional area) of the tendon between groups. More US PD (ultrasound power Doppler) was present in tendinopathic patients than in healthy controls, indicating hypervascularization in the early phase. However, analysis of patella tendon biopsies showed only discrete changes in anabolic signaling but increased Substance P. There was no change in biomechanical properties of the tendon or force production between the tendinopathic and healthy leg of the same person, indicating an intact whole tissue without tears.

An animal model was developed to study the effects of fibrillin-1 deficiency and subsequently compromised elastic fibers on tendons under voluntary aerobic exercise. Despite high training-loads, no signs of tendinopathy were observed, and mechanical properties remained intact. However, mice with compromised elastic fibers had smaller tendons regardless of exercise.

In conclusion, early tendinopathy presents in the clinic with soreness, hypervascularization and increased thickness but normal biomechanical properties. These changes suggest that

tendinopathy may be an inflammatory rather than a degenerative condition. One of the earliest signs of tendinopathy from 0-1 months in study I was increased tendon thickness and hypervascularization, indicating a component of an inflammatory process. The animal-model in study II indicated that elastic fibers did not play a major role in developing tendinopathy.

Dansk resumé

Sene overbelastningsskader (tendinopati) opstår i forbindelse med overbelastning af vævet og kan ske i fritiden såvel som under arbejde. Tilstanden udgør en stor socioøkonomisk byrde. Patienterne, herunder oftest sportsatleter, klager over smerte, hævelse og følelsen af stivhed i senen. Ydermere fremstår den skadede sene med øget vaskularisering, uorganiseret kollagenfibriller og øget udskillelse af glykosaminoglykaner (GAGs). Det er stadig uklart hvorledes tendinopati opstår, og det er uklart hvilke tidlige mekanismer, der fører til disse kliniske symptomer. Man har tidligere antaget at tendinopati skyldes en mekanisk nedbrydning af vævet med lidt eller ingen inflammation. Om inflammation opstår tidligt i tendinopati eller er til stede under hele forløbet er stadig uklart.

Formålet med det foreliggende arbejde var at undersøge det spatiotemporale forhold som ligger til grund for udviklingen af tidligt tendinopati med fokus på struktur og vævssignalering. Aktive atleter med ømhed i deres Akilles- eller patellasene indenfor de sidste 3 måneder blev inkluderet og undersøgt med kliniske metoder, spørgeskemaer, ultralydsskanning, biomekaniske tests, MRI (magnetisk resonans billeddannelse) og patellabiopsier. De blev opdelt i tre grupper baseret på varigheden af deres symptomer: 0-1 måneder, 1-2 måneder og 2-3 måneder. Alle tre grupper gennemgik de samme undersøgelser. Biopsier blev analyseret med PCR for mRNA-targets inden for inflammation, vævsremodellering og nociceptive substanser. Ultralydsskanninger viste kliniske forandringer svarende til øget tykkelse og CSA (tværsnitsareal) af senen imellem grupperne. Yderligere var ultralyds PD-signalet øget i tendinopatiske deltagere sammenlignet med raske kontroller, hvilket indikerer hypervaskularisering i den tidlige fase. Dog viste analysen af patellabiopsierne kun diskrete forandringer i anabolsk signalering og forhøjet Substans P. Der var ingen ændring i de biomekaniske egenskaber af senen eller kraftudviklingen mellem atletens tendinopatiske og raske ben, hvilket indikerer at hele senen er intakt uden tegn på mikroruptur.

En dyremodel blev udviklet til at studere effekten af fibrillin-1-mangel og deraf svækkede elastiske fibre i sener under frivillig træning. Trods høj træningsmængde var der ingen tegn på tendinopati, og de mekaniske egenskaber forblev uændrede. Mus med svækkede elastiske fibre fik mindre sener uafhængigt af træning.

Konklusionen er, at tidlig tendinopati præsenterer klinisk med ømhed, øget vaskularisering og større tykkelse, men normal biomekaniske egenskaber. Disse ændringer indikerer, at

tendinopati er en inflammatorisk proces snarere end en degenerativ tilstand. Et af de tidligste tegn på tendinopati fra 0-1 måneder fra studie I var øget senefortykkelse og hypervaskularisering, hvilket indikerer, at der er tale om en inflammatorisk proces. Dyremodellen fra studie II viser, at elastiske fibre ikke spiller en stor rolle i udvikling af tendinopati.

Introduction

Healthy tendons

Tendons

Locomotion has always played a vital role in human evolution and therefore places special emphasis on the importance of tendons[1]. Tendons are densely packed connective tissues with a hierarchical structure of collagen (80 % dry-weight) and elastic fibers (2 % dry-weight) together with extracellular proteins[2]. A majority of these fibers are collagen 1 and are packed in fascicles resembling cables, emphasizing the importance of tensile strength that tendons have during force transmission from muscle to bone[3] (Figure 1). Among these fibers reside fibroblasts, endothelial cells, immune cells, nerve cells and proteoglycans[2,4,5]. While collagen fibers in tendons have been studied extensively, there is still little knowledge of elastic fibers in tendons since most studies have focused on elastic fibers in blood vessels[6–8]. However, elastic fibers may provide other mechanical properties to tendons than collagen fibers and therefore should not be disregarded. Tendons must also show viscoelastic properties and be capable of storing elastic energy for efficient function during different loading situations, thus reducing overall energy expenditure during movement[9]. Much of this has been attributed to the inter-fascicular matrix where an abundance of elastic fibers exhibits low stiffness and high fatigue resistance[10,11]. While collagen fibers provide stiffness to tendons there is also a need for elastic recoil provided by elastin, making force generation more economical[12]. Both types of fibers are produced by fibroblasts that are regarded as power-horse cells of the tendon.
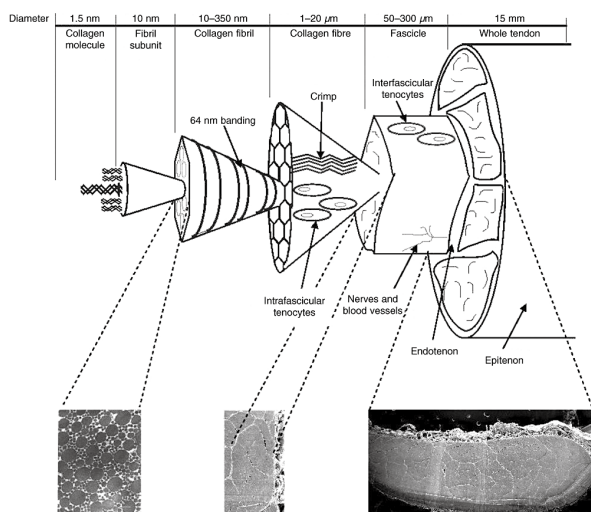
Figure 1 Structure of the tendon. From Thorpe[13].

As physiological demands are different for the two fibers, collagen and elastin, they also differ from each other in their composition. Collagen is made from procollagen intracellularly but is believed to form mature fibers in the extracellular matrix and requires ascorbate acid for this process[14]. There are at least 16 types of collagen, but all share a common structure of triple helices with repeating amino acid motifs and microfibrils. Post-translational modifications of collagen fibers include hydroxylation and cross-linking to increase fiber stability[15].

Elastin is the most abundant protein in elastic fibers and accounts for more than 90 % of the mature structure. Elastic fibers are cross-linked to provide stability during stretching and are therefore responsible for the resilience behavior of elastic fibers in tissues (skin, lungs, tendons, etc.)[16–18]. The core of elastic fibers are elastin and fibrillin-1 proteins in series connected to each other by MFAP-4. Gene-expression of elastin declines with age and is shown to compromise elastic fibers in the inter-fascicular matrix (IFM) of tendons[19–22]. This is especially relevant for the increasing number of master athletes but also for the elderly population in general. Despite their sparse distribution in tendons, elastic fibers have also been suggested to play an important role for shear stress and tendon length through interactions with collagen[23,24]. Thus, elastic fibers may allow collagen to stretch and recoil efficiently[11] (Figure 2).
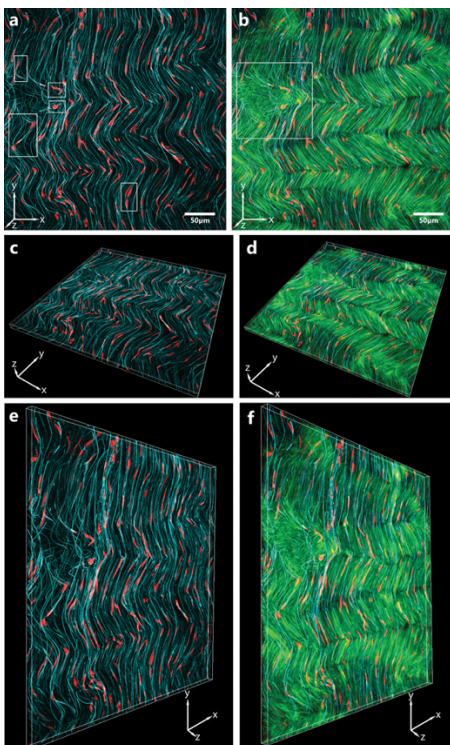
Figure 2 Three-dimensional representation of a longitudinal AT tendon at rest. Collagen fibrils (green) and elastic fibers (cyan) are crimped. The tenocytes (red) are in close pericellular relationship with elastin. From Pang[25].

Recently, animal studies on elastin knockout have shown that degradation of elastic fibers causes signs of chronic tendinopathy (overuse-injuries) while fibrillin-1 is upregulated in torn tendons[26–28]. However, elastin knockout mice (Eln^{-/-}) are unsuitable for models of chronic tendinopathy as the die perinatally. Also, most studies use elastase treatment on Eln^{+/-} mice that may have off-target effects and/or may not resemble chronic tendinopathy[29,30]. Another protein relevant for elastin is fibrillin-1; A glycoprotein that surrounds elastin to form a sheath and has been known to guide elastogenesis by providing a scaffold for elastin to be laterally packed inside[31] (Figure 3). Fibrillin-1 and elastin are therefore known to be associated with each other in almost all tissues (including tendons) except for some such as the ciliary zonule of the eye[32]. Overall, collagen and elastic fibers work in concert to meet the physiological requirements of the given tissue whether it be the positional tendons (e.g. the patellar tendon) or energy-storing tendons (e.g. the Achilles tendon).
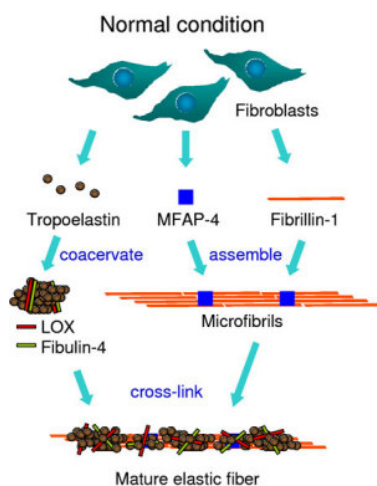
Figure 3 Elastogenesis involving fibrillin-1. From Kasamatsu[33]

Overall the Achilles tendon (AT) and patellar tendon (PT) are important for two different purposes; AT is capable of storing potential energy to be used for forward propulsion while the PT withstands strong forces and is part of the anti-gravity muscles to maintain human bipedalism[34]. Microscopic changes also occur within the tendon itself during force generation. As a tendon is pulled by muscles, the fibers are being stretched in a three-dimensional manner but mainly uniaxial to any force applied. Fascicles slide independently of each other to cope with changing joint-angles but do increase with shear stress[35]. Elastin may help collagen fibers recover to their hierarchical and undulated form, thereby reducing shear stress on the tissue[36]. Certain cells in tendons, e.g. fibroblasts, are more active than previously thought, as they are constantly sensing and responding to changes in mechanical loading[37]. Cells and proteoglycans

are abundant in the inter-fascicular matrix, the same compartment that is believed to facilitate the sliding movement of fibers[20]. More and more evidence now points to a complex of cells, proteins and fibers responding to mechanical stimuli for this humble (but yet complex) tissue.

Biomechanics

The physiological demand of tendons is to transmit force through pulling, and therefore tendon mechanical properties are important to consider when looking at function in vivo. Two major characterizations of mechanical properties exist: intensive and extensive properties[38]. Intensive properties display volume-independent mechanical properties whereas extensive properties are volume-dependent. Any differences in mechanical properties between two tendons that disappears after normalization to tendon dimensions is interpreted as a result of extensive properties such as size, volume or mass. Thus, mechanical differences caused by tendon quantity is called extensive properties while intensive properties can explain the mechanical differences in tendon quality. The stress-strain graph has strain (%) on the x-axis and stress on the y-axis (MPa), both values normalized to the object's dimensions (Figure 4). The physiological range (also called elastic region) defines a region of reversible deformation, meaning that no damage has occurred within the material. Any applied force within the elastic region will only cause transient deformation and once the force is released the material will revert back to its original shape following the same stress-strain curve. For the AT, elastic fibers contribute mostly at 0-1 % strain while more collagen is involved at higher strains[21]. Thus, collagen fibers also contribute to the toe region as they unpack from their wavy/undulating pattern while elastic fibers make it possible for collagen to recover once force is released[22,39]. In contrast, the plastic region causes accumulation of damage or micro tears and thus, believed to play a major role in the development of over-use injuries[40]. Beyond the plastic region comes a failure point in which the stress suddenly drops to zero as the object is now completely broken[41]. There are two interesting mechanical values in this region: The modulus (E) as a normalized value and material stiffness as an absolute value. Modulus is another measurement for the maximum slope of a stress-strain curve and therefore a point of maximum highest resistance from the material against any applied force:

$$Modulus = \frac{L_0}{CSA} \cdot Stiffness$$

and stiffness is given by:

$$Stiffness = \frac{Force}{L_1}$$

L_1 is the new length upon deformation and L_0 is the initial length of the material before any force is applied. Thus, modulus is stiffness normalized to the dimensions of the object and

therefore represents an extensive property while stiffness is independent of dimensions (intensive property). A small partial rupture in the tendon would therefore indicate changes in modulus and/or stiffness, indicating a loss of resistance from the tissue.

Cyclic load/unloading can determine how much mechanical energy is lost in the system as heat[42]. Prolonged cyclic loading *in vivo* might introduce irreversible damage to the tendon tissue even though the strain is very low (material fatigue). However, it is not always possible to achieve material fatigue in the lab due to the experimental setup and time constraint (requires many cycles of load/unload). Extrapolating the results from a laboratory experiment to real-life situations requires additional factors such as rate of loading (viscoelasticity) and duration of stress at a given strain (stress relaxation). Furthermore, it is nearly impossible for any machinery to load/unload a tendon (≈0,05 Hz) as fast as a person who is running (2 Hz) and therefore *in vitro* mechanical testing has its flaws by technological advances or lack hereof[43].
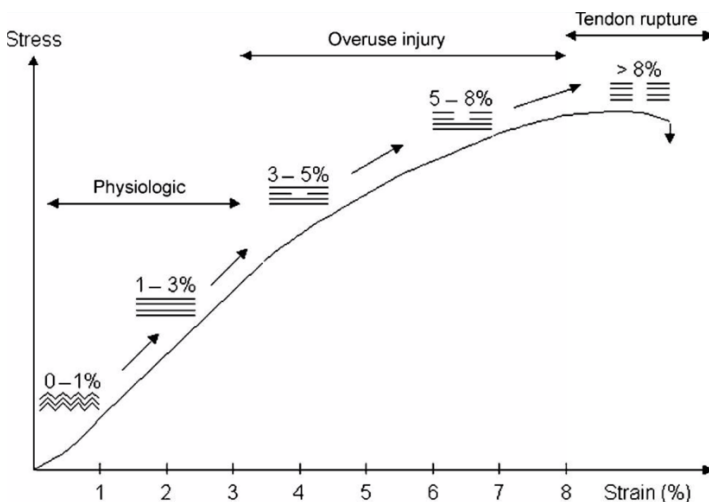

Figure 4 Stress-strain graph. From Arnoczky[40].

Early tendinopathy

Loading and overloading

Overloading of tendon tissue (tendinopathy) is a common disorder among all people, poorly understood and therefore difficult to treat[44,45]. Tendons can be overloaded in all situations but frequently observed in occupational-, leisure- and sports-activity to cause impaired performance and occupational capacity[46]. However, loading of tendons is absolutely necessary for a high life-quality and therefore balanced loading is a viable approach to healthy tendons. Tendon tissue can withstand very high loads (approximately 8-13 times body weight), far exceeding the physiological demands as a fail-safe mechanism[47,48]. In most cases, it is not even possible for athletes to reach failure point by normal neuromuscular activation. In healthy human tendon it is known that single fibrils run throughout the entire tendon length and the whole tendon is loaded homogenously in uniaxial direction[49,50]. It has been very difficult to document any imaging signs of tendon discontinuity in human tendon, and only 10 % of patients with tendon rupture had pre-existing Achilles tendinopathy although many more could have sub-clinical tendinopathy[51,52]. Loading of tendons involves all its fibers within the tendon tissue but the evidence of a connection between tendon rupture and tendinopathy is still very weak. Different compartments within the tendon have various degrees of turnover and could contribute to tendon adaptations based on how high the local turnover is. Although previously known to be an inert tissue it is now widely accepted that tendons are active tissues and may even have a high turnover in specific compartments, at least in inter-fascicular matrix (IFM)[53]. Evidence now supports that IFM plays a major role in both positive adaptations and pathology[11,20,22,54]. Loading may also occur at the cellular level with cytoskeleton and cells becoming pathologic whenever overloading is applied[55]. Physical activity has been shown to increase both stiffness and cross sectional area (CSA) of tendons but may require years before these adaptations occur, whereas immobilization quickly deteriorates mechanical properties within weeks[56–59]. Other structures, like the tendon core, is primarily formed during childhood and adolescence and shows no signs of renewal afterwards[60]. Thus, adaptations to physical activity can influence both tendon tissue quality and/or quantity without one necessarily affecting the other. In regard to sports-performance there is no preference for a bigger tendon over a tendon of higher quality and vice versa. What matters more is whether the tendon meets the physical demands of the chosen sport [the reader is referred to the story of two high

jumpers: Stefan Holm and Donald Thomas][61]. However, it is hypothesized that positive adaptations does not occur a cell or tissue if a tendon is being continuously loaded without adequate restitution (Figure 5)[40]. Thus, physiological adaptions occur when homeostasis is transiently violated but then allowed enough time for adaptions to take place. Overloading occurs when there is insufficient capacity or recovery to achieve a new level of homeostasis (adaptation), potentially leading to tendinopathy. However, tendinopathy could also be interpreted as another level of homeostasis although it is as a pathological condition since life-quality has now declined. The concept of insulting a tissue to achieve adaptation or supercompensation in training seems reasonable based on observations of all biological systems in nature[62].
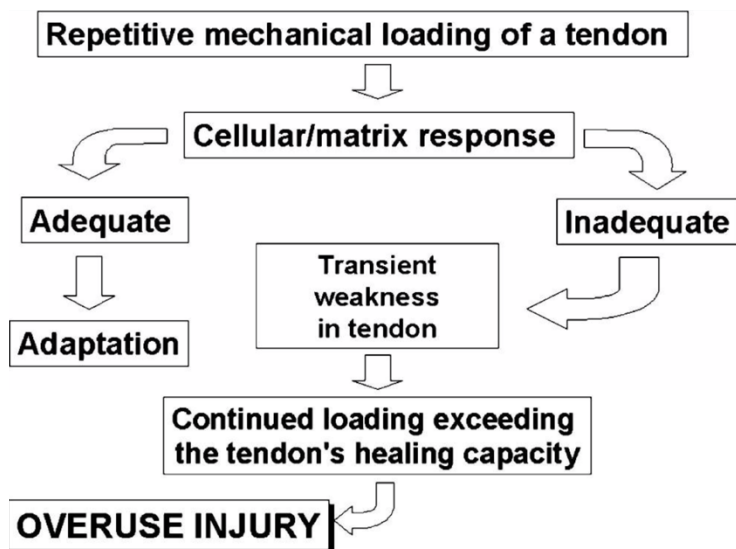

Figure 5 Loading and adaptation. From Arnoczky[40].

Aetiology of tendinopathy

The etiology of tendinopathy still remains unknown, but several factors have gained interest: Flouroquinolone, temperature-induced, genetic disposition, metabolic syndrome and running gait[63–68]. The etiology of tendinopathy seems to be both complex and multifactorial, involving a combination of internal (e.g. age, gender and genetics) and external factors (e.g. shoes, training-surface and BMI). This is also reflected by the various treatments for tendinopathy such as platelet-rich plasma, shockwave, high volume injection, acupuncture and massage but heavy slow resistance (HSR) training has been proven to be most effective[69–71]. Other treatments than HSR are therefore frequently offered in conjunction with HSR[72,73]. Rehabilitation from tendinopathy may take months, years or may never resolve, depending on the aetiology, type of tendinopathy and how effectively it can be treated. It is important to detect early signs or risk factors of tendinopathy to start rehabilitation/prevention-training as soon as possible. Any delay in diagnosis and treatment could cause tendinopathy to become chronic. This is further complicated by the fact that tendinopathy might already be present long before clinical symptoms appear[74]. Thus, preventive measures are always preferable and detecting risk factors plays a crucible role in this algorithm. However, limitations by state-of-art technology and lack of knowledge makes it impossible to determine very early signs of tendinopathy even before the patient becomes aware of any soreness or pain in their tendons. Certain substances are suspected to increase the risk of tendinopathy. Fluoroquinolone of particular interest as it interferes with DNA gyrase that might be vital in the formation for collagen fibers[64]. NSAIDs (non-steroidal anti-inflammatory drugs) can indirectly prolong tendinopathy because they inhibit the inflammation process needed for healing and decrease symptoms from the injured tendon[75]. In addition, NSAIDs are known to relieve the pain from tendons and thus, motivate the patient to exercise beyond their natural pain-tolerance and further worsen tendinopathy.

Temperature has been suggested to cause tendinopathy by affecting the resident cells and fibers[76]. It has also been suggested that systemic factors from heat-induced processes may influence the Achilles tendon negatively[65]. During physical activity the core temperature is increased beyond the physiological range of 36,5°C - 37,5°C. Given the narrow range of optimal body-temperature it is fair to assume that tendon tissue can suffer from intra-tendinous hyperthermia, especially when the tendon core-temperature has been predicted to increase to over 41°C in runners[76]. Elastic and collagen fibers experience temperature-fatigue caused by

16

repetitive tissue loading and could also be accelerated by intra-tendinous hyperthermia. However, temperature-induced tendinopathy may be confounded by other factors as seasonal variations in the incidence of tendinopathy has not been proven[77–79].

Human genetic diseases can provide insight into the consequences of abnormal fiber formation, leading to clinical manifestations. Ehlers-Danlos syndrome (EDS) is a classic example of inherited connective tissue disorder with clinical symptoms such as aortic dissection, joint hypermobility, scoliosis and early osteoarthritis[80]. Patients with EDS typically have mutations in type V collagen encoding genes which gives rise to lower tendon stiffness and structural tendon pathology[81–83]. Of special interest are patients with Marfan syndrome (MFS) because of the increased risk of aorta dissection followed by aortic rupture. The outcome for patients with MFS can therefore be fatal if preventative measures are not taken[84]. MFS is an inherited connective-tissue disorder caused by a mutation in the fibrillin-1 gene of chromosome 15 and has systemic symptoms related to compromised elastic fibers that becomes weakened under stress[85,86]. Other than the cardiovascular system patients with MFS can experience problems with eyes, bones and lungs, but cases of tendinopathy have not yet been reported. This is probably due to the fact that MFS patients are advised to refrain from strenuous activities once diagnosed in order to prevent further progression of cardiovascular complications[87–89]. Most recommendations for physical activity range from low to moderate intensity and thus, MFS patients may never truly overload their tendons enough to develop tendinopathy. The tendons of MFS patients have not yet been studied but could provide details on the role of elastic fibers in tendons if they are compromised. However, it is speculative whether MFS patients have completely non-functional elastic fibers.

People with HLA-B27 defects are at risk for IBD (inflammatory bowel disease), and there is a strong association between IBD and enthesopathy of the AT[90]. Enthesopathy is a type of tendinopathy that is restricted to the insertion-site of the tendon into the bone and provides some evidence that systemic factors may be involved in tendinopathy[91]. People with metabolic syndrome or diabetes have been of interest because of their dysregulation of blood-sugar[92]. Prolonged elevated blood-sugar or hyperglycemia have been shown to influence nerves, organs and blood vessels[68]. Diabetic patients were shown to have increased tendon stiffness as a result of intra-tendinous hyperglycemia. Having a high concentration of sugar in the tendon has been shown to increase end-glycation products and potentially weaken the tendon, but whether this leads to tendinopathy is unknown[93]. Hyperglycemia can also directly influence the resident cells

17

to become dysregulated or even pathological. This is also directly related to diet if it contains caramelized sugar or proteins[94]. However, studies on lifestyle factors for tendinopathy often establish correlations - not causation and therefore pose a "chicken or the egg" problem. People with metabolic syndrome or diabetes may be generally unhealthy with little physical exercise and therefore predisposed to tendinopathy. Thus, there is need to conduct cohort-studies on lifestyle factors and follow individuals over a longer period with frequent check-ups of their tendons.

Lastly, running gait is still a hot topic within sports-science and continues to be of considerable research interest. More and more drills, e.g. core-exercises, are now being implemented by athletes as auxiliary training to their main sport[95]. Within patho-mechanics, it is believed that correcting imbalances or improving running technique will prevent injuries and improve running economy[96]. Running barefoot or with minimalist shoes are an example of the latest trend to run more "naturally"[97]. However, it must be noted that neither core nor running technique drills have been proven to effectively prevent injuries. Different running-forms are often displayed across the elite field, but a common denominator for them all is that they look very efficient when running at top speeds[98]. There is still much research to be done in this field so that drills can be developed in a way that benefits athletes without injuries and the athletes with specific imbalances at risk for tendinopathy can train these drills to prevent injuries.

Clinical changes in patients with early tendinopathy

The term tendinopathy is used to describe painful over-use injuries in tendons and covers a both objective and subjective findings. Objective findings on ultrasound include increased tendon size, hyper-vascularization and loss of fibrillary structure[99]. Subjectively, patients in the clinic complain about soreness or pain related to their tendon. Daily-life activities are usually affected and range from mild soreness to severely painful situations, often accompanied by tendon swelling[100,101]. The structural integrity of the AT may be compromised and show signs of tendinopathy on US but it does not correlate with the degree or severity of clinical symptoms[102,103]. There is still much controversy whether abnormal findings on US or magnetic resonance imaging (MRI) are necessary and sufficient for diagnosing tendinopathy in the very early phase. Furthermore, discrepancies also occur when attempting to correlate histological findings, degree of inflammation and pathological US findings with severity of symptoms experienced by patients and vice versa[103–105]. For example, hyper-vascularization is found to be associated with chronic tendinopathy, but how early this develops in the disease phase is unclear[106]. Athletes with moderate symptoms of tendinopathy have increased sustained blood flow for prolonged time after exercise (measured by US), but this hyper-vascularization is not shown to be correlated severity of symptoms[74,106]. In fact, hyper-vascularization has been found in symptom-free runners without any history of tendon pain and was even protective of tendinopathy following exercise[107,108]. Thus, most studies have reported objective findings that at most fulfil a necessary condition for tendinopathy but none are sufficient [the reader is encouraged to read about necessity and sufficiency][109]. Biochemical changes in AT tendinopathy correlate even more poorly with clinical symptoms and again reflect the complex nature of this condition[110,111]. Some correlations between clinical symptoms and objective findings are more pronounced than others, but none are necessary nor sufficient for tendinopathy. Thickness of the tendon has shown promising results; Increased thickness of the injured tendon compared to the healthy side might indicate tendinopathy[112,113]. Recently, studies have focused on changes in sono-elastography as proxy for altered biomechanics in AT tendinopathy compared to healthy AT although more data is needed to confirm this[114,115]. Many of these objective findings are found to be confounded by the fact that some athletes have a dominant leg in their discipline and therefore naturally bigger and/or stiffer tendons on their dominant leg[56,116].

There are numerous clinical tests to diagnose tendinopathy, but pain or soreness is a certain criteria[117]. However, symptoms are difficult to quantify or qualify objectively. The interpretation of symptoms varies not only between individuals but also within each individual. Factors like socio-economic status, age, pain in other places and mood can severely affect how patients report their experience of pain[118,119]. The patients self-reported experience of pain is included in the criteria of tendinopathy and therefore makes a valid point of treating the symptoms first - not the tendinopathy itself. Also, relieving symptoms might even pose more relevance to the patient than improvements in any biomarkers. In research, understanding complaints from patients requires a method to qualify and quantify subjective pain and soreness. Two systems are widely used and accepted in the research area: the Numeric Rating Scale (NRS) and the Visual Analog Scale (VAS)[120]. Both provide quantification of perceived pain but no information on the quality (what kind) of pain. Another limitation is that their two-dimensional build-up only allows patients to quantify pain in a specific situation and not how it has progressed through time. Viable information may be lost by not creating a coherent picture of how the pain has progressed through time, especially if soreness is one of the most promising early signs of tendinopathy[117]. Full-blown tendinopathy occurs in cases where the patient is reluctant to reduce training load or seek treatment. At this stage the pain is so severe that walking gait is affected, the patient may walk down the stairs "backwards" and some exercises would be extremely painful. As tendinopathy progresses the pain can also be present during rest and when transitioning from sit-to-stand. There is usually a small relief when the tendon is warmed up during the day, but the pain worsens again in the evening because of accumulated tendon-overloading and exhaustion of supportive muscles[63,121]. Sometimes the patient becomes aware of how much larger the affected tendon has grown compared to the healthy side. The patient might even have trouble fitting into their regular shoes. Unfortunately, symptoms of tendinopathy can as well develop on the contra-lateral side, and the reason for this is unknown[122]. Possible explanations are thought to over-compensation by the healthy side or systemic factors released from the chronic tendinopathy[63,123]. Either way there seems to be an increased risk of bilateral tendinopathy once a patient has been diagnosed with unilateral tendinopathy.

Healthy tendons gone tendinopathic

Inflammation and degeneration

It is still unclear how and what mechanisms cause tendinopathy but patients typically present with soreness, swelling and stiffness in the tendon[124]. Based on clinical observations it seems plausible that tendinopathy involves some kind of inflammation. Histological findings shows signs of tissue calcium deposits and altered cell morphology, indicating degeneration of the tissue[125] (Figure 6). These pathological changes support the oldest hypothesis of collagen tearing, favoring a degenerative nature of tendinopathy. A central feature in the degeneration model is that partial ruptures or tearing causes tendinopathy by under-stimulating resident tendon cells (due to the lack of load transmission) and causing the cells to pathologically proliferate or even undergo apoptosis[40,126]. Supporting this hypothesis is the increased collagenase activity with MMP-2 and 3 (metalloproteinases), deteriorating the quantity of the collagen network[127,128].
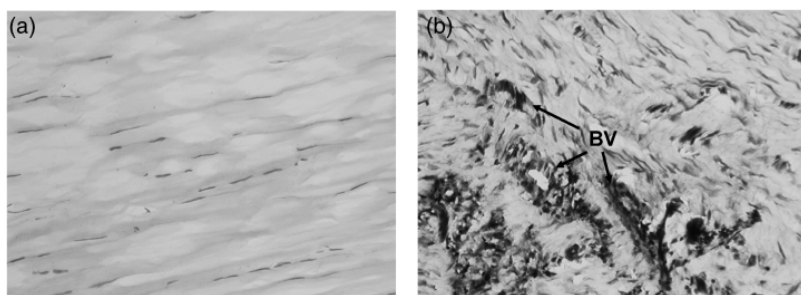
Figure 6 Normal AT from a cadaver (a) compared to a surgical specimen from a patient with chronic tendinopathy (b). Major findings in chronic tendinopathy involves blood vessel (BV) infiltration, cellular proliferation, tissue degeneration and disorganization. However, no inflammatory cells are visible. From Riley[129].

Another hypothesis involves inflammation in response to overloading. Although being a newer hypothesis, a growing number of studies now supports that inflammation is present in tendinopathy. Inflammation is used to describe clinical features of calor (heat), dolor (pain), rubor (redness), tumor (swelling) and functio laesa (loss of function)[117]. Some studies might indicate the presence of inflammation in tendinopathy as they reported symptom-relief with anti-inflammatory drugs such as NSAIDS and glucocorticoids, but the effect is usually short-term, not curative[130]. From a histological perspective there is still much controversy for the existence of an inflammatory component in tendinopathy. Inflammatory cells (e.g. macrophages) and mRNA markers of inflammation have been found in tendinopathy, at least in

the early phase[131]. Studies on early AT tendinopathy are scarce, but the time pattern of inflammation in early tendinopathy has been addressed in shoulder tendons[131], demonstrating inflammatory mRNA signs and cell infiltration during the early phase. Furthermore, different stages of disease from tendinopathic to fully ruptured has indicated inflammatory activation signatures present to varying degrees throughout stages of tendinopathy and included signs of resident stromal cells maintaining chronic inflammation[132–134]. Signs of inflammation was also found in biopsies from patients with chronic AT tendinopathy (symptoms over three months). However, another study found no significant change in inflammatory cells in patients with AT tendinopathy compared to healthy controls[135,136]. The paucity of inflammatory signs in chronic tendinopathy is further supported by studies that show no long-lasting effects of anti-inflammatory medical treatments in treating these often protracted conditions[75,137,138]. Thus, it is likely that inflammation is only present in the early phase of tendinopathy. Increased expression of important key cells in the early phase of inflammation includes (but is not limited to) macrophages, tenocytes and mast cells[139]. More importantly, the resident stromal cells from AT tendinopathy were found to be "primed" for inflammation[132]. The authors speculate that stromal memory cells appeared to be sensitized or primed to pro-inflammatory mediators more than cells from healthy hamstrings, potentially lowering the threshold for an inflammatory condition in the AT. It may be that inflammation peaks in the early phase of tendinopathy and then subside to a more low-grade chronic matter, thereby causing the discrepancy between the findings of various studies. Thus, resolution normally follows inflammation, but the two events are not always coupled[140]. Chronic inflammation is known to cause extravasation of macrophages into the tissue in an inflammatory environment (primed by other cells) to become pro-inflammatory[141]. This state of "inflamma-scence state" in the tissue alters the phenotype of local macrophages; Chemo attractants are being secreted by macrophages to attract more cells, and so the cascade continues. Blood vessels begins to invade the tissue in order to provide nutrients and cells for healing. However, the cascade becomes a closed circuit of inflammation and therefore the blood vessels keep growing. Thus, tendinopathy could represent a condition of failed healing as well as non-resolving inflammation: the pathological transition from stability to an "unstable" equilibrium or homeostasis[142,143] (Figure 7).
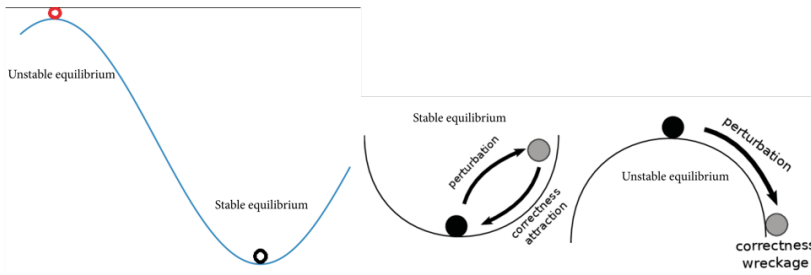
Figure 7 Stable vs. unstable equilibrium. From Danglot[143].

Nerves have been hypothesized to follow blood vessels as neo-angiogenesis becomes more pronounced during AT tendinopathy[144]. Thus, newly formed blood vessels invades the tendon tissue and are then later accompanied by nerve ingrowth, ultimately leading to a painful sensation[145]. The ingrowth of blood vessels and nerves seems to be detrimental for proper tendon function, but this has not been clinically confirmed. Asymptomatic athletes with hyper-vascularization in their tendons perform without any problems and equally as well as their peers[108]. Hyper-vascularization in the tendon can also be seen after physical activity without any other sign of tendinopathy, suggesting that in some cases blood vessel may already be present in the tendon and not as a result of neovascularization[107,146]. Whether these blood vessels have accompanying nerves or not remains unclear. Also, it is not clear whether such blood vessels differ from blood vessels present in tendinopathy. People with hyper-vascularization may are already at risk of developing tendinopathy. However, the detrimental effects of neovascularization or hyper-vascularization is being challenged by the fact that hyper-vascularization within the tendon after physical exercise have also being shown to protect against tendinopathy[108]. It is unknown to what extent the quality of nerves and nociceptive substances are related to blood vessels during early tendinopathy, but sympathetic innervation has not been shown to increase[147]. Vascularized paratendinous tissue may become hyper-sensitive to neuronal input and drive abnormal tenocytes to produce neurotrophic substances, thereby by-passing normal sympathetic innervation[148,149].

In summary, two main hypotheses predominantly exist about the origins of tendinopathy, but they are not necessarily mutually exclusive. In addition, inflammation could be an umbrella-term that covers multiple events, including e.g. hyper-vascularization and degeneration. Whether inflammation and degeneration can be regarded as separate events or in conjunction is still debatable but regardless of definitions they have both been shown to occur in tendinopathy[150]. Available data now supports at least one or more events occurring in the

development of tendinopathy that differentiate an affected tendon from a normally functioning tendon, even though some may signs more controversial than others[151]. The sequence of development regarding these changes in the early phase of tendinopathy is currently unknown. Overall, tendinopathy could start with degeneration which evokes an inflammative response and then subside to an inflamma-scence state. This pathological state damages the tissue over time by accelerating a degenerative condition. This further lowers the threshold for the tissue to withstand loading, and without sufficient restitution from exercise it will become even more degenerative. Ultimately, more inflammatory cells will enter and cause further degeneration of the tissue.

Pathophysiology of tendinopathy

Although substantial knowledge has been accumulated regarding how healthy tendons respond to physical activity[53,58,75,150,152,153], the pathogenesis of tendinopathy is poorly understood and therefore tendinopathy represents a challenge to treat[44,45]. With emphasis on the multiple causes of tendinopathy, there is now compelling evidence to suggest that the pathophysiology of tendinopathy is also more complex than first anticipated. In addition to pain, chronic tendinopathy is associated with imaging findings by e.g. ultrasonography, MRI or electron microscopy such as increased tendon size, hyper-vascularization, altered fibrillary structure, increased ground substance and areas of altered cell morphology[137,154,155]. There are more definitive changes regarding the extracellular matrix (although not an exhaustive list): Cell rounding, increased cellularity, proteoglycan accumulation and loss of collagen organization[154]. In accordance with these 'degenerative' changes it has been hypothesized that tendinopathy results from partial rupture followed by altered biomechanical stimulation of resident cells and little to no inflammation[40,156]. As previously discussed, more compelling evidence now supports inflammation in tendinopathy, but the process is complicated as it depends on systemic factors, time, region, clinical symptoms and etiology[139,155,157]. Furthermore, different definitions and nomenclature among researchers may contribute to the mixed results. All of which could alter the mechanisms of tendinopathy. This may explain heterogeneities in observations made during decades of research in an attempt to understand the development of tendinopathy even though the two hypotheses, degeneration vs. inflammation, could be intertwined[126].

A qualified guess of the pathophysiology of tendinopathy must take into account all these variables, and that is what is attempted in the following. Even though the aetiology of tendinopathy is multi-faceted, there is evidence that shear stress plays an important role in proper tendon function and that multiple factors can influence shear stress, possibly by deterioration of elastic fiber in IFM[11,21,158–160]. IFM has one of the highest turnovers of any compartment in the tendon and therefore makes it a viable target for pathological processes to occur[60,161,162]. One hypothesis could be that overloading of tendons increases shear stress, putting stress on the fibers and cells within the inter-fascicular matrix. This mechanical stimulus might be vital for tendon homeostasis while too much repeated stress may be detrimental[163,164]. Studies have shown that repetitive cycles of tendon-loading, resembling running or walking causes an increased inflammatory response[20,158,165]. However, inflammation is important for any tissue to adapt and overcome. It is thought to clear out old cells or damaged tissue that occurred during loading and thereby allowing new tissue to be formed during resolution[132,166] (Figure 8). It resembles building a new house: The old house has to be destroyed (inflammation) and cleared (resolution) before the new house can be built on top. Therefore, the inter-fascicular compartment plays a major role being as metabolically active that it is. Special emphasis has been placed on elastic fibers in IFM, as they contribute to the initial toe-region and therefore are expected to withstand long stretching as the collagen fibers unfolds[22,39]. Accumulated damage of elastic fibers needs to be repaired and improved to overcome new levels of mechanical stress. Interestingly, prior tendinopathy does not increase the risk of tendon rupture substantially (4 %), indicating structurally damage in other compartments such as the IFM[167]. It may be that elastic fibers are improperly repaired if there is insufficient rest between training sessions. Consequently, it can alter the mechanical stress of adjacent cells and increase the stress on collagen fibers. Elastic fibers or other proteins in the IFM may be important for the resident cells to experience the right mechanical eustress through modulation of shear stress[40,55,168]. One of the resident cells, fibroblasts, are normally spindle-shaped but in tendinopathy become irregular- or even round-shaped[154,155]. This could be a process of reduced mechanical loading, as the cells no longer sense a uniaxial direction of mechanical stretching. It could also be overloading of other cells, since they now have to take the load and respond by becoming more active. Regardless of the discussion on over-stimulation or the under-stimulation causing tendinopathy there are morphological changes in tendon cells, indicating changed cell metabolism.
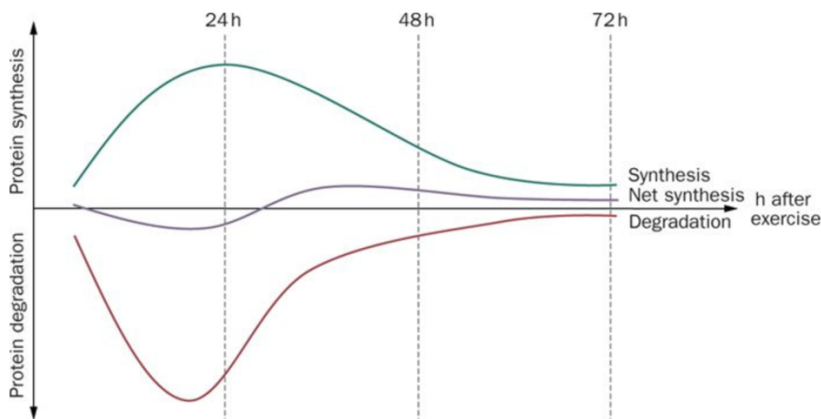
Figure 8 Process of adaptation to mechanical stress. From Magnusson[150].

The cells start the inflammatory process to remodel the tissue[132,140]. If the inflammatory process never resolves then it creates a sustained pro-inflammatory environment for the cells[169]. Resident cells are in limbo; on the one hand the overloaded tendon needs repair, but on the other hand there is not sufficient time and/or nutrients to repair. Repairing intra-tendinous injuries requires the full package of inflammation, resolution and repair. Thus, resolution is not solely about restoring tissue homeostasis after injury but introduce adaptation to another level of homeostasis or baseline of function. The first step (inflammation) takes time, cells and nutrients that may require blood-vessels to deliver, but problems occur if insufficient time for recovery is given between injuries. Over time, resident cells become primed to inflammation, as resolution never occurs. Autocrine and paracrine pro-inflammatory factors keep this environment self-sustained and chemo-attractants start to leak to nearby blood vessels, promoting angiogenesis[170–172]. Among the secreted factors from the primed cells are VEGF (vascular endothelial growth factors) and ANGPTL4 (angiopoietin-like 4) to encourage neo-vascularization of the tendon[173,174]. However, later stages might depend on other factors for blood-vessels to persist in chronic tendinopathy[175]. This process resembles a demolished house that is not cleared while the roads accessing the construction-site (blood-vessels) are cleared in order to recruit more workers (cells and nutrients) for rebuilding the new house, but they are unable to do so because the old house is still in ruins. To speed up the adaptation-process, even more roads are built, and more workers are recruited to the construction-site. The problem is that the foundation (injured tissue) has not been cleared and that new blood vessels invade tissues without respecting tendon-architecture, showing somewhat sporadic-sprouting behavior. However, it is important to distinguish between acute and chronic events;

e.g. acute hyper-vascularization following tendon-loading in exercise is a vital part of an adaptation-process but detrimental in situations with overloading. The same is true for inflammation in general[176–178].

Proteoglycans are osmotically active molecules that have been shown to be increased in tendinopathy, thereby attracting water to cause swelling[179–181]. Whether proteoglycans are increased as a by-product of inflammation or as leaks from newly formed intra-tendinous blood-vessels is still unclear. The preferred substrate of choice for energy metabolism in nerves is glucose, and therefore nerves are often found near blood-vessels that have an abundance of glucose[182–184]. Glucose-uptake in nerves is independent of insulin, relying on passive diffusion and facilitated transporters like GLUT1 [185,186]. The close proximity between nerves and blood-vessels coupled with the complex neuronal glucose up-take spikes two hypothesis about nerve ingrowth: 1) It occurs after angiogenesis, and 2) It follows the path of angiogenesis in the tendon. Assuming nerve ingrowth occurs late in tendinopathy, this could explain the latency of clinical symptoms despite having developed tendinopathy. It is the nerve ingrowth that makes a patient feel something is wrong with their tendon – they start to experience soreness or pain[187–189]. Thus, inflammation can precede clinical symptoms but is part of an early-stage disease with some events leading up to inflammation (e.g. hyper-vascularization), while others are present in later stages when inflammation subsides (e.g. nerve ingrowth and swelling), and therefore the timing of these events is crucial[137,190].

In summary, physical activity and/or exercise increases tendon-loading that causes repetitive stress to elastic fibers within IFM. Insufficient recovery results in degradation of elastic fibers, compromising the IFM while collagen fibers remain intact. This narrows the physiological range of the stress-strain curve and therefore increases the range for tendinopathy (Figure 4 and Figure 9). Intra-tendinous resident cells become pathological: Cells are affected by damaged elastic fibers and become under-stimulated while unaffected cells get over-stimulated by consecutive tendon-loading. Lack of sufficient recovery and nutrients to a certain "point of no return" causes the cells to be in limbo between inflammation and resolution. There is now a self-sustained "inflamma-scence" state. Subsequently, a process of angiogenesis starts in order to deliver sufficient nutrients and cells for repairing damaged tendon tissue. Nerve ingrowth follows blood-vessels into the tendon, and therefore the patient will experience tendon-related symptoms. Meanwhile proteoglycans are upregulated during inflammation and leak out from newly formed blood-vessels, contributing to tendon-swelling. Tendinopathy is now full-blown

and seen as narrowing of the physiological area (stress-strain curve) which again increases the stress on the tissue, thus repeating a viscous cycle (Figure 4 and Figure 9).
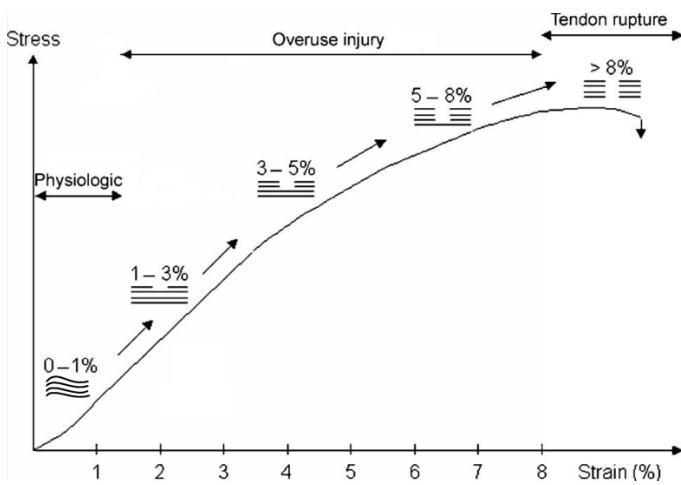
Figure 9 Narrowing of the physiological range in tendons with inadequate recovery. With damaged elastic fibers, the collagen fibers are no longer crimped at lower strain but wavy, indicating a slack in collagen fibers. Also notice the narrowed physiological range as the range for overuse injuries keeps expanding during overtraining. Compare this figure to Figure 4. Reproduced from Arnoczky[40].

As with every other biological tissue these events are expected to be present on a continuum of cells and proteins with changing roles [the reader is encouraged to read about Schrödinger's cat and Heisenberg's uncertainty principle][191,192]. Thus, the proposed model follows a sequence of events only for simplicity and better understanding (Figure 10). It is not a compelling nor comprehensive model of the pathophysiology of tendinopathy.
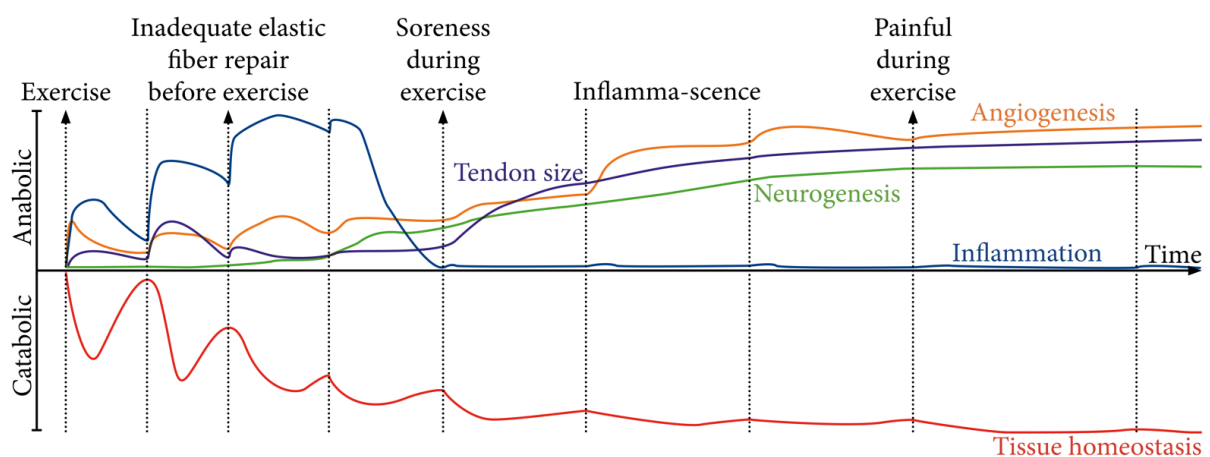
Figure 10 Pathophysiology of tendinopathy. Schematic representation of how tendinopathy could develop in a runner with inadequate recovery between exercise sessions. The dotted lines indicate each exercise session and as soreness in the tendon is experienced by the runner, exercise sessions become increasingly spaced out.

Bridging the gap

Numerous animal models and lab techniques have been suggested in order to study the pathophysiology of tendinopathy in humans. It is noticeable that the majority of these studies use different and often vague definitions of early tendinopathy with emphasis on an early event based solely on the time frame. While time could play a role in progression of injuries or disease, progression may as well be time-independent but related to other factors such as activity level, pain scores or biochemical markers. Generally, physicians agree that early tendinopathy is related to symptoms under three months while chronic tendinopathy is over three months[193].

Another challenge is the different definitions of tendinopathy, reflecting different understandings of this complex condition. Experiments in the laboratory provide a safe and controllable environment to study early tendinopathy but often lack translational purpose. Thus, a deductive approach that includes injecting known substances into cells *in vitro* and studying the end result is not translatable to clinical tendinopathy in patients given the complexity of tendinopathy. Another reason why early tendinopathy is difficult to study seems to be that no good animal or *in vitro* models exist for tendinopathy – besides athletic horses [140,194,195]. Athletic racing horses may develop tendinopathy in the same way as humans (by overloading) but, studies relying on racing horses face two major challenges: i) It is difficult to interpret symptoms from horses and thus determine if the tendinopathy is early or not and ii) Horses are an extremely labor-intensive and expensive model to work with.

Often rodent are chosen for these methods as rodents are easier to handle and cost-effective. However, studies on rodents also present potential problems when extrapolating to humane studies. First, their quadrupedal locomotion causes different tendon- and muscle-loading compared to bipedal primates. Worth mentioning is that the soleus muscles in rodents do hypertrophy much more than humans[196]. Second, rodents have a unique physical activity level and do most of their running during the night as they are nocturnal creatures. Some rodents can run 10 kilometers per day, and with a stride length of 5-6 cm this equals 100.000 steps[197–199]. This is incomparable to humans, as that many steps would equal 150 kilometers per day given a stride length of 1,5 m, and even the greatest Danish distance-runner of all time, Henrik Jørgensen, reported "only" 150 - 200 Km per week[200,201]. Thus, rodents achieve very high levels of physical activity without tendinopathy and it would be unrealistic to any human run the same quantity without injury or overtraining. Lastly, tendinopathy is a painful condition, but

rodents may never show any signs of discomfort. A vital part of their survival is based on agility and any form of limping would expose their weaknesses to potential predators. Charts have been designed to evaluate rodents' facial expressions to give a suffer score, but these can be inaccurate and observer-biased[202].

Human studies have looked into supraspinatus tear for a better understanding of tendinopathy. A partial tear or rupture of a tendon can serve as a model for tendinopathy, but it may not apply specifically to AT tendinopathy. The cellular signaling of matrix regulating proteins is very different between tendinopathy and tendon rupture[203]. Overuse-injuries in the supraspinatus tendon are often caused by mechanical impingement against the osteoclast ceiling of acromion and therefore could represent a degenerative condition[204]. This may not be comparable to AT tendinopathy that has a completely different loading mechanism and physiological demand. Other treatments of tendinopathy include: Heat-ablation, surgery, collagenase injection and running downhill. Surgery, collagenase and downhill running all introduce structural damage within the tendon and have been classified as tendinopathy, probably as part of a degenerative model. Whether these conditions represent *bona fide* tendinopathy (an over-use injury) or solely a mechanical injury is debatable. In fact, it may be advisable to distinguish between an acute injury caused by mechanically destroying collagen fibers versus overloading the tendon without adequate rest. The mechanisms could be entirely different depending on how the insult was introduced and therefore so would the outcome. Thus, a consensus on the term early tendinopathy has to be achieved among researchers before models for tendinopathy can become effective[193].

In summary, the goal is to bridge the gap between characteristics of a healthy tendon with those of tendinopathy; two conditions of a tendon on each side of the continuous biological spectrum. The most important objective is to find inflammation in early tendinopathy and thereafter assemble the puzzle with jigsaws of clinical symptoms, tendon swelling, hypervascularization and nerve-ingrowth.

Aims and Hypotheses

Aims

The primary goal of this PhD thesis was to demonstrate the time pattern and magnitude of pathological changes in structure and tissue signaling in the early development of tendinopathy. This includes tendon size and stiffness, angiogenesis and inflammatory activity as well as tissue anabolic/catabolic and nociceptive signaling. Further, we aimed at testing an animal model with disorders in extracellular matrix (Marfan Syndrome) for early development of tendinopathy.

Hypotheses

In study 1 on humans we hypothesize that a mismatch between anabolic and catabolic pathways causes tendinopathy by triggering the following processes in this specific order: 1) Inflammatory activity with hyper-vascularized tissue and inflammatory signaling markers; 2) Increased nociceptive and anabolic/catabolic signaling, and 3) Increased tendon size.

We hypothesize that inflammation peaks within the first month of early tendinopathy and subsides in a time-dependent matter from one to three months. Inflammation leads to accumulation of osmotically active proteoglycans to cause tendon swelling and induce production of cytokines to promote angiogenesis. Newly formed blood-vessels then invade the tendon tissue. This process is later accompanied by nerve ingrowth, ultimately leading to a painful sensation. In accordance with this, we hypothesize that clinical symptoms are minimal during inflammation and that clinical symptoms are therefore expected to peak at later time points from two to three months. It is our primary goal to demonstrate the spatiotemporal distribution of these events as well as what tendon-related measurements are vital for the development of tendinopathy. In the present cross-sectional study, we included 200 patients with soreness lasting no later than three months in either the AT or PT to investigate early tendinopathy. These 200 patients were unique in that they had no history of tendinopathy or prior soreness in their tendons, providing the opportunity to study first-time occurrence of these events in the development of tendinopathy.

Study 2 on mice with Marfan Syndrome will reveal if compromised elastic fibers cause overloading of the tendon, thereby lowering the threshold for tendinopathy. We hypothesize that the lack of elastic fibers does contribute to tendinopathy in this model, and that this could play a role for some of the mechanisms seen in early tendinopathy of study I. We used three different genotypes of 3-4-week-old mice: Wild type (n=25), less-severe (n=27) and more-severe (n=43) in two groups. The mice were then randomized to either a sedentary control group (n=54) or voluntary aerobic exercise (n=41) for 4 weeks before being euthanatized when 8 weeks old.

Methods

Study I

Study design

Two hundred patients with soreness in either AT (n=155) or PT (n=45) were included in this cross-sectional study. Furthermore, 50 healthy controls with no history of tendon soreness were included in the control group. The three main criteria for AT or PT tendinopathy regarding soreness were: 1) Soreness in relation to physical activity, 2) Tendons must be sore on palpation during clinical examination, and 3) The soreness had to be within the last three months (not lasted >3 months). Patients were divided into three groups: symptoms for 0-1 months (T1), 1-2 months (T2) or 2-3 months (T3). They were excluded if they had any history of chronic tendinopathy or had previously experienced pain on the affected side. Any patients who had undergone invasive treatments such as corticoid injections, HVI (High volume injection), PRP (plasma rich platelet) or dry needling were also excluded. Oral or topic NSAIDS were acceptable but had to be noted. Those who tried HSR training for their tendons were evaluated according to their symptoms, what exercise they had been doing, for how long and the intensity. Some patients who had used HSR systematically because they had previously experience pain in their tendons were also excluded. All participants were physically active but ranged in sports-level from recreational amateurs to Olympic elite athletes. Recruitment was done face-to-face, through the internet and by hanging up flyers in Copenhagen-based running-clubs such as Sparta, KIF and Runners United. Many recreational runners and triathletes were reached by Facebook groups. Some of the investigators were avid runners themselves and therefore recruited patients directly after races or even training. Participants were screened through a self-administered online questionnaire followed by a telephone interview (Figure 11). Since the study was on early tendinopathy the investigators strived to include relevant patients as fast as possible. Therefore, the experimental day was often placed a few days after the telephone interview. The experimental day consists of: Clinical examination, ultrasound-scanning, filling of questionnaires (including VISA A or P) and blood samples drawn from v. cubiti antebrachi. Participants were then MR-scanned and followed two pathways depending on whether they were experiencing early AT or PT tendinopathy. Patients with AT tendinopathy were offered rehabilitation training by a physiotherapist at Bispebjerg Hospital after MR-scans

while PT tendinopathic patients would return after MR-scans for PT biopsies (bilateral) and ultrasound isometric for biomechanical testing of their PT. Finally, patients with tendinopathic PT were also offered rehabilitation training after mechanical testing. Healthy controls followed the same procedures as patients with AT tendinopathy and thus did not undergo biopsy or biomechanical testing. There was no follow-up after rehabilitation training, but patients were offered extra sessions with a physiotherapist if their symptoms persisted. The rehabilitation training was performed according to best practice for all regular patients with tendinopathy submitted to a physiotherapist at Bispebjerg Hospital.
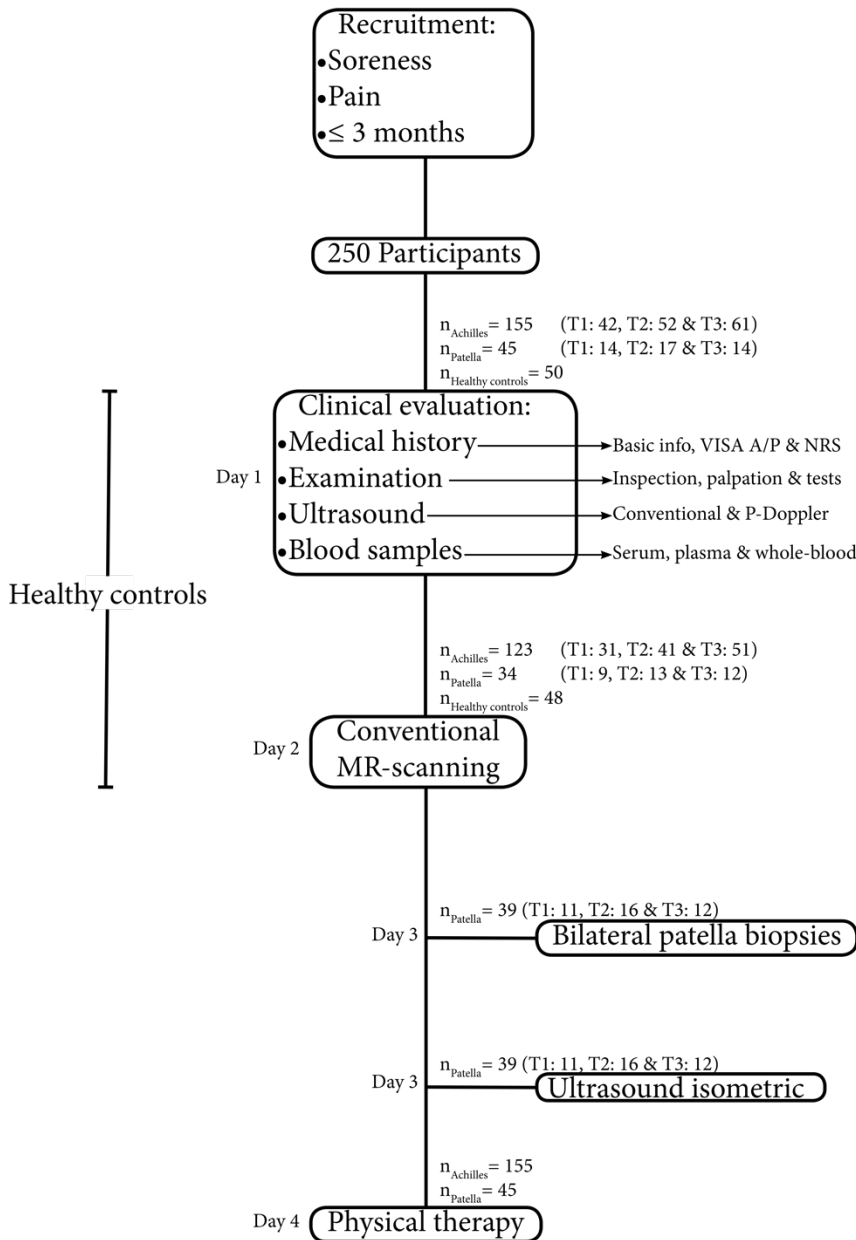
Figure 11 Flow-chart of study I, TESINaC. The project is a cross-sectional study on physically active individuals with soreness in either their AT or PT within the last three months. Healthy controls had never experienced any soreness in the respective tendons.

Clinical examination

Clinical examinations were performed by the investigators at Bispebjerg Hospital. Participants were asked about their training history, symptoms and treatments. Other relevant information was filled by the patients themselves in questionnaires (e.g. medical history, family history, medication, etc.). After medical history they were asked to take off shoes and pants in order to allow visual inspection of the tendon. Height and weight were measured. Participants were

35

then asked to perform two tasks to test core-stability: Half-squats on one leg and pelvic thrusts in supine position. Core-stability is emphasized by many physiotherapists as crucial for stability during running to prevent injuries including tendinopathy. It would therefore be relevant to include observations of any asymmetry or pelvic drop during testing. The most important criteria for diagnosis of AT or PT tendinopathy was soreness on palpation of the tendon and this was sufficient for inclusion. Thus, patients were included in the study regardless of any findings on other clinical tests or US-scanning if the soreness had persisted under three months (taking into account no exclusion-criteria were met). For AT-tendinopathic patients the tendon-area with maximum soreness was marked as area of interest and measured as a distance from the calcaneal insertion. For PT tendinopathic patients the distance was measured from tuberositas tibiae to where the soreness is (area of interest). Some patients had symptoms on both legs (bilateral symptoms), but then the leg with the most severe symptoms was chosen as primary. If both legs had equally severe symptoms, the one with most longevity within three months was chosen as primary. In a few rare cases, if symptoms and longevity were equal on both affected legs, the ultrasound findings would determine the primary leg. This assessment was based on pathological findings of tendon morphology: Dimensions, fibrillary structure, vascularity and hyper- or hypoechoic areas. Patients with bilateral symptoms were examined with US PD and MRI as these measurements were not normalized to the healthy contra-lateral leg within the same patient.

Ultrasound-scanning

A protocol was developed to ensure standardized settings for all ultrasound machines and to ensure that all investigators followed the same procedure. For AT: The participant must be in prone position (on their stomach) with both ankles floating freely off the examination table. The ultrasound (US) machine was set according to protocol prior to scanning, and the affected leg was always chosen first for scanning. Both ATs were fully scanned in order to establish the area with most US PD-signal and where the tendon had the largest dimensions. US PD area was determined only within the tendon substance, and tendon dimensions were measured on the ultrasound-screen as a perpendicular line from one border of the tendon to its opposite site (Figure 12). US PD was chosen over conventional color flow Doppler as the method is more sensitive. However, while the method is more sensitive, US PD also enhances background noise,

requiring the probe to be held completely still during examination. Holding the probe completely still allowed the investigator to more reliably distinguish pulsating signals in blood vessels from background noise. Both US PD-signal and tendon dimensions relied on the investigator's observation and interpretation to find the best angle for visualizing these findings within area of interest (maximum soreness). The area of interest was defined as the point of maximum soreness during clinical examination. Often, this would correlate with the area of maximal soreness that had been marked previously (within 5 mm), but not always. If there was a discrepancy between the area of most pathological findings on US and soreness, the latter would be considered most important (area of interest). The area of interest was recorded on the US machine and composed to a video-film of 5-10 sec in length or at least 3-4 heartbeats. This was done in both a longitudinal and transverse plan on the same area of interest to give more reliable 3-dimensional confirmation of the findings. The contra-lateral leg was scanned and video-filmed according to the affected leg's area of soreness. Thus, recordings of US-findings for the contra-lateral leg was performed at the same anatomical level as the affected level. PT participants were instructed to assume supine position with their knees at 90°-flexion. This position of the knee was chosen as a middle ground for optimal visualization on ultrasound. Too much flexion would stretch the PT too much and risk compressing any fine blood vessels. Too little flexion can make the PT slack, and it will often appear "bulging" on US, making measurements of tendon-dimensions challenging. Healthy controls were scanned on the thickest part of their tendon, often mid-portion and then on the same anatomical level on the contra-lateral leg. A pilot study determined the mid-portion region to be the most representative area for whole tendon morphology. Healthy controls usually had no area with US PD-signal and therefore the thickest mid-portion region was chosen for US-scan (both US PD and tendon dimensions). Tendon dimensions and US PD-recordings on the contra-lateral healthy tendon of patients were determined at the same anatomical level as on the tendinopathic side within the same patient. The anatomical level on the tendons of patients and healthy controls were measured by a ruler. US videos were analyzed in Fiji ImageJ2 (freeware) by using a PD macro. An area of interest (maximum soreness in patients during clinical examination or thickest mid-portion measured by US in healthy controls) was marked for analysis and the macro would quantify the number of pixels that corresponds to US PD-signals. The area of interest was chosen inside the tendon borders. The macro analyzed all video-frames within the chosen borders and automatically selected the frame with the most

relevant US PD-signal. Sometimes the zoom on US was changed, but this had been taken into account during data-analysis and corrected for so it could be compared to the standardized 15 mm zoom. Some video films had too much noise within the tendon and therefore would give higher false-positive pixels of US PD activity. Since the video films are 5 – 10 seconds in length it was possible to: 1) Valuate if the signal was from blood vessels or background by scrolling through frames looking at pulsation, and 2) Make a substack (stack of a stack) in ImageJ to select a range of relevant frames without much background. This ensures that only the *bona fide* US PD-signal was quantified, leaving behind any background-noise. US measurement of thickness, diameter and CSA were performed on the frame with most US PD activity chosen by ImageJ (macro Doppler). If it was not possible to appropriately measure the tendon dimensions, another frame closest to maximum US PD-signal was chosen. This was done on longitudinal and transverse sections for both legs.
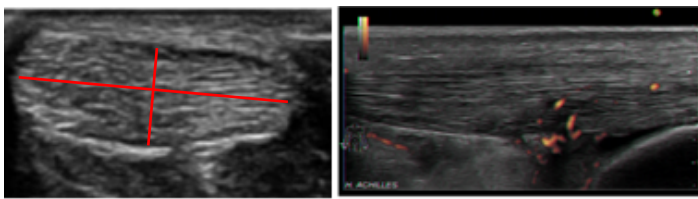
Figure 12 US-scanning of AT. Left image: Red lines indicate measurement of width and A-P thickness on transverse section. Right image: US PD-signal seen as yellow color near insertion in a longitudinal section.

Questionnaires

Three types of questionnaires were handed to the participants: Basic info, VISA A or P and NRS. The questionnaires were filled in entirely by the participants themselves, but if required, assistance was provided by the investigators. Participants were encouraged to answer all questions although some questions were unclear and therefore required further elaboration by the investigators. Investigators were briefed on the interpretation of questionnaires so that a consensus was achieved. If participants left questions unanswered and were out of reach, they would be reached by telephone later on. In such cases the investigator would fill out the blank spaces in collaboration with the participant. The first questionnaire involved basic information about the participant: Medical history, family history, any ongoing infections, medication and job. Furthermore, there were questions regarding physical activity and injuries during the

participant's lifetime: Previous sport, level of previous sport, current sport, how much time is dedicated, intensity, previous injuries and how long they had to recover from previous injuries. The "Basic info" questionnaire was completely designed by the investigators and has not been used in any other studies. It was created to provide an overview of health and training status in the past and present. VISA A or P are validated questionnaires that have been used in numerous studies. However, participants were often confused by the questions in VISA and often relied on the investigator to help elaborate. The NRS questionnaire consisted of a few questions regarding the participant's average pain-score at present using NRS (Numeric Rating Scale): How much does it hurt during training/after training/rest, rating from 1 – 10, and does the tendon feel stiff in the morning?

NRS graphs are a concept developed by the investigators to quantify the symptoms over time to provide a more coherent picture of the symptom-history (Figure 13). The y-axis consists of values from 1 – 10 and resembles traditional NRS to quantify symptoms. The x-axis represents time and is entirely dependent on when the symptom was first felt. Patients had to remember the start-date of their symptoms to place it on baseline while the end-date is an arbitrary distance on the x-axis but often as far away as possible from start-date. Patients are then encouraged to draw a line, representing the average NRS-score of their symptoms, from start- to end-date. The constructed graph made it possible to back-track how symptoms had developed through time. Since NRS graphs were invented and used only in this study, it has not been validated in any studies, but a pilot-study conducted by the investigators seems to be reproducible. Patients made a plethora of different graphs that seem unique to their situation, but this was expected given the heterogeneity by freehand drawing a graph. Analyzing NRS graphs required several steps. First, graphs were divided into a grid using transparent millimeter paper by superimposing and aligning two papers with each other. Second, every square related to the graph was noted as an x- and y-coordinate. The distance between start- and end-date on the y-axis was divided into squares and would correspond to a day on the x-axis. Third, graphs were reproduced in Excel with an accuracy to 0,25 Units on the NRS. All graphs made in Excel closely resembled the original graphs drawn by the participants. The x-axis was then normalized to the duration of symptoms [Graphs from each patient are represented in the front page of this thesis]. E.g. if a patient A had symptoms for 10 days and the graph expanded over 50 squares on the x-axis, it would amount to 0,2 days per square. This could be compared to another patient B with symptoms for 7 days over 50 squares (0,14 days

per square). If the two patients are compared it would only be 35 squares from patient A (7 days from patient B divided by 0,2 squares per day from patient A) vs. 50 squares from patient B. Thus, it would only be possible to compare the first seven days of patient A with the total seven days from patient B. Comparing beyond seven days has little relevance, as patient B only had symptoms for seven days and therefore the patient with the shortest time-frame of symptoms determines the maximum limit for days to compare.

Despite all efforts to have the participants answer every question, there would still be some blank spaces left. If a participant's writing was unclear, or if the questions were left unanswered, it would appear as N/A and therefore not be included in the final dataset. Also, questions that were misunderstood by participants and answers that did not make any sense were noted N/A. Sometimes participants would answer "A few per week" in e.g. drinks and tobacco consumption. Such entries were converted to 2-3 by investigators to have a numeric value for comparison. If there were multiple answers, the investigators would choose between listing the most severe first or take the average depending on what was relevant for the question. Several assumptions had to be made for "Basic Info": 1) Family history was only confined to siblings, parents and grandparents; 2) Medication was listed with the active pharmaceutical ingredient by searching pro.medicin.dk on specific advertised brand or generic names, and 3) Working in office is an umbrella-term for jobs that include sitting in front of a desk for prolonged time (e.g. student, operating telephones and software-related jobs). Sometimes participants had multiple primary sports, in which case the one with the highest impact on the Achilles or PT was prioritized as most important.
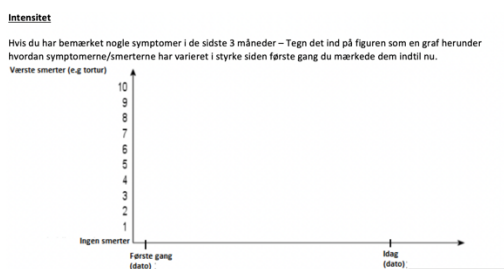

Figure 13 Blank NRS-graph in Danish. Patients were asked to draw a graph starting from their first symptom to today focusing on NRS 0 - 10. For simplicity, patients rated only on average how NRS had developed in the specific period.

Blood samples

All participants had approximately 10 milliliter blood drawn from v. antebrachi cubiti into three vials for plasma, serum and whole blood. Participants were observed by staff in the waiting room while blood samples were processed according to protocol. Three different tubes were used to store the blood samples: 1) BD 362753 Vacutainer CPT for whole blood; 2) Vacuette 454204 Serum Clot Activator for serum, and 3) Vacuette 454217 K3EDTA for plasma. Serum was always transported in ice-bath. The vials were left to rest in the laboratory for 30 minutes before centrifugation at their required rpm (rotations per minute): 1) Whole-blood required centrifugation at 20° Celsius at 3000 rpm for 20 minutes, and 2) Serum and plasma were centrifuged at 4° Celsius at 4000 rpm for 4 minutes. Vacutainers would develop a cloudy substance in the supernatant above the gel that would be extracted as it represents leucocytes. Whole-blood, serum and plasma were divided into several Eppendorf tubes and stored at -80° Celsius before analysis. Blood samples are used for genome analysis and routine tests (triglycerides, cholesterol, HbA1c, etc.) but laboratory results are expected to be released after submission of this thesis.

Magnetic Resonance

Participants were screened for MR (Magnetic Resonance) contraindications at their first visit and were then instructed not to perform any strenuous activities right before MR-scanning at Frederiksberg Hospital. Experienced radiologists worked with at least one investigator from the project to ensure a comparable process for participants and standardized MR-scans within the tendon's area of interest (maximum soreness in patients during clinical examination or thickest mid-portion measured by US in healthy controls). Only the affected leg was MR-scanned because the whole protocol, with multiple sequences, took approximately 40 minutes. Having both legs scanned would expose the participants to too much SAR (Specific Absorption Rate). Even though MR-scans do not emit X-rays, they do produce strong radiofrequencies to align magnetics fields, and therefore they increase the core-temperature of the participant. SAR is an expression for the radiofrequency power disposition in the participant given as watts per kg. Participants with reduced thermoregulatory capacity (elderly, diabetes, fever, etc.) are at increased risk for hyperthermia with subsequent fatal outcomes if SAR is not reduced in time. However, none of our participants were at risk as the majority were young and healthy. Some

had metal implants but of non-magnetic type and were MR-scanned without any problems. Whenever the investigators and radiologists were in doubt of contraindications it would be the responsibility of the investigator to contact Philip Hansen, the primary radiologist on the project. Conventional MR-scans were performed on participants. Conventional is excellent for measuring tendon dimensions such as length, thickness, width and cross-sectional area. Analysis of conventional MR-images was performed on Horos 3.0 (freeware) and according to standard configurations: 1) The zoom was set to 2000 %; 2) WL/WW: 0 – 1500, and 3) Conventional images of type pd_tra_lille was used. In-program functions like "Pencil" and "Repulsor" were used to measure the tendon dimensions (Figure 14). Patients were measured at the tendon's specific area of interest (maximum soreness in patients during clinical examination or thickest mid-portion measured by US in healthy controls) that was observed from clinical examination as a distance from tuberositas tibiae or calcaneus insertion to the area of maximum soreness. The images represent slices of patients, but sometimes the area of interest was between two slices in which the two nearest slices (one before and one after) were measured and averaged. Healthy controls were measured in the middle of their tendons; a position that typically corresponds to the thickest area measured by US. Compositional MR-images were converted with a custom-made program coded in C++ and was run by Homebrew on Mac OS X. ITK-SNAP was then used to mark ROI (region of interest) to be analyzed in Matlab using a macro developed by René Svensson. The whole procedure is highly experimental, but there may be a considerable potential for future studies; Compositional MR-scans are minimally invasive and offer insight on a macromolecular level of tissues without taking a biopsy.
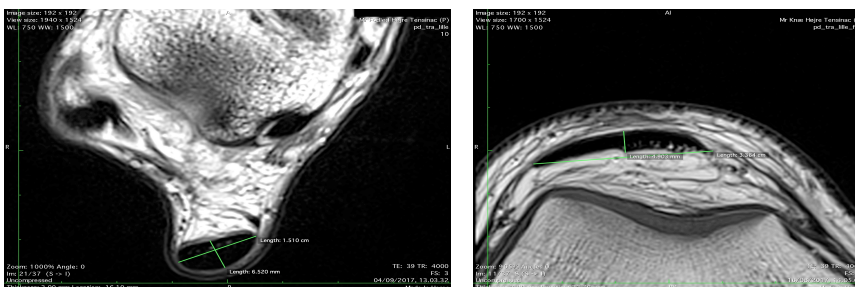

Figure 14 Tendon measurements on Achilles patients (left) and Patella patients (right) were performed in Horos. In this example, A-P thickness for AT and PT is 6.52 mm and 4.90 mm, respectively. Width for AT and PT is 15.10 mm and 33.64 mm, respectively. Measurements were performed only on transverse images.

Patellar biopsies

PT biopsies were performed at M81, Bispebjerg Hospital. Patients with soreness in PT received PT biopsies on both legs after informed consent. 45 PT patients were included but six refused biopsies, leaving 39 patients for bilateral PT biopsies. The response to biopsies was mixed and ranged from some pain to pain-relief. Interestingly, some patients reported an immediate relief of their symptoms after a biopsy was taken and experienced reduced soreness during their sports. However, most patients were unaffected by the biopsies and could return to regular daily activities within a day. The PT is easily accessible because it is located right under the skin. There are no vital structures nearby and therefore performing a biopsy does not pose any health risks for the patient. The department is very experienced in biopsies: procedures are performed routinely and have been described as safe in previous publications[205,206]. In this project, a semi-automated biopsy instrument (Bard Magnum, Bard Biopsy Systems) with caliber 14G was used for PT biopsies. PT patients were in supine position with their knees flexed at 45°, and a pillow was placed under the knees to keep the position fixed (Figure 15). Using sterile procedure and local anesthesia an incision of 15 millimeters in width was made through the skin 10 millimeters distally from the PT apex. The semi-automated biopsy instrument was angled at approximately 25° to the knee before insertion. This was to align the biopsy needle along the tendon and to allow it to "scratch" a sample of the tendon, thereby increasing the chance of a successful biopsy. A high-pitched clicking sound would indicate that a biopsy had been taken and would cause a brief discomfort in the patient. Sample size was normally 10 – 20 milligrams of tendon tissue per biopsy, and only one biopsy was taken from each leg. Even though biopsies can provide detailed information about the tissue ranging from tissue architecture to gene expression, it must be noted that 20 mg only represents a small part of the whole tendon and therefore not necessarily precisely from the area of interest. Thus, it may be difficult to sample from a pathological area if tendinopathy is a very localized process. Sometimes acquiring an acceptable biopsy would require two or three trials if no tissue was visible under the microscope, and the same incision was used multiple times. However, multiple biopsies from the same tissue might induce several acute inflammatory markers following the first biopsy. The investigators did not use ultrasound-guide biopsies because it was not possible to determine an exact ROI within the tendon at this early stage of tendinopathy. After successful biopsies on each leg, the incision was ligated by Steri-strips and a plaster was applied. Patients were told to change plasters every day and after bathing.

Samples were taken according to the department's standardized procedure for PT biopsies. One investigator took the biopsy and handed it over to another investigator to analyze the sample under microscope. Samples were dissected to free tendon tissue from fat and subcutaneous tissue. It was then divided into two pieces: A small sample for EM analysis of the ultrastructure and the rest for biochemical analysis later on. The latter was instantly frozen in liquid nitrogen and stored at -80° C until biochemical analysis was performed.

Figure 15 Position of patient before biopsy. A 15 mm incision was made under local anesthesia near the PT apex and a biopsy needle was inserted at 25° relative to the knee for obtaining a sample of PT tissue.

Biochemical analysis of Patellar biopsies

PT biopsies were analyzed for levels of mRNA expression with real-time-RT-PCR by Peter Schjerling at M81, Bispebjerg Hospital, Denmark. PCR (Polymerase Chain Reaction) uses a temperature-stable polymerase, primers, cDNA and SYBR green to amplify target sequences through cycles of different temperatures. High temperature dissociates double-stranded cDNA while low temperature allows primers to bind and be elongated by polymerases. This process is repeated with the newly made double-stranded cDNA, and the number of target molecules created is given by the formula: $X_n = X_0(1+E)^{(N-2)}$, in which X_n: Number of target molecules; X_0: Number of starting target molecules; E: Efficiency of reaction from 0 – 1; and N: Number of cycles. Although it resembles exponential growth at first, the reaction becomes saturated and efficiency is expected to decline. SYBR green is a fluorescent dye that binds all double-stranded DNA, enabling quantification of X_n. Afterwards, melting curves are used to test if SYBR green actually did bind target molecule. Three step-wise procedures are used to obtain mRNA from PT biopsies: 1) Dissection of samples; 2) RNA extraction, and 3) Real-time RT-PCR[75]. Briefly, tendon samples are cleaned for subcutaneous tissue, cut into 0.5 mm slices and then weighted. Stainless-steel balls of 2.3 mm together with sharp particles are used to homogenize the slices of the tendon tissue. Bromo-chloropropane separates the sample into an aqueous and organic phase, but only the former is used. Glycogen is then added to the aqueous phase to facilitate RNA precipitation. Finally, extracted tendon RNA is converted into cDNA, using reverse transcriptase, and loaded on real-time PCR with primers for specific targets (Table 1). The same procedure is used to analyze Achilles tendon tissue in study II. Expressed values are normalized to the internal control, RPLP0, an mRNA that codes for the large ribosomal protein P0. GAPDH mRNA was then used to validate RPLP0. The rationale for using two unrelated housekeeping mRNAs is the lack of *bona fide* constitutive RNA. While an increase in target mRNA could mean a decrease in housekeeping mRNA and vice versa this method of validation minimizes the risk of such a situation. Increased target mRNA expression would be an actual upregulation, because downregulation of RPLP0 is unusual. This assumption is further strengthened by using a second housekeeping mRNA to validate RPLP0. Given that no relationship exists between RPLP0 and GAPDH, it was expected to see no significant difference between the two. However, it remains debatable if mRNA expression actually reflects gene expression and/or protein production.

Target	Primer name	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGTCTTGGTGGTTTT
COL3A1	NM_000090.3	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC
Tenascin C	NM_002160.3	CAGCCAAGATCCAGGCACTCAA	GTCCTTGGGGAAGGGGTACAGG
Decorin	NM_001920.5	GGTGGGCTGGCAGAGCATAAGT	TGTCCAGGTGGGCAGAAGTCA
Fibronectin	NM_212482.3	TTTGCTCCTGCACATGCTTT	TAGTGCCTTCGGGACTGGGTTC
Tenomodulin	NM_022144.3	GCGCCAGACAAGCAAGTGAAGA	TCTCATCCAGCATGGGGTCAAA
MMP2	NM_004530.5	CCGCCTTTAACTGGAGCAAAAACA	TTGGGGAAGCCAGGATCCATTT
MMP3	NM_002422.4	GATCCTGCTTTGTCCTTTGATGCTGT	CTGAGGGATTTGCGCCAAAAGTG
TGFb1	NM_000660.6	GAGGTCACCCGCGTGCTAATG	CACGGGTTCAGGTACCGCTTCT
TGFB2	NM_003238.4	CCCAAAAGCCAGAGTGCCTGAA	ATGTAGCGCTGGGTTGGAGATG
TGFb3	NM_003239.4	TGCCAAAGAGATCCATAAATTCGACA	CCTTAGAGGTAATTCCTTTGGGGCA
IGF1-Ea	NM_000618.3	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
CTGF	NM_001901.3	TGCGAAGCTGACCTGGAAGAGA	GCCGTCGGTACATACTCCACAGAA
ITGB1	NM_002211.4	GGATATTACTCAGATCCAACCACAGCA	TCAATGGGATAGTCTTCAGCTCTCTTG
EGR1	NM_001964.3	GCAGCCCTACGAGCACCTGACC	AACTGGTCTCCACCAGCACCTTC
PDGFRA	NM_006206.6	AAGTTGCCCGAGGAATGGAGTTTTT	AGCCAGATCACGGTGGACACAA
SubstP	NM_013996.2	TGGTACGACAGCGACCAGATCAA	TCTCTGCAGAAGATGCTCAAAGGG
HSP70	NM_005345.5	GTGGCTGGACGCCAACACCTT	TTACACACCTGCTCCAGCTCCTTC
ATF3	NM_001674.4	GCTGCAAAGTGCCGAAACAAGA	TGAGCCTTCAGTTCAGCATTCACA
ATF4	NM_001675.4	GGTCAGTCCCTCCAACAACAGCA	TCCAAGTCGAACTCCTTCAAATCCA

COX-1	NM_000962.3	GGTTTGGCATGAAACCCTACACCT	CCTCCAACTCTGCTGCCATCT
PTGFR	NM_000959.4	TGTATTTGTTGGAGCCCATTTCTGGTT	GGAGAGCAAAAAGTGTTGTTTCACAGG
VEGFA	NM_001025366.2	ATGACGAGGGCCTGGAGTGTGT	CTCCTATGTGCTGGCCTTGGTG
ANGPTL4	NM_139314.3	GGGACAAGAACTGCGCCAAGA	ACTGGCCGTTGAGGTTGGAATG
ATG12	NM_004707.4	CCAGACCAAGAAGTTGGAACTCTCTAT	GCAAGTTGATTTTCTTTGTGGTTC
Beclin1	NM_003766.4	CGCAGCTGGATAAGCTGAAGAAAACC	CGACCCAGCCTGAAGTTATTGATTG
MAP1LC3B	NM_022818.5	CAGATCCCTGCACCATGCCGT	TTGGTTGGATGCTGCTCTCGAAT
HBB	NM_000518.5	CTCAAGGGCACCTTTGCCACAC	ATGGGCCAGCACACAGACCAG

Table 1 Primers for real-time RT-PC.

Ultrasound isometric

Ultrasound isometric was used to evaluate tendon biomechanics *in vivo* and was performed at M81, Bispebjerg Hospital. It is a non-invasive procedure, but one that requires physical effort from PT patients, and which can be challenging after having biopsies taken from both PT. Only PT patients had to perform ultrasound isometric. The principle is a simultaneous tracking of tendon movement with generation of force. The setup is complex and often involves several trials before the patient was comfortable with the given instructions (Figure 16). Most tests were carried out by the same two investigators in order to minimize variation, and one of the main investigators was always present together with an assistant because of the labor-intensive procedure. First, the patient had to change clothes to shorts and warm up on a stationary bike for 10 minutes. This was to mimic a usual warm-up procedure before any sports-event to prevent injuries and loosen up muscles. It has also been proven to precondition the tendon, making results more reproducible[207]. Second, after warm-up the patient was placed in a custom chair resembling an in-flight seat, and the patient was then told to buckle up. Backrest was adjusted for knees to just come over the chair's edge and allow the legs to hang loosely over the ground. An ultrasound-machine was used to visualize the deformation of PT *in vivo*. The ultrasound-probe was fixed on the patient's knee using a holster with straps. To visualize the whole PT from origin to insertion, a 100 mm probe was used. An ankle shackle with force-

measurement capability was attached to the tested leg, and only one leg was tested at a time. Third, the investigator would slowly count to eight and the patient was instructed to make one single long kick for eight seconds, increasing the force every second. Since their leg was fixed movements were minuscule, but the measured force would increase (seen on PC) together with tendon deformation (seen on ultrasound). After eight seconds the patient was told to relax before another round of contraction. This was repeated at least three consecutive times on one leg with 1-2 minutes of pause before testing the other leg. Some patients complained of soreness in their PT as they had just been through the biopsy procedure a couple of minutes ago. In some cases, there was blood on the plaster, complicating visualization of the tendon. In such cases the plaster had to be changed and the patient had an extended break before the next maximal contraction. Finally, femur and tibia length were measured to determine knee-joint moment arm.
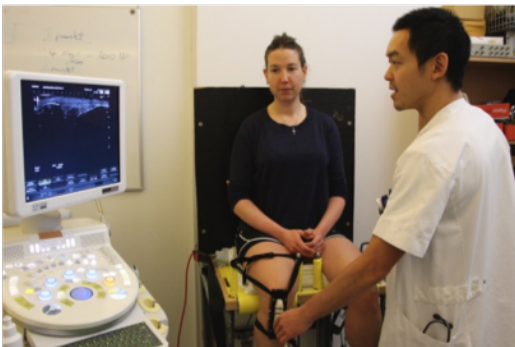
Figure 16 Setup of ultrasound isometric. One investigator is holding the ultrasound probe in place while another investigator (not seen) triggers the measurement of force and tells the patient to start one slowly increasing isometric kick over 8 seconds.

Tendon force was analyzed in Matlab R2016b with a macro made by René Svensson to track tendon movement by using dots (Figure 17). Tracking was performed two times on apex of the PT and tibia on separate occasions, representing the tendon origin and insertion respectively. Around 10 – 20 tracking dots, evenly spaced, were placed within the area of the PT. Each video was tracked four times: Two times on PT apex and two times on tibia. The best videos were selected by the investigator based on three criteria: 1) Full visualization of PT origin from apex Patellar to insertion on tuberositas tibiae; 2) Synchronized force and deformation data, and 3) Natural movement of the tendon and bones to produce smooth curves with minimal noise. Some videos were difficult to track due to biopsy bleeding that blurred relevant areas and/or static objects on the video that would interfere with the tracking dots. The investigator tried to

overcome these problems by finding alternative places within the PT to track; however, the problem could not be solved, leading to seven out of 45 patients being omitted due to bad videos. Tracking-results were copy-pasted to an Excel template made by René Svensson: "US Force + Def Template_uitrack_1". Recorded force data was also copy-pasted into the same template. Under the "Results" tab it was possible to see the correlation between force and deformation. Tibia and femur length were included in the template to calibrate the tendon force.
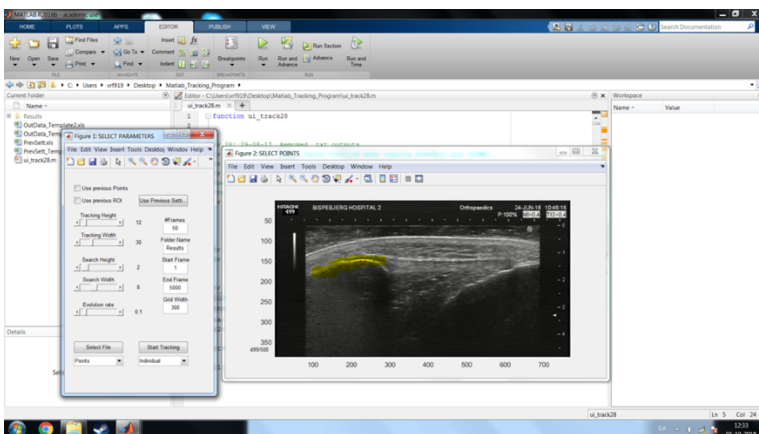
Figure 17 Tracking of PT using Matlab. Red tracking dots in the yellow area on PT mark points that follow the movement of the PT during contraction.

Deformation and tendon force data were synchronized as there would be a slight lag from the equipment used to measure force (Figure 18). Data from the template was copy-pasted to SigmaPlot 10.0. Force-curves were generated by second degree polynomial in SigmaPlot, and values for the graph were copy-pasted into another template made by René Svensson: "Tendon_properties". The same template required length and CSA of the PT. MRI scans were only performed on the affected leg, and ultrasound is unreliable for length measurements. A compromise was made to choose MRI for length on the affected side, and it was then assumed that the length on the unaffected side was the same. CSA was expected to be changed between the two sides and therefore ultrasound from ultrasound isometric videos was used as the whole tendon was visualized with this probe. Length was measured on the most superficial part of apex Patellar to a hyperechoic plateau on tibia. Since not all patients (only the first 50) had MR-scans, length was measured on ultrasound. For patients for whom both MR and ultrasound data were available, the two datasets were compared with each other using IMAGEJ. Length and CSA was used for the template to calculate appropriate tendon biomechanics.
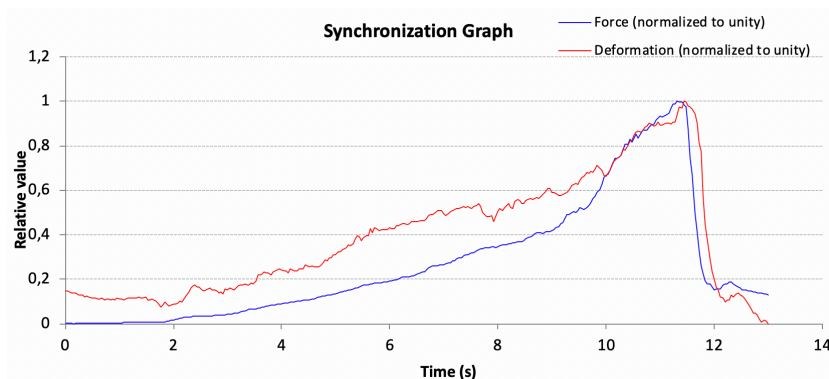
Figure 18 Force-deformation curve from ultrasound isometric. Ultrasound tracking provides data on deformation while the ankle-cuff provides data on force. The two, deformation and force, are synchronized in the Excel template.

Statistics

In study I, data was separated over AT and PT according to duration of symptoms, i.e. T1, T2 and T3 with mean ± SEM (standard error of mean) indicated in graphs and text. Patients with normal contra-lateral leg was pooled into group C while healthy controls were pooled into group HC. MRI tendon dimensions were normalized to an average of T1. US tendon dimensions, tendon mechanics and mRNA-expression were normalized to the normal contra-lateral leg (C) of patients. Gene expressions were log-transformed before statistical analysis and represented on graphs with geometric means ± back-transformed SEM. A one-way ANOVA was performed between groups T1, T2 and T3. Following a significant one-way ANOVA, individual differences between T1, T2 and T3 were tested with a post hoc test (Tukey). A paired t-test was performed between normal contra-lateral leg (C) and affected leg (T all), while an unpaired t-test was performed between healthy control participants (HC) and tendinopathic patients (T all). As a US PD area for the healthy legs was often not present, comparisons to those were performed with non-parametric equivalents to the t-test, Mann-Whitney U-test and Wilcoxon. Differences were considered significant when $P < 0.05$. All statistical analyses were performed using Graphpad Prism 8.

Study II

Study design

Study II was performed at Icahn School of Medicine, Mount Sinai in New York City, USA while most of the data analysis was done at M81, Bispebjerg Hospital, Denmark. Three different genotypes of mice were used: One more-severe type similar to MFS patients, $Fbn1^{mgR/mgR}$ (n=43), one less severe, $Fbn1^{+/-}$ (n=27), and one healthy wild-type group (n=25). For each genotype, mice were separated into either a voluntary running wheel exercise group (n=41) or served as sedentary controls (n=54) for 4 weeks (Figure 19). The running wheels were programmed to measure rotations per second and were used to calculate running kinetics. The mice had access to food and water ad libitum. Bedding was also provided to increase comfort. A veterinarian attended the mice once a day. Mice in the sedentary control groups were pooled in the same cage according to their genotype while those randomized to running wheels were single-housed. Mice were studied after 4 weeks of exercise intervention (8 weeks old). After euthanasia by carbon-dioxide, the extremities were collected for analysis. Histology was done on the AT in situ, i.e. the AT stayed connected to muscle and bone. The two back hind limbs were used for biomechanical testing with Inströn, mRNA and AFM; FDL for biomechanical testing and AT for mRNA and AFM (waiting for analysis). During the 4-week intervention some of the mice were selected for ultrasound-scanning of the aortic arch since there was a risk for aortic dissection among severe MFS-mice. Some tendons from the mice were discarded due to damages during sampling or lost data from a power cut (Figure **20**).
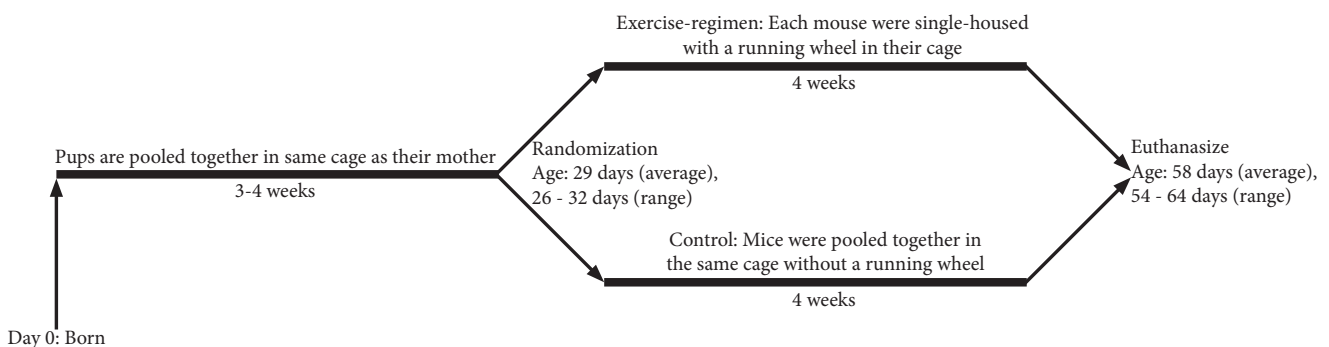
Figure 19 Flow-chart of study II on MFS mice. Tendons from two different genotypes of mice were compared to wild type after 4 weeks of aerobic exercise. Also, tendons from exercising mice were compared to control mice with no access to a running wheel. Exercising mice were single housed with a running wheel while controls were pooled together in the same cage without running wheel.
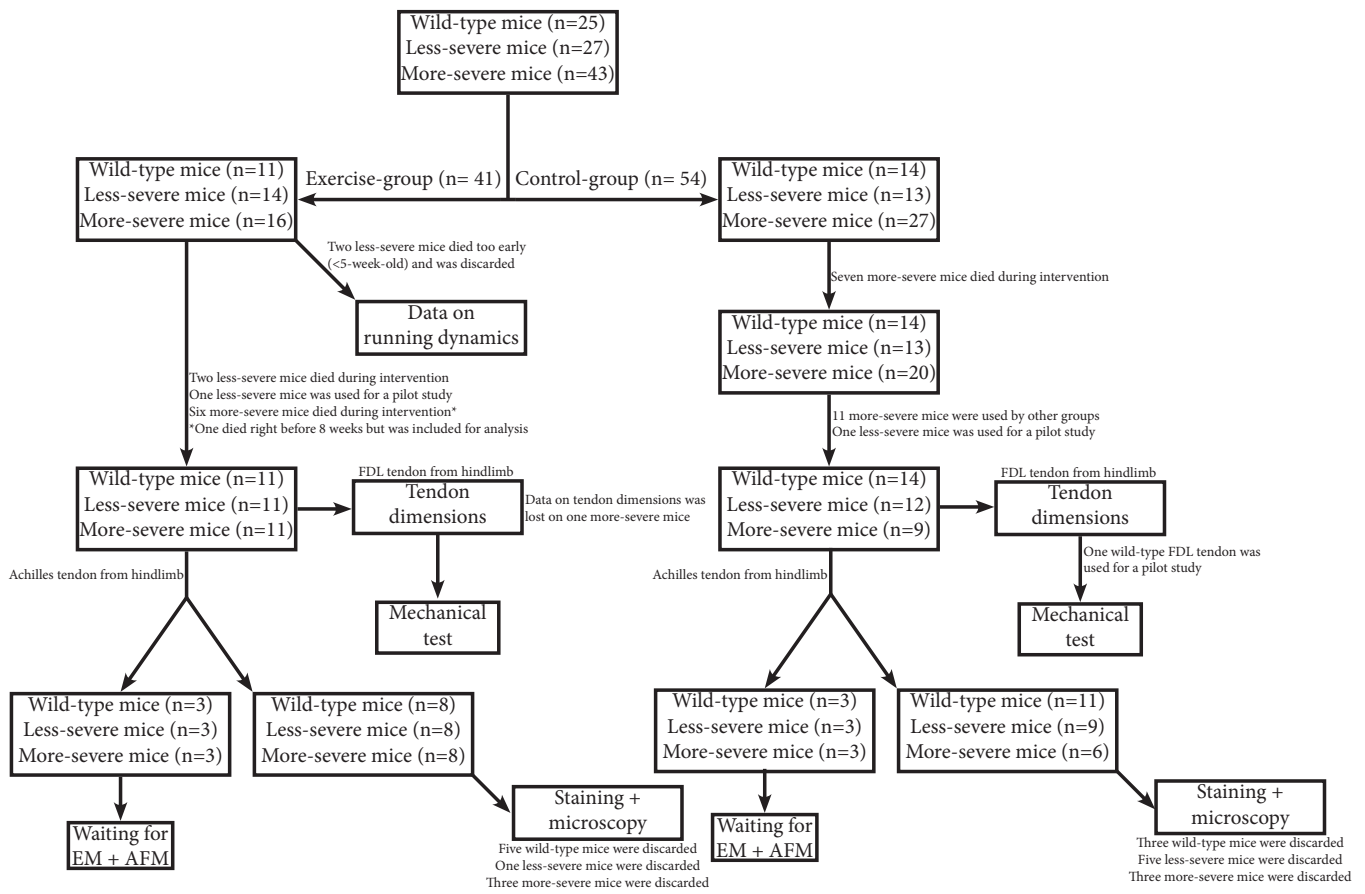
Figure 20 Overview of how the samples were treated and how many samples were available for each experiment. A noticeable number of samples were discarded during tissue preparation for picrosirius red straining (a more detailed description is provided under the method section for study II). Some mice were used for pilot studies to test different procedures and design optimal protocols.

Mice genotypes

Study II in NYC used three different genotypes of mice: One more-severe similar to MFS (Marfan Syndrome) patients, Fbn1$^{mgR/mgR}$ (n=43), one less-severe, Fbn1$^{+/-}$ (n=27) and one healthy wild-type group (n=25). The Institutional Animal Care and Use Committees of the Icahn School of Medicine at Mount Sinai in New York City reviewed and approved all animal studies. Fibrillin-1 deficiency was a global knock-out and not specifically related to tendons. Fbn1$^{+/-}$ or Fbn1$^{+/mg\Delta}$ are heterozygous hypomorphic mice with a 6kb neomycin-resistance expression cassette in exons 19-24 of the *Fbn1* gene[208]. In-frame deletion of these exons causes a fall of normal fibrillin-1 production-level to 50 %. Fbn1$^{+/mg\Delta}$ were created by injection of mgΔ-cells into blastocysts to produce a chimeric mouse and then mating it with a C57BL/6J female (Jackson Laboratory, B6(Cg)-Tyr^{c-2J}/J, stock no: 000058). The mice in this project were backcrossed for at least 5 generations into the C57B1/6J background. Fbn1$^{mgR/mgR}$ is a homozygous hypomorphic mouse that is identical to mgΔ-line but still has exon 19 intact and produces normal fibrillin-1 at 20 % capacity[209]. It must be noted that Fbn1$^{+/-}$ and Fbn1$^{mgR/mgR}$ produce normal Fibrillin-1, only the amount is smaller than in the wild-type. They are expected to have some Fibrillin-1 as the most severe, Fbn1$^{mgR/mgR}$, still has 20 % production-level and therefore elastic fibers are not completely compromised. The reasons for not using Fbn1$^{mg\Delta/mg\Delta}$ relates to their survival. Mice with Fbn1$^{mg\Delta/mg\Delta}$, 0 % production of fibrillin-1, dies perinatally and thus, were not suitable for the intervention in this project. In contrast, Fbn1$^{mgR/mgR}$ are born with normal frequency, show no phenotypic abnormalities at birth and have a 3-month median survival rate. The project used 3-4-week-old mice to run on running wheels for 4 weeks (week 4 to week 8), totaling 8 weeks of survival before euthanasia. The mice had to be at least 3-week-old for two reasons: 1) They needed to be able to support themselves without their mother, because those with running wheels would be single-housed and; 2) They needed to be old enough to learn how the running wheel works and be strong enough to keep using it. However, the mice may even be too young, but a compromise must be made because they do not survive long either. The investigators settled for 8 weeks in total as a realistic goal given the circumstances and for practical reasons. Fbn1$^{+/-}$ are sub-clinical and were not expected to show any traits similar to MFS-patients. They have a median survival rates equal to those of wild types (24 months). In contrast, Fbn1$^{mgR/mgR}$ are prone to develop severe clinical manifestations such as kyphosis, elongated musculo-skeletal system and aortic aneurysm. Most Fbn1$^{mgR/mgR}$ die because the aortic aneurysm progresses to aorta dissection and ultimately aorta rupture. A

rare cause of death is diaphragmatic hernia because of respiratory distress that leads to respiratory failure. Wild types show survival rates comparable to normal mice in cages, and these mice typically live for 24 months. They were litters of Fbn1$^{+/mg\Delta}$ and Fbn1$^{+/mgR}$. The average weight (~23 gram) did not differ between genotypes.

Running wheel

Running wheels were custom-made by Annesofie Thorup Olesen at M81, Institute of Sports Medicine, Bispebjerg Hospital. A rod connects the wheel with a plastic plate with four holes (Figure **21**). On one side of the plastic plate is a diode, and the other side has the light-receiver. Every rotation of the wheel will simultaneously rotate the plastic plate which allows light to pass through the holes. One full rotation will cause light to pass through the holes four times, making each hole a 45° degrees rotation. Since the light-receiver detects changes in light intensity there are eight signals during one rotation or two per 45° degrees rotation (light – no light). The running wheel had a diameter of 11,5 cm and was connected to a sampling box of 15x5 cm that could fit in the cage if the roof was lifted by 1 cm. It was advised not to lift the roof further, as the mice would then be able to escape the cage. However, by using this method the running wheel could rotate freely, even though there was bedding at the bottom. In some cases, the mice would gather bedding at the running wheel, but the investigators checked on the mice daily and removed any excess bedding that would hamper the running wheel. The wheels had an internal resistance of 1,45 g on average (min: 1,03 g and max: 2,00 g), and therefore the investigators chose to calibrate all the running wheels to 2,00 g in order to minimize variation. The cages were Polycarbonate regular-temperature mice cages with dimensions 257mm x 483mm x 152mm (PC10196-RT, Allentown) with wire bar lids in stainless steel (WBL 1019 MMB, Rim Rod Design, Allentown).

For every second the data would be stored on SD-cards in Notepad format. There was a single event of power-shortage that caused the SD-cards to be overwritten by new data because the running wheels were restarted. Another technical issue caused the SD-cards to stop storing new data. There seems to be a limited amount of read/write cycles for SD-cards, and since it was told to read/write every second this would amount to 2.592.000 million times for 30 days. However, this was quickly discovered by the investigators who attended the mice every day, and the SD-cards were replaced with new ones on the same day. This happened during the day

when the mice were not normally active and only a few hours of running were left unregistered. A few hours of data were lost on the seven mice, but they were included in the analysis since the power cut would have minimal effect on total running dynamics over 30 days. These data can be converted to distance and velocity: Distance = 2*PI*R*#rotations. By dividing the number of signals by eight to arrive at rotations and using the formula above it was possible to calculate running distance. The velocity was calculated by dividing the distance by time. This was automatically calculated using a code made by Annesofie Thorup Olesen.
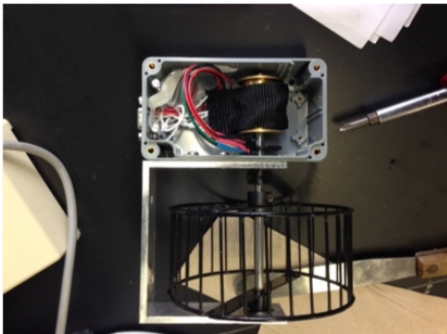
Figure 21 Running wheel. The black band was adjusted to provide minimal friction in order to achieve the same resistance on all running-wheels.

Tissue preparation

At 8 weeks the mice were euthanized by 100 % carbon-dioxide at flow 3 L/min followed by cervical dislocation. Mice that died before 8 weeks were dissected in order to determine the cause of death but were not included for analysis (Figure **20**). However, one mouse died just 2 days before being 8-week-old (lived to 54 days) and was included in final analysis. None of the wild-type mice died, but more-severe and less-severe MFS mice did. For most-severe MFS we found blood in the thorax, indicating that they died of aortic rupture. To prepare the mice for tissue analysis we removed the limbs and carefully disinfected instruments after procedures. From the right hindlimbs we dissected the AT and flexor digitorum longus tendons (FDL) free of other tissues (skin, muscle, blood, etc.) while the left hindlimbs were saved with other tissues intact for histology. Two less-severe mice were used in a pilot study to create protocols for correct staining and streamline flow-capacity of samples (Figure **20**). It was determined to use less-severe mice in the pilot study as they represented a realistic middle-ground between wild type and more-severe mice.

Biomechanical testing

FDL of mice were shipped to Denmark for biomechanical testing at M81, Bispebjerg Hospital. They tendons were stored with PBS (Phosphate Buffered Saline) at -80° C during transportation using dry -ice and were kept at the same temperature in the freezer until use for analysis. It was preferred to use FDL instead of the AT because the FDL is longer and therefore easier to test on the mechanical rig. On average, the FDL was 15 mm long (average) while the AT was only 8-10 mm long (average) and the mechanical rig required the sample to be at least 10 mm. Although they are different tendons, it was assumed that the physiological demands are more or less the same. FDLs were soaked in PBS at room temperature to quickly thaw them and then placed between two clamps. A thin piece of gauze with PBS was wrapped around the middle part of the tendon to keep the tendon moist. Tendon ends were left to dry in order to be glued on the clamps. Regular superglue was used to glue both sides of the tendon ends: One side for the clamp and the other side for a metal plate. Mechanical tests cause stress on the tendon, and therefore it was necessary to ensure that each end was properly fixed. This was achieved by super-gluing the tendon between two pieces of metal. The clamps are a part of the custom-made Deben mechanical rig (20N tensile stage, Petri dish version, Deben Ltd, Stuffolk, UK) and can be pulled or retracted by the machinery (Figure 22). This was done in PBS after the ends were tightly glued and completely dry because dehydration could affect the biomechanical features of the tendon. The Deben software was used to operate the mechanical rig in regard to the velocity of clamp-movement. The Deben software displays force on the y-axis and length on the x-axis. One loading/unloading cycle consists of pulling the clamps away from each other to a certain distance and then retracting the clamps back to their initial starting position. The testing protocol was: 6 preconditioning cycles, 10 testing cycles, finishing with a maximum test. Before the preconditioning phase, the tendon dimensions were measured and recorded using an Olympus light microscopy (20x zoom) with Leica camera attached. These measurements included diameter, thickness using a mirror prism and length. The length had to be re-measured after preconditioning, because the 6 cycles of loading and unloading had changed the tendon characteristics. Preconditioning was necessary because freezing and thawing can alter the tendon. There are three reasons for warming up the tendon before the actual testing: 1) Freezing and thawing can misalign the fibers within the tendon; 2) The tendon is elongated (slack) after the first few cycles, but the length remains constant afterwards, and 3) The zero-offset or baseline force is changed after preconditioning, meaning that a new calibration of

force was made to 0,1 N. It is uncertain why these changes occur during preconditioning, but after thorough testing it was observed that no more changes were induced after 3-4 cycles. For both preconditioning and testing cycles the investigators were determined to use a 2.5 % strain and 4 mm/second speed after trial and error in a pilot study on a wild-type mouse (Figure **20**). It was not possible to increase the speed further to mimic a tendon (< 2 mm/second) under physiological conditions due to limitations within the machinery itself. The strain was calculated by Extension (L_1)/initial length(L_0). Too much strain and the tissue would break. Too little strain and it might not be enough to see any biomechanical differences between the mice. A compromise was made based on the most-severe mice, assuming that they had the weakest tendons. The purpose was to find the maximum strain that could be tolerated by these mice before they would break. The pilot study confirmed that 2.5 % was ideal, as it allowed most tendons to last through the cycles while still applying significant stress on the tissue. If the preconditioning-phase was omitted, it would result in a slacker tendon during loading to 2.5 % strain, as the tendon was now permanently longer and therefore 2.5 % would not be relative to its current length but much less. The maximum test involved moving the clamps away from each other until the tendon would break into two separate pieces and the measured force would drop to baseline.
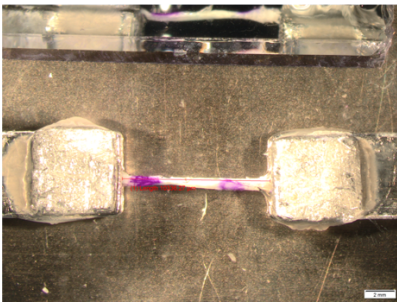
Figure 22 Setup in Deben. The two clamps holding FDL was immerged in PBS after the glue was completely dry. A side mirror was placed in order to measure the tendon diameter.

Data analysis was performed by the main investigator with the help of René Svensson. The cross-sectional area was calculated from following formula: pi*0,5*thickness*0,5*diameter. René Svensson made an Excel template to graph the stress-strain curve using force- and position-data from Deben during the mechanical test (Figure 23). Since stress on the y-axis is given by Force/CSA it was necessary to use the calculated CSA to calibrate the y-axis. Obtaining normalized stress-strain graphs for each mouse made it possible to calculate biomechanical properties which could then be compared across genotypes and training groups. There are

mainly two types of properties: Intensive and extensive. Intensive are independent of dimensions and include: Max stress, strain at max stress, max modulus, stress at max modulus, strain at max modulus, yield stress, yield strain, energy density at yield, energy density at failure and energy density total. In contrast, extensive properties are dependent on material properties: Length at onset, max force, deformation at max force, max stiffness, force at max stiffness, deformation at max stiffness, yield force, yield deformation, energy at yield, energy at failure and energy total. Thus, intensive properties are the normalized values, in respect to tendon dimensions, of extensive properties. Although not an exhaustive list, the investigators chose to focus only on relevant properties that could be meaningful to the athlete: Tendon dimensions, max force, max stiffness and max modulus. Lastly, the hysteresis was calculated from repeated cycles and defined as how much energy is lost in the system during those consecutive cycles. This was calculated by the area under the loading-curve subtracted by the area under the unloading curve. It was sometimes difficult to calculate hysteresis if the graphs for each cycle did not overlap. Typically, this problem arose due to technical errors in the equipment or the software. The investigators tried to include as many cycles as possible, aiming for the maximum of 10 cycles without compromising the quality of data. This would be a compromise between the quantity of cycles versus the quality of data (overlapping graphs). However, it was possible to achieve data from at least 5 overlapping cycles for every tendon and this would be sufficient for calculating hysteresis. Given that the graphs overlap each cycle, the difference in area between the loading and unloading curves is the energy lost in the system, for instance as thermic energy (heat). Hysteresis is relevant to the athletes as well since their tendons can be under tremendous loading/unloading cycles during training. Hysteresis was calculated using the same template by René Svensson and is given by Kj/m^3.
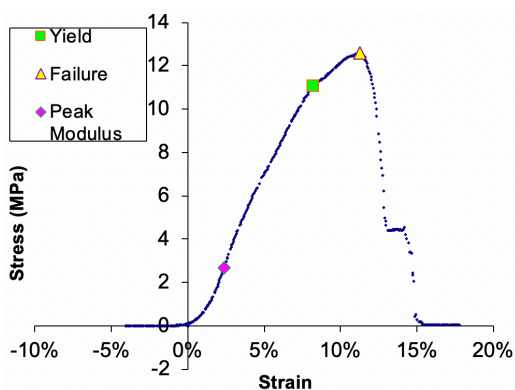

Figure 23 Stress-strain curve obtained from Deben data.

Preparation of tendon tissue

The AT were prepared for histology at Icahn School of Medicine, Mount Sinai, USA. The samples were later shipped to Denmark for analysis. Three protocols were used: Formalin was used for tissue fixation, EDTA to decalcify the bones and paraffin to embed the tissue. Formalin-fixation was performed according to protocol and uses the technique of immersion fixation with neutral buffered formalin. Whole hindlimb from mice was used for formalin fixation and contains foot, ankle, tibia, fibula and femur with all connective tissues intact. The samples were fixed in 4 % paraformaldehyde for 48 hours at 4°C to prevent the tissue from decaying, but the process also masks epitopes. Previous experience from the department had shown that it was possible to preserve the tissue without masking epitopes too much using this protocol[210]. Even though this project uses different antibodies than what the lab had previously worked with, it was still relevant to use the same protocol given their experience with the tissue in general. It was decided to prepare the Achilles tendon *in situ,* meaning that the tendon would still be attached to the muscles and heel-bone. This way of preserving the tissue provides important landmarks during microscopy as the heel bone and muscles are easier to stain. Another important feature is the preservation of the AT's fibers between calf muscle and heel bone in a natural way. However, this approach requires an extra step of decalcification to soften the bones enough for microtome-slicing, and that may mask epitopes even more. Decalcification is a process of extracting calcium out of bones with EDTA. The tissue is immersed in EDTA solution at pH 7.4 for several days until all the bones are soft. This process takes approximately 72 hours, but the time required varies among tissues and therefore the tissue was tested by injecting a needle into the femur bone. The investigator would feel the feedback during needle injection, and if it felt soft, the tissue was ready for paraffin embedding. Lastly, paraffin embedding involves progressive dehydration with ethanol followed by immersion in melted paraffin for 60 minutes at 65°C. The AT was placed in a vertical position with the ankle in neutral position and femur in 45° to tibia. A microtome was used for obtaining samples from paraffin blocks and was set to 2 micrometers. Setting the thickness to 2 micrometers was chosen as the middle ground regarding thickness of sections. Very thin sections are hard to cut and especially if the tissue contains bones. Even though EDTA decalcification softens the bones it is still difficult for paraffin to completely infiltrate bones, resulting in shredding during microtome-sectioning. In contrast, thick sections are easier to obtain but more difficult to stain due to masking of epitomes. Sections obtained from the microtome were transferred to a 37°C water bath to be

sampled on glass slides (Superfrost, Thermo Fisher Scientific) and then left to dry at 32°C. It was necessary to place the sections into water baths for two reasons: 1) The surface tension of water was used to unfold and stretch out the sections, and 2) It was easier to collect the sections with glass slides once they were floating on the water. There were 2-4 sections on each glass slide and 30–35 glass slides for every hind limb. One major challenge was to obtain intact sections that were not shredded during microtome handling. A few times, the investigators would hear an odd sound while cutting the sections, which may indicate bones that were not fully decalcified. It is worth noting that the sections would then shred into strips when approaching tibia. Why some samples were tougher to decalcify than others remain unclear. Another critical step in histology preparation was to transfer the sections to the water bath as it involved using two non-toothed forceps with steady but quick movement. If the pace was too slow, the paraffin slides would melt on the forceps. Dry ice was used to cool the paraffin blocks during sectioning and freezing the tips of forceps for better handling of sections. Despite efforts to minimize loss, it was estimated that around half of the sections were lost during this process. Thus, preparation of tendon tissue proved to be extremely difficult and therefore many samples were unfortunately discarded (Figure **20**). Either there were no slides to stain with picrosirius red or the investigators were unable to identify any relevant structures under the microscopy.

Histological staining

Histology was performed at Icahn School of Medicine Mount Sinai, USA. Three different stains were used for paraffin histology: HE (hematoxylin and eosin), picrosirius red and Alcian Blue. Histology was performed according to protocol. HE was combined with alcian blue as alcian blue HE while staining with picrosirius red was done separately. Alcian blue HE stains three things: 1) Hematoxylin stains nuclei/purple; 2) Eosin stains cytoplasm red, and 3) Alcian Blue stains proteoglycans blue. There are more resident cells in tendinopathic tissue and their shape changes from spindle shape to be more rounded. Proteoglycans are increased as well, probably due to more stress on the tissue and therefore reflect ongoing adaptation to mimic a cartilage phenotype. Staining with hematoxylin makes it easier to count the number of cells, while eosin provides information about morphology. Staining with alcian blue for proteoglycans would reveal proteoglycans in the tendon but was also expected to stain cartilage and bones. The main focus was tendons, but bones were assumed to be an important landmark because identifying calcaneus would help locate the Achilles tendon. The investigators tried alcian blue HE on sagittal and transverse sections, but the staining produced odd colors: Cell nuclei were difficult to spot, cytoplasm stained blue and the tendon tissue became purple. Bones and cartilage were correctly identified by blue color with large cells. It was difficult for the investigators to distinguish tendon tissue from skin because of a predominantly purple color in both tissues. At transverse sections the tendon was stained blue and infiltrated by large cells, indicating the insertion of Achilles tendon to calcaneus. This part of the Achilles tendon is characterized as a fibrocartilage-like phenotype. However, despite multiple protocols to optimize the alcian blue HE protocol, there was no improvement observed and the investigators ultimately decided to not proceed further. Problems with alcian blue HE staining was rooted back to three possible problems: 1) Some batches of staining might have been exposed to light, have been contaminated or be too old, although alcian blue seemed to have worked based on the color of the cartilage; 2) EDTA decalcification can complicate the staining procedure by masking the tissue, and 3) HE are well-tested basic stains, but can be altered when combined with alcian blue. The investigators attempted to stain without alcian Blue, and the odd colors persisted, indicating that the problematic batch was probably HE. Histology was performed at the end of the investigators' stay in New York, and therefore it was not possible to wait for a new batch of HE to arrive. The investigators managed to stain all genotypes in the two groups, both sagittal and transverse section. It was decided to save the remaining slides for

future staining. Picrosirius red (Direct red 80, CAT#365548, Sigma-Aldrich) was used to stain sections for visualization of collagen fibers and was performed after standard protocol [211–213]. In contrast to alcian blue HE, picrosirius red proved to be successful on all samples. The sections were deparaffinated with xylene two times before hydration in ethanol of decreasing concentration. Afterwards sections were left to stain in picrosirius red working solution (i.e., 1 mg Sirius red added to 1L 1.3 % (w/v) picric acid solution in diH$_2$O) at room temperature for 1 h and then washed two times in 5 % glacial acetic acid. Sections were rehydrated with ethanol before being mounted with Cytoseal® and coverslips. At CFIM (Core Facility for Integrated Microscopy, Panum) a brightfield microscope equipped for polarization microscopy (Axio Lab.A1, Zeiss, Germany) was used for visualization of particularly two colors under 20x zoom (20x/0.45 Pol, N-Achroplan, 0.227 7µm/pi, Zeiss, Germany): Red for mature collagen fibers and green for unmatured collagen fibers. The stage of the microscopy was manually rotated in incremental degrees of 10° from 0° - 180° to observe changes in color intensity, and pictures were then taken for every 10° using a mounted 6Mpx camera (AxioCam 506, Zeiss, Germany). Pictures were taken with the software ZEN Lite (Zen Lite 2.3, Windows, Zeiss) with the following configurations: Camera acquisition ROI size 2048 and offset 352 80. Exposure set to 20.000 ms with 10 cycles (time series). Data-analysis of the pictures was done in Fiji ImageJ2 (2.0.0-rc-69/1.52i, macOS) by measuring the color intensity of red and green. An intensive red light was observable for fibers that were aligned under polarized light, while fibers arranged 90° to the light would become dim. Rotating from 0° - 360° would vary the light intensity of the sample and therefore samples could be plotted as a sinus-curve with 2π period. Maximum light intensity is given value 1 or -1 on the y-axis while 0 is for minimum light intensity (dim). Given that every periodic π (180°) would give the same signal as 0°, $\pi/2$ would reveal maximum light intensity, and the percentage of light intensity could be measured as relative to 2π. This takes into account the fact that an absolute lack of light or dimness is not achievable because some fibers will always be aligned to a given angle. An absolute 100 % light intensity is an expression of completely unreflective light passing through the sample and is impossible to achieve due to some fibers being out of alignment.

Statistics

In study II, survival rates of mice in the exercise and control groups within genotypes was analyzed using a Mantel-Cox test (logrank test). Running data, distance and velocity were analyzed using a one-way ANOVA. For other data a two-way ANOVA was performed to determine any effects of genotype, groups and their interaction with tendon composition, dimensions and mechanical properties. If the two-way ANOVA was significant, individual differences within genotype and groups were tested with a post hoc test (Tukey). Statistical analysis was performed in Prism (GraphPad, Prism 8.0.1, MacOS), and in all cases, a p-value less than 0.05 was considered significant. Data are presented as mean ± standard error of the mean unless otherwise stated in the text under the figure or table.

Results

Study I

The results from study I and study II are meant to complement each other. Study I demonstrate increased anterior-posterior thickness and vascularization but normal biomechanical properties of tendons during early tendinopathy.

Demographic data

The study included 200 patients with soreness in either the Achilles (n=155) or PT (n=45). In addition, 50 healthy controls were included with no soreness in the respective tendons (Table 2). Patients were grouped into T all (45.8±1.6 days (mean and SEM)), T1 (17.3±1.0 days), T2 (43.9±1.1 days) and T3 (70.6±1.0 days) according to their duration of symptoms measured in days. Patients were predominantly males (Achilles: 110 males and 45 females; Patellar: 30 males and 15 females) (Figure 24). 12 % of Achilles patients, 22 % of Patellar patients and 14.5 % of healthy controls had suffered an ongoing infection within three weeks before inclusion. Their working hours involved minimum physical activity as the majority were students or worked in an office-based setting. In general, participants were in good health with no ongoing diseases or infection. Patients were significantly older (age: 37.99±0.75 years (mean and SEM)), and had higher BMI (BMI: 24.18±0.20 kg/m^2) than healthy controls (age: 30.58±1.09 years, BMI: 21.12±0.39 kg/m2) but had the same training experience (Table 3). Within the three variables to estimate training-load (duration, frequency and intensity), only frequency was significantly higher in patients, with 5 sessions per week compared to 4 sessions per week for healthy controls. Training intensity was on average 15 for all participants given by the Borg scale, ranging from 7 (very, very light) – 20 (very, very hard). Participants provided the investigators with an average training intensity program for their whole week despite the fact that intensity did vary from session to session. Sports-activities ranged broadly from diving to team sports, but most participants were predominantly runners. Participants with running as their primary sport did 15 - 35 kilometers per week and ran most of their sessions on asphalt. The majority of patients increased their training-load within three months of inclusion compared to healthy controls (Figure 25). Participants increased their training-load by spending more time on each session, followed by intensity and then frequency (Figure 25).

	Tendinopathic patients (n=200)		Healthy control persons (n=50)
	Tendinopathic tendon	**Contra-lateral healthy tendon**	**Healthy tendon**
Achilles tendon (n=155)			
US	n=155[a] (T1: 42, T2: 52 & T3: 61)	n=118[a] (T1: 32, T2: 41 & T3: 45)	n=35
MRI	n=123 (T1: 31, T2: 41 & T3: 51)		
Patellar tendon (n=45)			
US	n=45[a] (T1: 14, T2: 17 & T3: 14)	n=34[a] (T1: 11, T2: 12 & T3: 11)	n=15
MRI	n=34 (T1: 9, T2: 13 & T3: 12)		
Mechanical test	n=39 (T1: 11, T2: 16 & T3: 12)	n=28[a] (T1: 8, T2: 11 & T3: 9)	
Biopsy	n=39 (T1: 11, T2: 16 & T3: 12)	n=29[a] (T1: 9, T2: 11 & T3: 9)	

Table 2 Overview of tendinopathic patients and healthy control persons. In total, 250 participants were included in the study. Patients with Achilles or Patellar underwent US and MRI investigations. In addition, patients with Patellar tendinopathy had tendon biopsies sampled and mechanical properties examined. Healthy control participants underwent examination of their tendons bilaterally.

[a]In 37 patients with Achilles tendinopathy (10 in month one, 11 in month two and 16 in month three) and in 11 patients with Patellar tendinopathy (3 in month one, 5 in month two and 3 in month three) bilateral symptoms were present, thus excluding the use of the contra-lateral tendon as healthy control participants. Patients with bilateral symptoms were examined only with US PD and MRI.
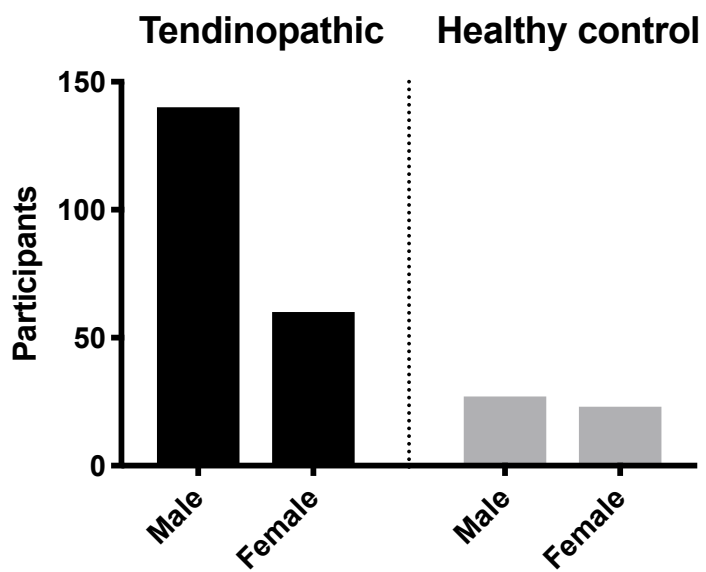
Figure 24 Distribution of gender in Achilles (110 males and 45 females) patients, Patellar (30 males and 15 females) patients and healthy controls (27 males and 23 females). Achilles and Patellar patients are pooled together under Tendinopathic.

Overall tendinopathic (T all, n=200)	Mean	Min	Max	SEM
Age (years)	37.99*	18	66	0.7583
BMI (kg/m^2)	24.18*	17.51	37.88	0.2096
Training experience (years)	10.15	0.500	50.00	0.7542
Training duration (hours/session)	7.46	0	30	0.3038
Training frequency (sessions/week)	5.50*	1.00	22.00	0.1893
Training intensity (Borg: 7 – 20)	15.50	12.00	19.00	0.1010
Healthy control (HC, n=50)	**Mean**	**Min**	**Max**	**SEM**
Age (years)	30.58*	22	53	1.093
BMI (kg/m^2)	21.12*	16.47	27.63	0.3938
Training experience (years)	7.25	1.00	20.00	0.9211
Training duration (hours/session)	6.48	1.00	13.00	0.4567
Training frequency (sessions/week)	4.42*	1.00	8.00	0.2576
Training intensity (Borg: 7 – 20)	15.26	12.00	18.00	0.2046

Table 3 Basic information. Mean, SD: Standard deviation, Min: Minimum, Max: Maximum, and SEM: Standard error of mean. *P < 0.05.
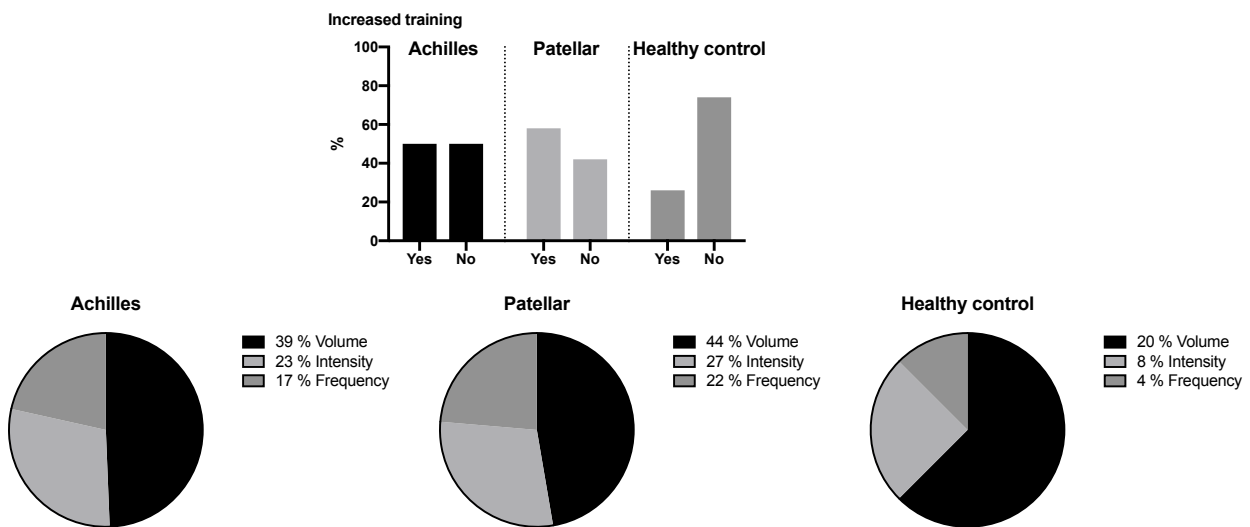
Figure 25 Increased training. Participants were asked if they had increased their training recently within three months of inclusion. Those who increased their training were further asked how they did so based on volume (duration of each training session), training intensity (average Borg-scale of each training session) and training frequency (training-sessions per week). Results are means ± SEM.

Clinical symptoms

Achilles and Patellar patients had similar severity upon inclusion, measured on NRS as average pain throughout the day (Figure 26). Most patients reported problems in the mid-portion of their Achilles (4.19±0.19 cm (mean and SEM)) or Patellar tendon (3.21±1.07 cm) measured as distance from calcaneus or tuberositas tibiae, respectively (Figure 27). Patients frequently had other problems than their presenting complaint. Achilles patients mostly had knee problems while patellar patients had problems with their SIJ (sacroiliac joint). Healthy controls were relatively symptom-free. In patients, pain level measured on NRS was significantly higher during training (Achilles: 4.71±0.19 and Patellar: 4.69±0.32) and right after training (Achilles: 3.69±0.23 and Patellar: 4.16±0.44) compared to rest (Achilles: 2.48±0.22 and Patellar: 2.03±0.37) (Figure 28). When split into individual groups (T1, T2 and T3) both Achilles and Patellar patients reported a similar pain level measured as NRS on average during training and right after training (Figure 29, A-D). Only NRS at rest was significantly lower in T1 compared to T2 for Patellar patients (Figure 29, F). Progression of average symptoms from onset to present did differ by T2 having significantly fewer symptoms than T3 at day 44 (Figure 30). The mean VISA score was 70.29±1.11 points (of 100) and was similar in all three groups (Figure 31).
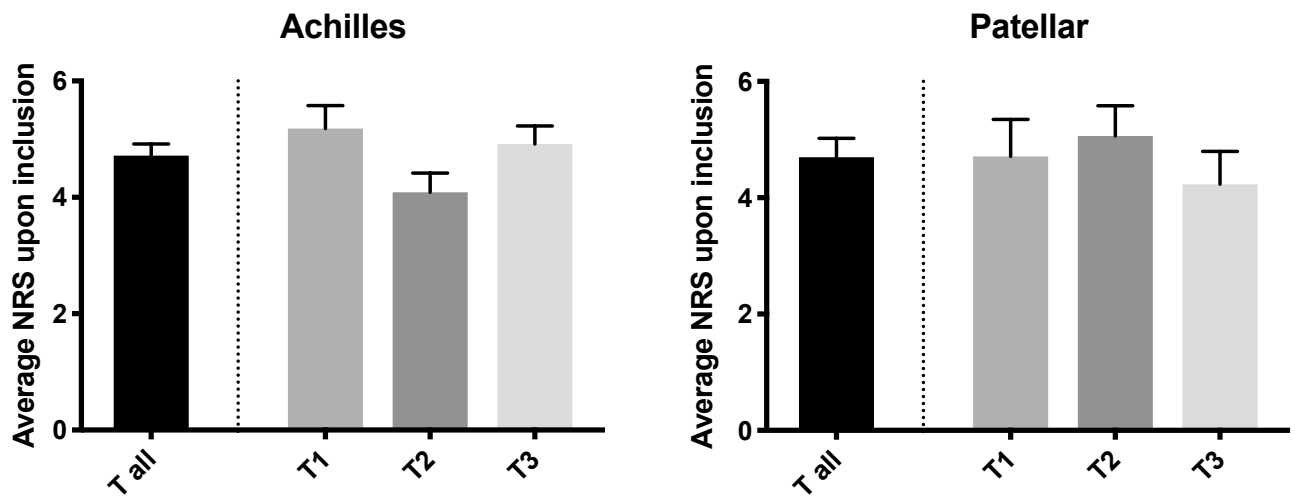
Figure 26 Subjective tendon pain score for Achilles (n=145) and Patellar patients (n=43). Average Numeric Rating Scale (NRS) for pain throughout the day was recorded for each patient (12 patients did not report NRS upon inclusion) during their first visit at the outpatient clinic. Severity of symptoms did not differ between the three groups (p > 0.05). Results are means ± SEM.
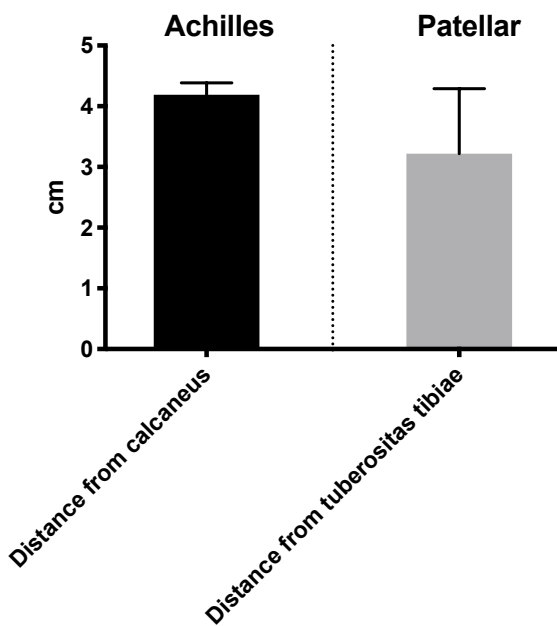
Figure 27 Maximum soreness in tendinopathic patients. Distance was measured from calcaneus (Achilles) or tuberositas tibiae (Patellar) to point of maximum soreness reported by the patient. Maximum soreness was found using bi-digital palpation along the uniaxial line of the tendon while receiving feedback from the patient. Results are means ± SEM.
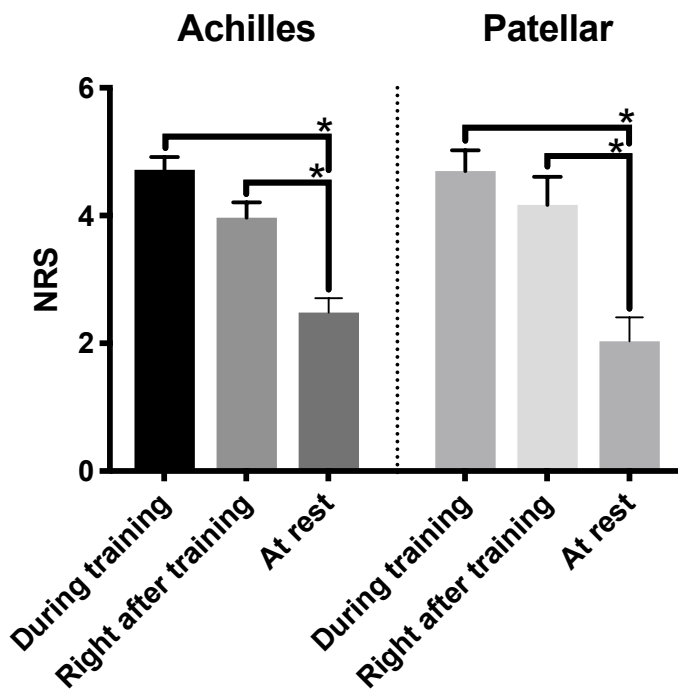
Figure 28 Subjective tendon pain score for Achilles (n=145) and Patellar patients (n=43) on NRS. All three groups were included for Achilles and Patellar patients in three situations: During training, right after training and at rest. *P < 0.05. Results are means ± SEM.
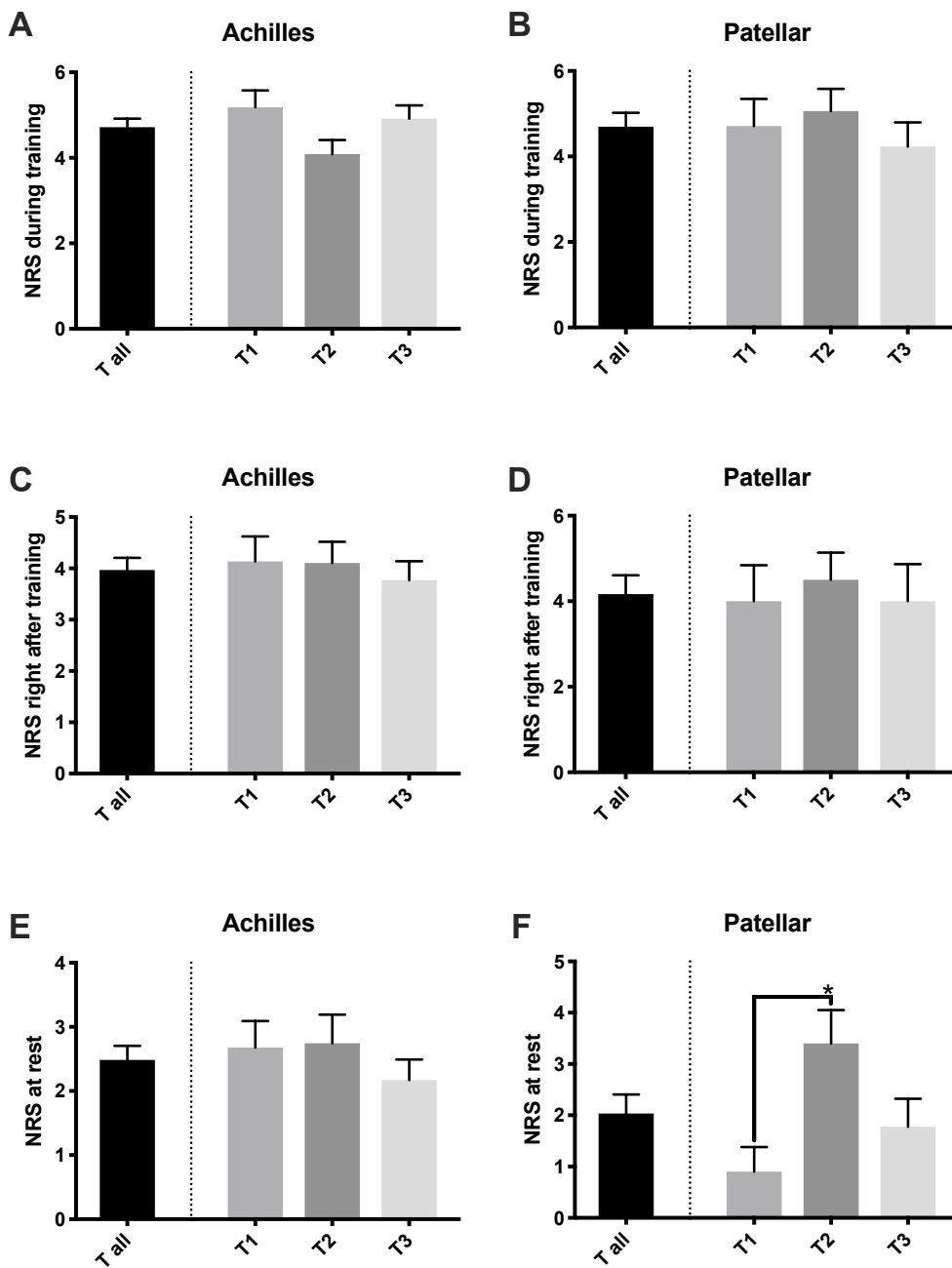
Figure 29 Subjective tendon pain score for Achilles (n=145) and Patellar patients (n=43). Patients were asked to rate their average pain level during training (A-B), right after training (C-D) and at rest (E-F) on the Numeric Rating Scale (NRS). *P < 0.05. Results are means ± SEM.
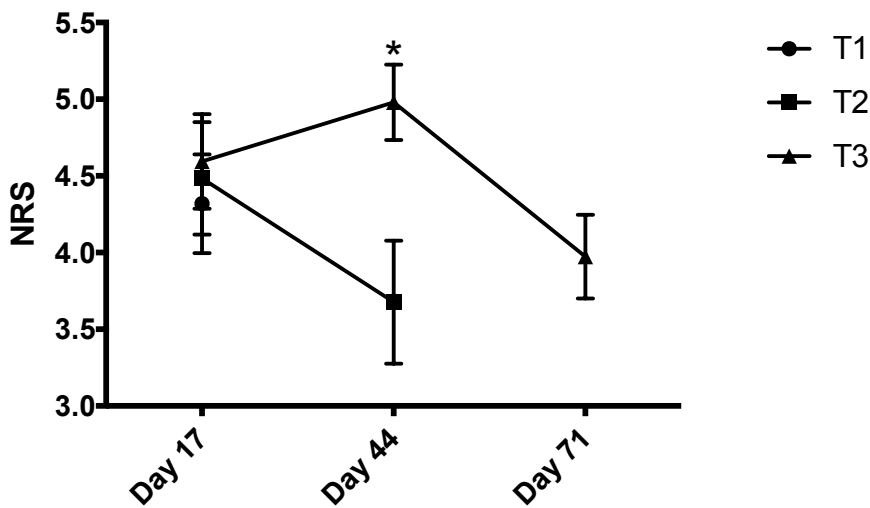
Figure 30 Progression of NRS for Achilles (n=145) and Patellar patients (n=41). Patients were asked to graph their average NRS throughout the day from onset of first symptom to present at their first visit in the out-patient clinic. Patients were grouped according to their duration of symptoms in either T1 (0 - 1 month), T2 (1 - 2 months) and T3 (2 - 3 months). The average duration of symptoms (x-axis) within T1, T2 and T3 were 17 days, 44 days and 71 days, respectively. Only patients in their respective groups (T1, T2 and T3) were used to calculate the average duration of symptoms within the same group. Average NRS (y-axis) for day 17 contained data from T1, T2 and T3 while day 44 used data from T2 and T3. Lastly, average NRS for day 71 used only data from T3. *P < 0.05. Results are means ± SEM.
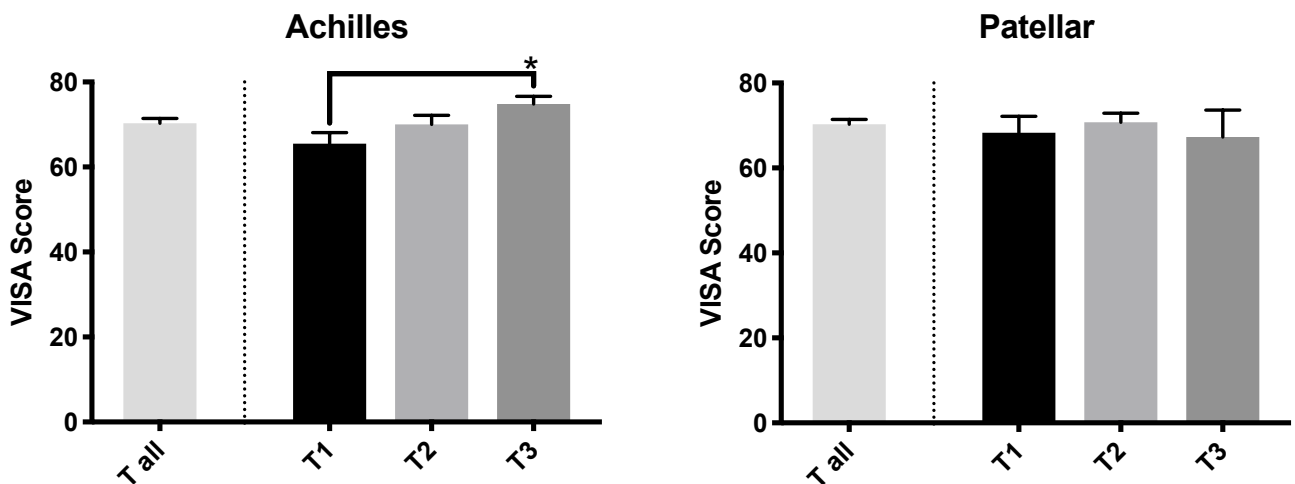
Figure 31 VISA scores for Achilles (n=145) and Patellar patients (n=43). VISA questionnaires were filled in by each patient during their first visit at the outpatient clinic. Points are given from 0 - 100 (where 100 represents an asymptomatic, fully performing individual). *P < 0.05. Results are means ± SEM.

Tendon morphology and hyper-vascularization

Ultrasound-scanning was used to determine tendon dimensions on participants (Figure 32 and Figure 33). Tendon cross-sectional area (CSA) was larger in Achilles tendinopathy patients overall (T all) compared to both contra-lateral healthy tendons (C) and tendons of healthy control individuals (HC) (Figure 32, A). A similar result was observed in Patellar patients when CSA was compared to C (except for T2), but Patellar patients did not have larger CSA compared to HC (Figure 33, A). In addition, there was an effect of time over T1, T2 and T3 in CSA of Patellar patients. CSA measured by MRI did not show any clear difference between groups (T1, T2 and T3) in early tendinopathy development for neither Achilles nor Patellar tendon (Figure 32, A and Figure 33, A). In both Achilles and Patellar patients there was an increase in tendon anterior-posterior (A-P) thickness determined by US in T all compared to HC (Figure 32, C and Figure 33, C). Thus, early tendinopathy does increase A-P thickness, although no difference was found over time between groups T1, T2 and T3. A-P thickness was larger than C in Achilles patients already at T1, whereas this occurred late in Patellar patients as only T3 was larger than C. However, T2 in Achilles patients was no larger than C. No difference between subgroups T1, T2 and T3 was found by MRI for Achilles or Patellar patients (Figure 32 and Figure 33). Interestingly, tendon width determined by US was elevated in T3 compared to C of both Achilles and Patellar patients. Overall width in tendinopathy (T all) was not elevated compared to HC in Patellar patients, while the width of tendinopathy was elevated in Achilles patients (Figure 32, E and Figure 33, E). Also, T all in Achilles patients was elevated compared to C, but this was not observed for Patellar patients. Tendon width/A-P thickness ratio remained unchanged (Figure 34). Measurements of CSA, A-P thickness and width in MRI was significantly larger than US (Figure 35). No correlation was observed between CSA and anthropometric measurements of participants such as weight, height and BMI (Figure 36). In addition, max force in Patellar patients did not correlate with CSA. Hyper-vascularized areas determined by US PD of tendinopathic tendons (all three groups of both Achilles and patellar tendons pooled) were always larger than the ones observed in contra-lateral control tendons (Figure 37).

Overall there was a significant increase in CSA, A-P thickness, width and US PD when compared to HC and C, but not between tendinopathic groups.

Achilles tendon
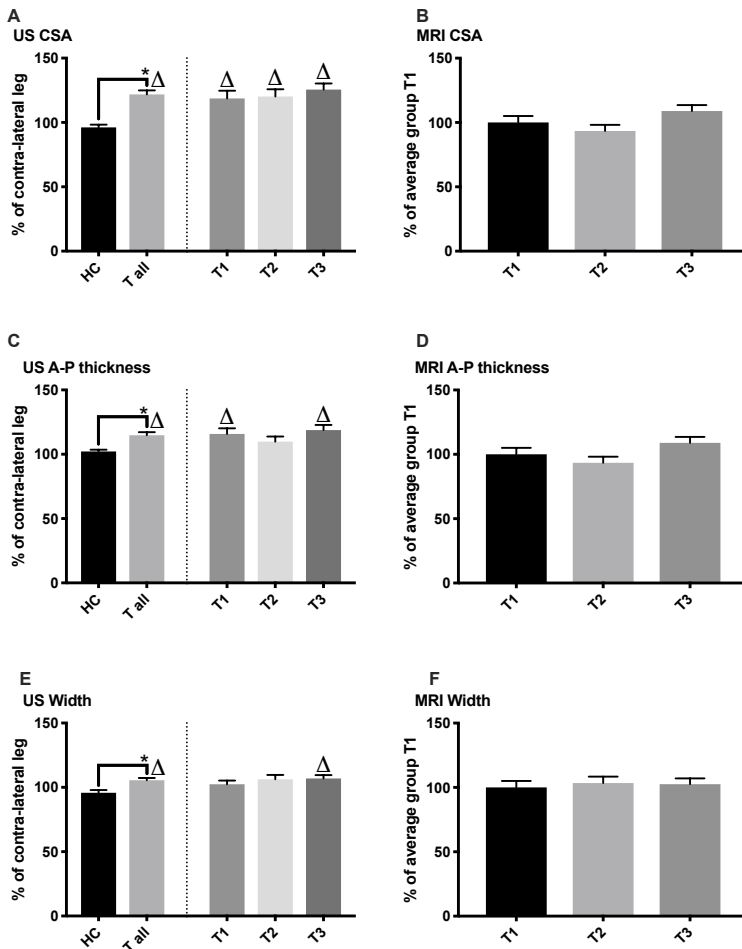
Figure 32 Tendon dimensions of Achilles tendinopathy patients with unilateral symptoms and healthy control participants (n=35). Ultrasound-scanning (US) was performed on both legs (n=155). The affected leg was then normalized to contra-lateral leg within each patient (A, C, E). Healthy controls were measured on the mid-portion of their tendon and then normalized to the same anatomical level on their contra-lateral leg. MRI was performed unilaterally on each patient (n=123, bilateral symptoms included) and normalized to an average of T1 (n=31; CSA: 89.28 mm^2; A-P Thickness: 6.76 mm, and Width: 16.08 mm). CSA (A-B), A-P thickness (anterior-posterior thickness) (C-D) and width (E-F) were measured on transverse images corresponding to the tendon area with most soreness. HC: Healthy control group. *P < 0.05 versus healthy controls and $^\Delta$P < 0.05 versus the contra-lateral leg. Results are means ± SEM.

Patellar tendon

A
US CSA

B
MRI CSA

C
US A-P thickness

D
MRI A-P thickness

E
US Width

F
MRI Width
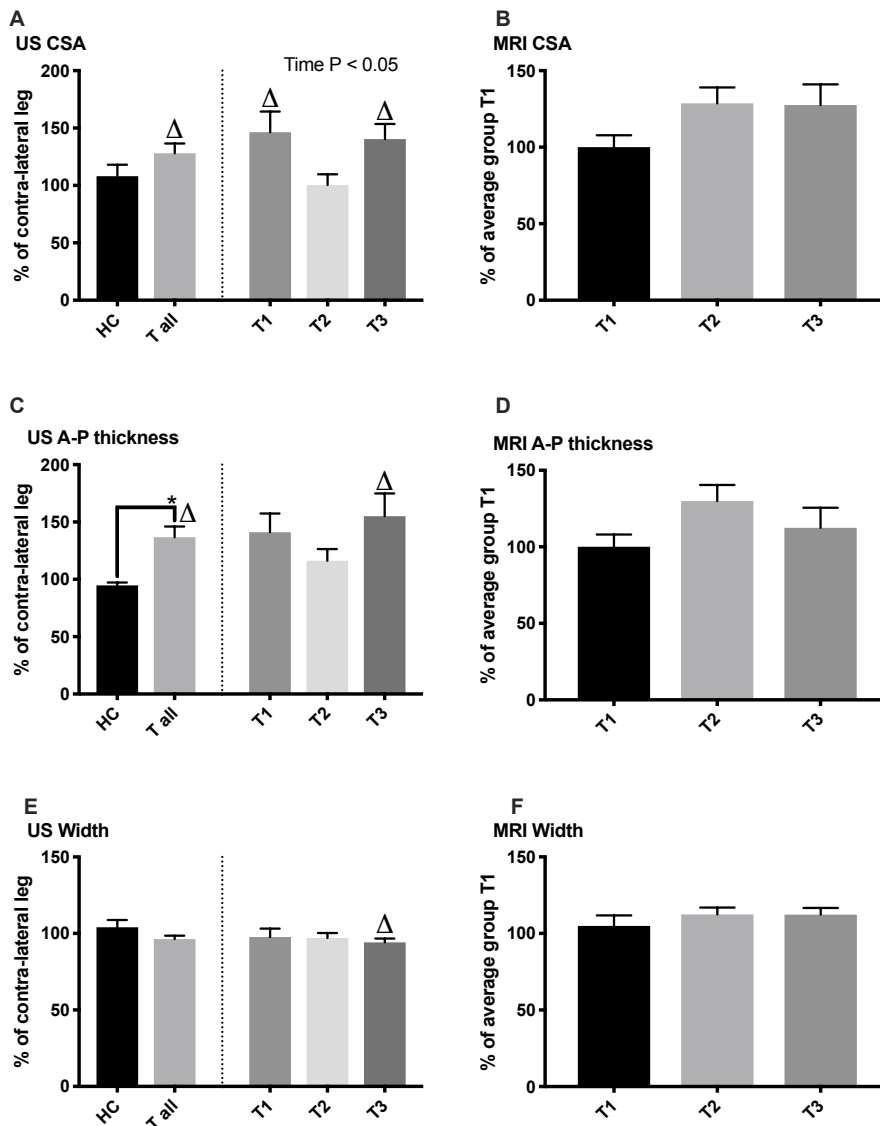
Figure 33 Tendon dimensions of patellar tendinopathy patients with unilateral symptoms and healthy control participants (n=13). Ultrasound-scanning (US) was performed on both legs (n=45). The affected leg was then normalized to contra-lateral leg within each patient. MRI was performed unilaterally on each patient (n=34, bilateral symptoms included) and normalized to an average of T1 (n=9; CSA: 90.07 mm^2; A-P Thickness: 4.35 mm and Width: 24.52 mm). CSA (A-B), A-P thickness (anterior-posterior thickness, C-D) and width (E-F) were measured on transverse images corresponding to the tendon area with most soreness. *P < 0.05 versus healthy controls and $^\Delta$P < 0.05 versus the contra-lateral leg. Results are means ± SEM.
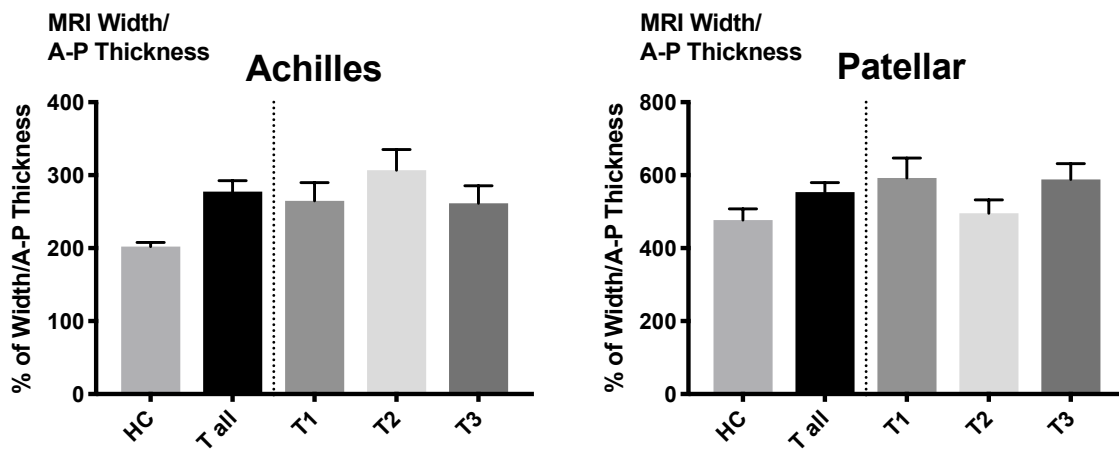
Figure 34 Tendon width/A-P Thickness ratio of Achilles patients (bilateral symptoms included), Patellar patients (bilateral symptoms included, n=34) and healthy control participants (Achilles: n=32 and Patellar: n=16). MRI was performed unilaterally on each Achilles patient, Patellar patient and healthy control. Width and A-P Thickness were measured on transverse images corresponding to the tendon area with most soreness. *P < 0.05 versus healthy controls. Results are means ± SEM.
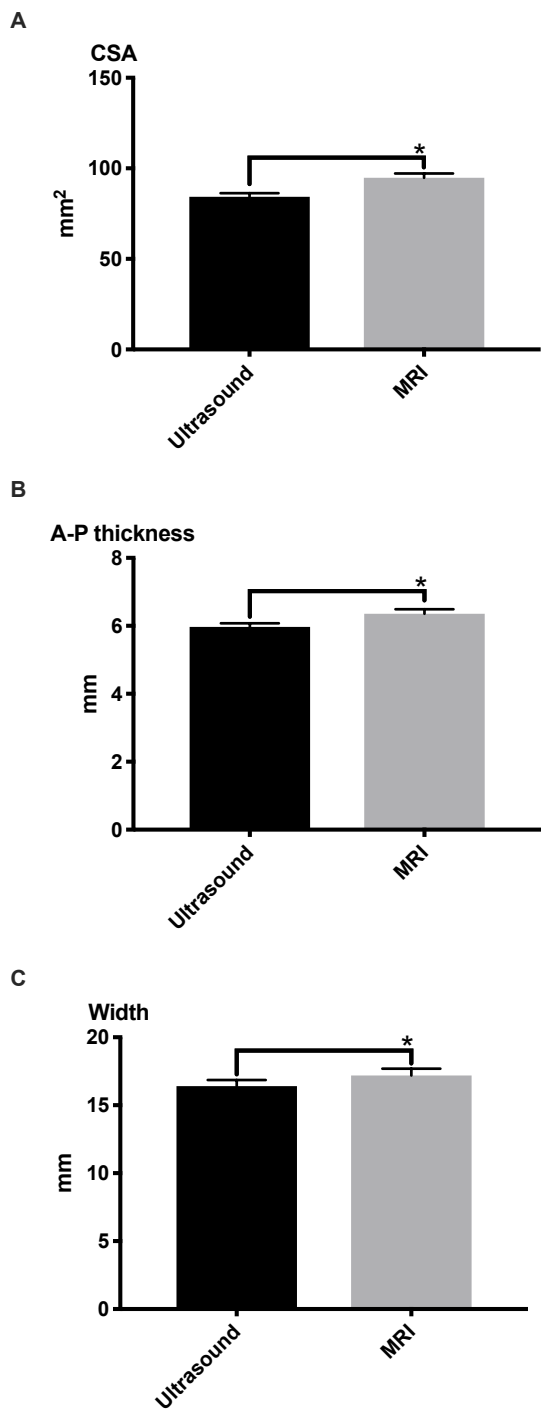
Figure 35 Difference in measurements between US (n= 235) and MRI (n= 157) in participants.
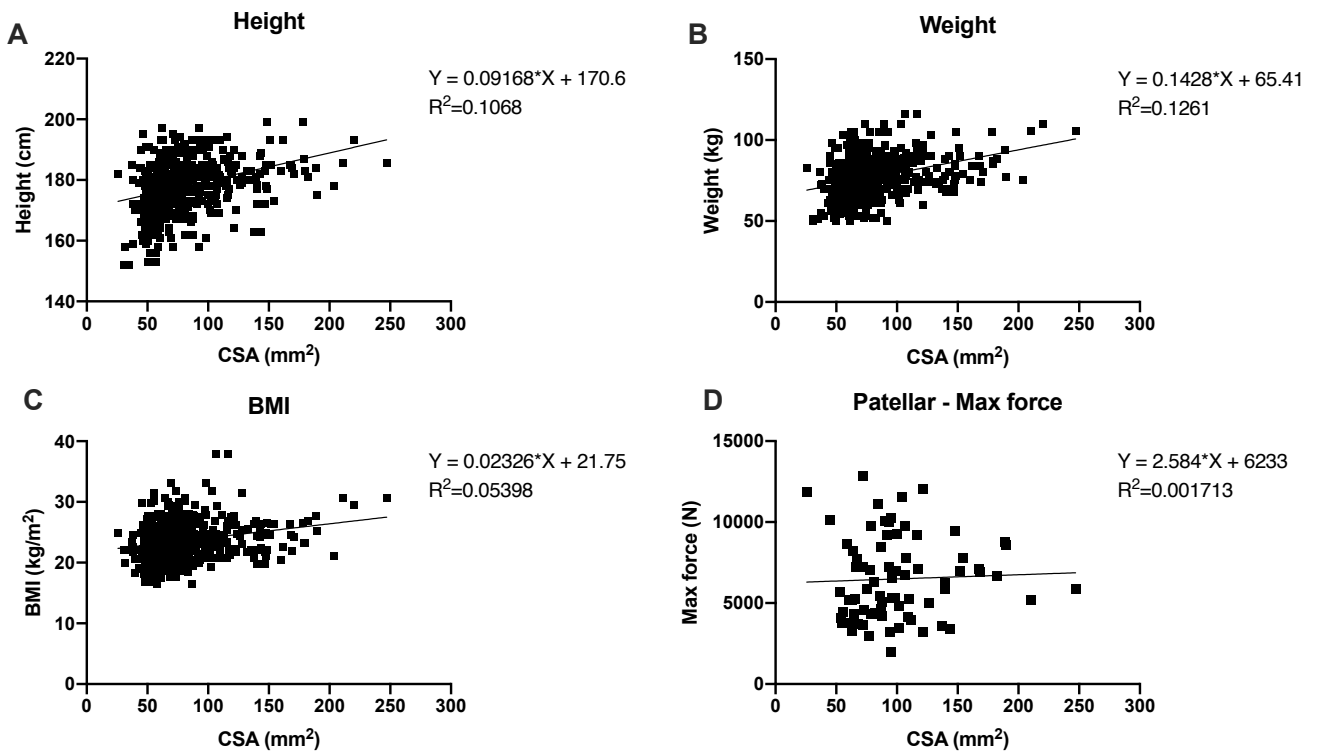
Figure 36 Correlation between CSA and anthropometric measurements in participants (n=250). Max force was measured during mechanical testing of Patellar patients (n=38). CSA was measured by US on both legs of each participant.
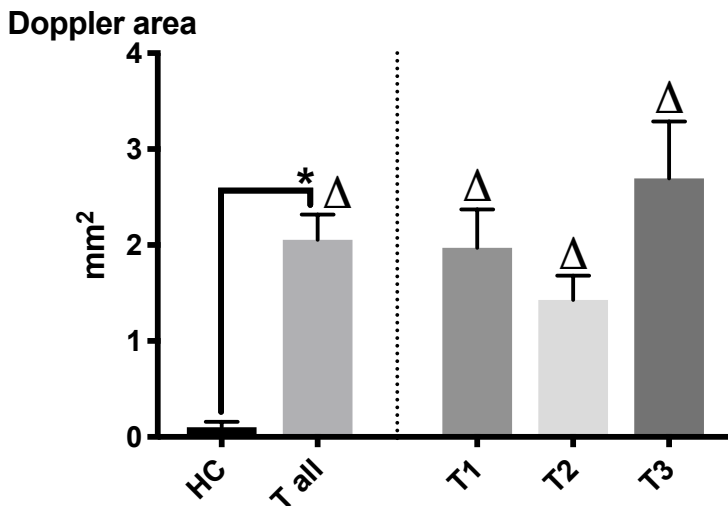
Figure 37 Ultrasound PD area on affected leg of patients (bilateral symptoms included, n=200) and normal leg of healthy control participants (n=48). US PD was visualized from the tendon area with most soreness in patients while it was visualized from the mid-portion of healthy control participants. US PD area is expressed as absolute values in cm^2. Statistically significant differences were calculated using one-way ANOVA for T1, T2, and T3 with Tukey's post hoc test. Differences between T all and HC (and between T1, T2 and T3, respectively, with HC) were examined with a Mann-Whitney U-test. A Wilcoxon was performed to compare T all, T1, T2 and T3 to their contra-lateral leg of C1, C2 and C3, respectively. *P < 0.05 versus healthy controls and $^\Delta$P < 0.05 versus the contra-lateral leg. Results are means ± SEM.

Gene expression

Biopsies taken on Patellar tendinopathy patients with unilateral symptoms demonstrated that mRNA for catabolic/anabolic signaling remained unchanged in the tissue of early tendinopathy. Only mRNA for TGF-beta 1 was significantly elevated in T3 compared to T2, and mRNA for MMP (matrix metalloproteinase) was significantly elevated in T all compared to group C (Figure 38). Further, markers of cell stress, inflammation, autophagy and angiogenesis remained unchanged in all three groups (Figure 39). Finally, the nociceptive marker substance P was significantly higher in T3 compared to T1 (Figure 39).

Tendon matrix proteins and turnover
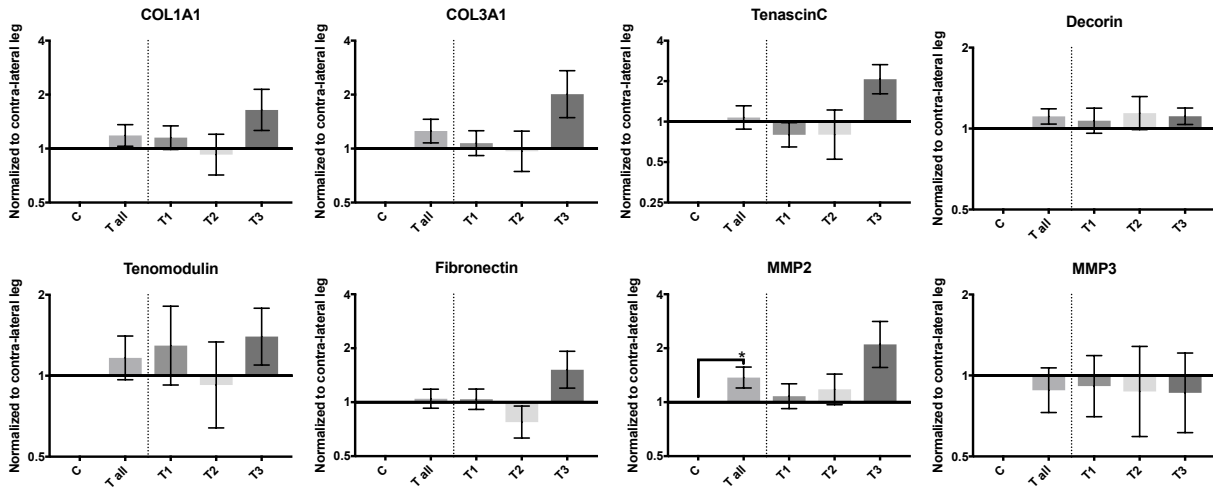
Tendon growth factors
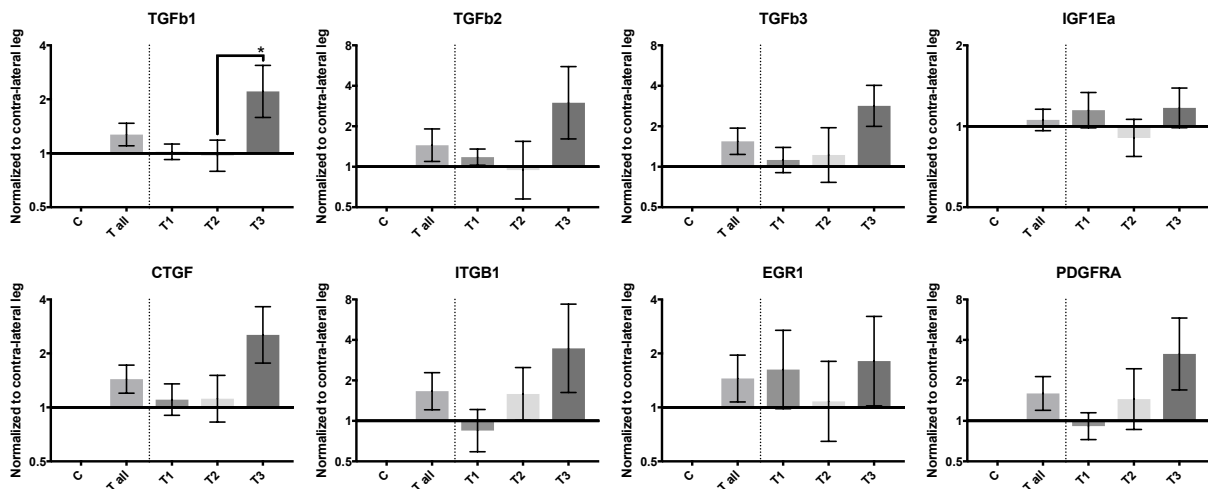
Figure 38 mRNA-expression in patellar biopsies for tendon turnover and growth. Values are normalized to normal contra-lateral leg within each patient (n=29, no patients with bilateral symptoms included). C: Normal contra-lateral leg of each patient. COL1A1: Collagen type I Alpha 1 chain. COL3A1: Collagen type 3 Alpha 1 chain. MMP: Matrix Metalloproteinase. TGFb: Transforming Growth Factor beta. IGF: Insulin-like Growth Factor. CTGF: Connective Tissue Growth Factor. ITGB1: Integrin beta-1. EGR1: Early Growth Response protein 1. PDGFRA: Platelet-Derived Growth Factor Receptor A. *P < 0.05 between indicated groups. Results are geometric means ± back-transformed SEM.

Nociception, cells stress and inflammation
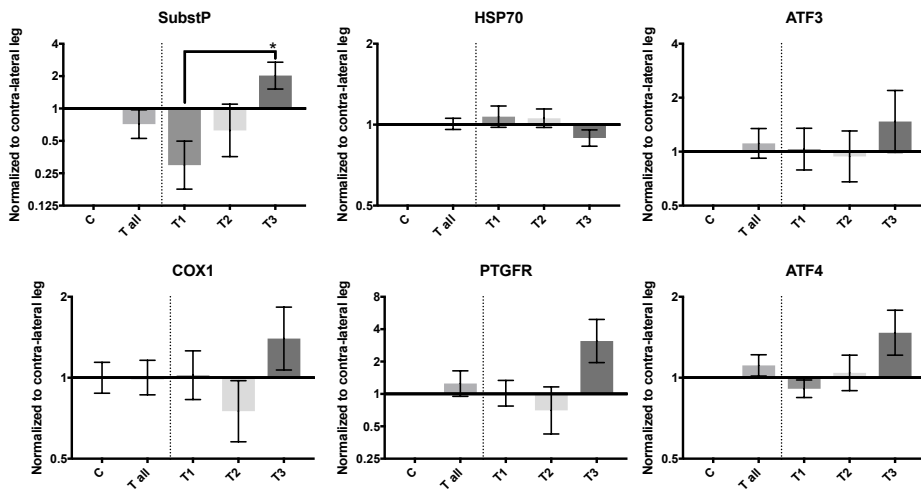
Angiogenesis and autophagy

Internal control
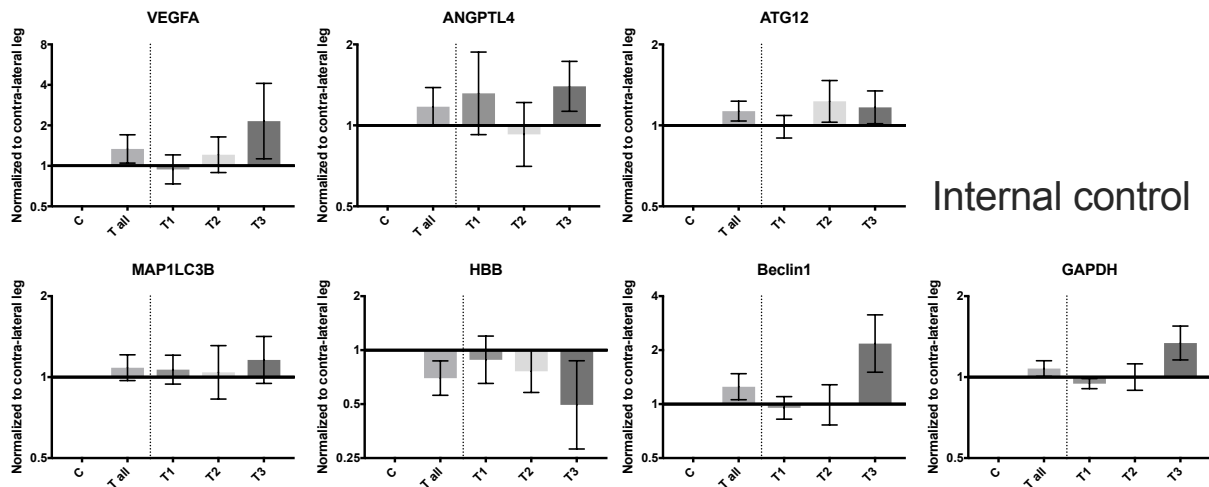
Figure 39 mRNA-expression in patellar biopsies for tendon inflammation. Values are normalized to normal contra-lateral leg (n=29, no patients with bilateral symptoms included). SubstP: Substance P. HSP70: 70 kilodalton heat shock proteins. ATF3: Activating Transcription Factor 3. COX-1: Cyclooxygenase 1. PTGFR: Prostaglandin F Receptor. VEGFA: Vascular Endothelial Growth Factor A. ANGPTL4: Angiopoietin Like 4. ATG12: Autophagy Related 12. MAP1LC3B: Microtubule Associated Protein 1 Light Chain 3 Beta. HBB: Hemoglobin subunit beta. *P < 0.05 between indicated groups. Results are geometric means ± back-transformed SEM.

Patellar tendon biomechanical properties

Biomechanical properties measured at max force revealed that tendon stiffness remained completely unchanged in the affected tendon compared to the contra-lateral healthy tendon in all groups (Figure 40, A). Absolute values for modulus was significantly different in T all and T3 compared to values in normal healthy contra-lateral leg, but not when normalized values were compared (Figure 40, B). Max force in T all was significantly higher compared to normal healthy contra-lateral leg in absolute values (Figure 40, C). However, no difference was observed in normalized max force between groups. In absolute values, deformation was higher in T all and T3 compared to their respective group of normal contra-lateral leg (Figure 40, D). Both absolute and normalized values for stress were significantly lower in T all compared to normal contra-lateral leg (Figure 40, E). In addition, absolute values for stress were significantly lower in T3 compared to normal contra-lateral leg, C3. Absolute values for strain were significantly lower in T all and T3 compared to C and C3, respectively (Figure 40, F). Overall, T all and T3 was significantly different from C in absolute values, while only normalized stress was significantly lower in T all compared to C.
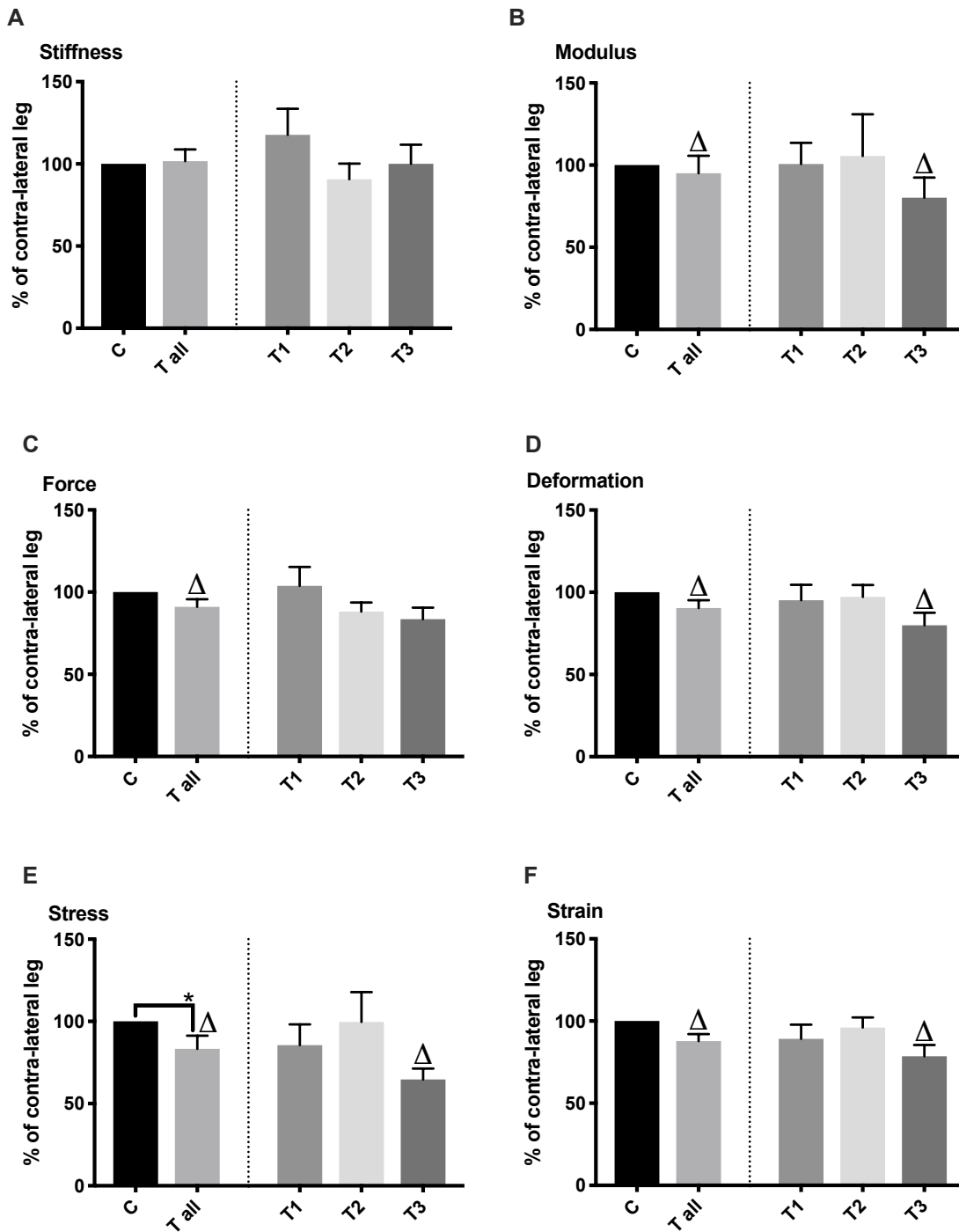
Figure 40 Mechanical properties of patellar tendons at maximal force. For each patient, measurements on the affected side were relative to the unaffected contra-lateral leg (C) (no bilateral patients included). All of the affected legs are grouped in T all (n=28). Patients were additionally grouped in T1 (n=8), T2 (n=11) and T3 (n=9) according to symptom duration. Values are expressed as a normalized value. *P < 0.05 versus healthy controls and ᐃP < 0.05 versus the contra-lateral leg. Results are means ± SEM.

Study II

Voluntarily aerobic exercise and survival

The mice performed voluntary aerobic exercise during 10 PM to 9 AM, approximately three hours after the lights of the animal facility were switched off, and all groups with access to running wheels ran 3-4 km/24 h (Table 4). It was observed that WT (Wild-Type) had the highest standard deviation in both distance and average velocity (Table 4). Some WT were capable of covering 214.6 km in 30 days while one WT covered only 31.87 km and especially two mice were reported by the investigators as extremely sedentary. In addition, two less-severe mice spent very limited time on the running wheel during the 4-week period (2.45 km and 6.84 km), but there was still no significant difference between genotypes when they were removed as outliers (P = 0.3213 and P = 0.5193 for distance and velocity, respectively). Also, no significant difference was seen when the most sedentary mice from WT were removed as outliers. There was no significant difference between genotypes for running distance and average running velocity (Table 4). After 4 weeks of voluntary aerobic exercise there was no significant change in survival of more-severe and less-severe mice when compared to controls (Figure 41). All WT mice survived without any decrease in survival regardless of exercise. Two out of 14 less-severe MFS mice in the exercise group died at days 35 and 36. Thus, the survival rate of less-severe mice was 85.71 % after 4 weeks of exercise but was not statistically significant when compared to the survival rate of the same mutant animals in the control group (Figure 41, B). Six and seven of the more-severe MFS mice died in the exercise and control groups, respectively. After 8 weeks the survival rate of exercised more-severe MFS mice was 62.5 %, while more-severe controls dropped to 74.07 % (Figure 41, C). More-severe MFS mice have a natural median life-span of three months and the cause of death is usually ruptured aorta aneurysm[209]. Dissections of the mice that died during this study before 8 weeks all revealed traces of blood in their thorax, indicating ruptured aorta aneurysm as a possible cause of death, although aneurysms could not be distinguished from rarer conditions such as intrathoracic hernia. However, there was a significant reduction in the survival rate of more-severe MFS mice when compared to WT (exercise: P < 0.0121 and control: P < 0.0427) regardless of exercise (Figure 41, D).

Distance (km)	Number of mice		Mean +/- SE
Wild-type	11		116+/-22
Less-severe	14		102+/-15
More-severe	14		89+/-13
Average velocity (m/s)			Mean
Wild-type	11		0.20+/-0.02
Less-severe	14		0.21+/-0.01
More-severe	14		0.22+/-0.01

Table 4 Running data over 30 days for mice on running wheel. Distance (km) was calculated as total distance during the 4 weeks period. Average velocity (m/s) is a measured distance for each exercise-bout that resulted in a rotation of the wheel more than 45° during 1 second divided by time until it no longer exceeded the threshold of 45° per second. SE: Standard Error of mean.
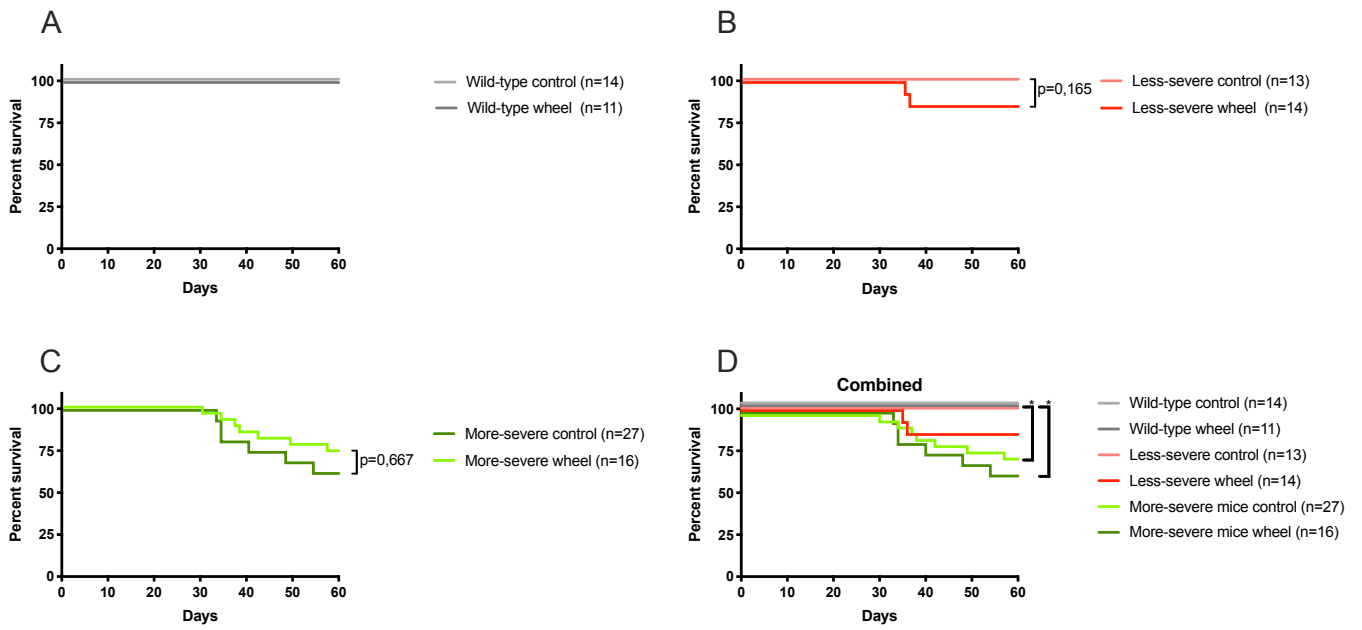
Figure 41 Survival curves for all three genotypes over 8 weeks. There was no difference in survival between the exercise group and controls. More-severe MFS mice were expected to have a median survival of 10 weeks regardless of exercise and therefore showed significantly lower survival rates when compared to wild-type.

Tendon morphology

There was a significant difference in tendon dimensions between genotypes but not within genotypes considering exercise versus control (Figure 42). More-severe mice had significantly smaller cross-sectional area (CSA) when compared to WT controls (Figure 42, C). This was due to a decreased anterior-posterior thickness of tendon of more-severe mice in the exercise group when compared to WT controls. Less-severe mice had smaller CSA when compared to WT controls in both the control group and exercise group due to larger diameter (data not shown). Thus, there was no significant difference in thickness of WT compared to less-severe regardless of groups. Immature collagen and fiber alignment were not significantly different between genotypes (Figure 43). Also, regular exercise had no significant impact on the quality of tendons in any of the groups, and even when genotypes were pooled together in either a sedentary control group or an exercise group, no difference was observed between active and sedentary mice (Figure 43, D).
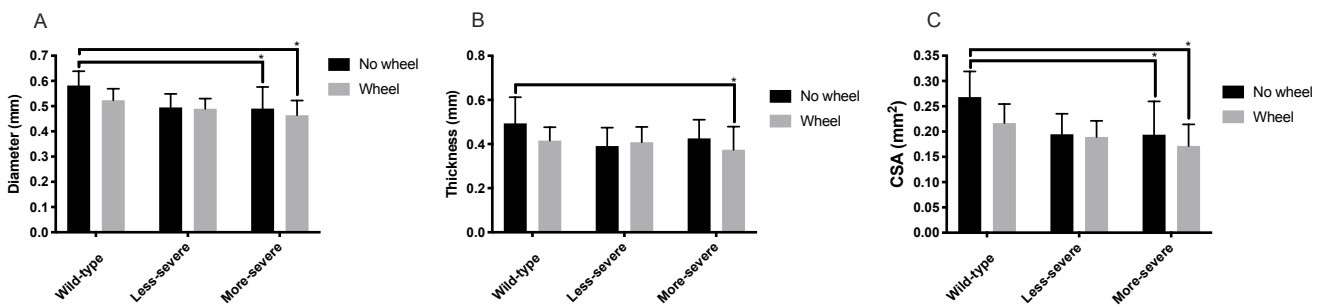
Figure 42 Tendon (FDL) dimensions of mice in both the exercise group and control group. Diameter of more-severe was significantly less than wild-type and the same with thickness. Therefore, cross-sectional area was significantly smaller in more-severe when compared to wild-type. There was no significant difference between less severe when compared to other genotypes regardless of groups. In control-group: $N_{Wild-type}=14$, $N_{Less-severe}=12$, and $N_{More-severe}=9$. In exercise-group: $N_{Wild-type}=11$, $N_{Less-severe}=11$, and $N_{More-severe}=10$.
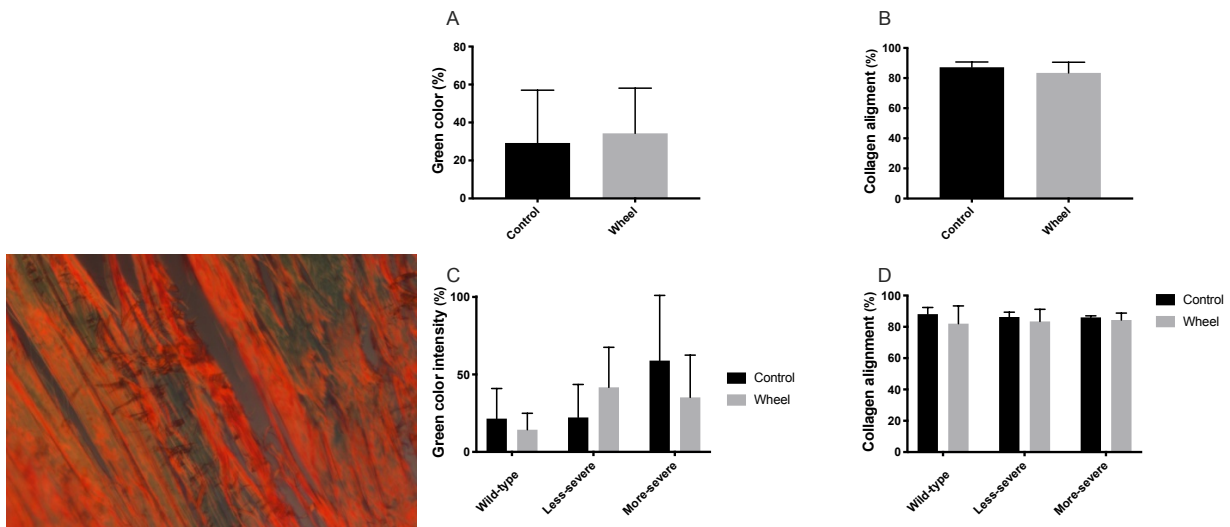
Figure 43 Results from picrosirius staining of the Achilles tendon in control group and exercise group. The green color is indicative of collagen dispersion, meaning that green color intensity increases the more immature the collagen is. Collagen alignment is seen in relation to other neighboring collagen fibers, thus 100 % represents completely aligned collagen fibrils in the picture. In control group: $N_{\text{Wild-type}}$=8, $N_{\text{Less-severe}}$=4, and $N_{\text{More-severe}}$=3. In exercise group: $N_{\text{Wild-type}}$=3, $N_{\text{Less-severe}}$=7, and $N_{\text{More-severe}}$=5.

Mechanical properties

More-severe mice had the weakest tendons of all genotypes regardless of exercise and were significantly lower in max force when compared to WT (control: P = 0.0083, and exercise: P = 0.0024) but not when compared to less-severe (Figure 44, A). Less severe did not differ in max force from WT in neither the control nor the exercise group. Thus, tendons of less-severe mice were not weaker than WT. Exercise had no influence on tendon strength. Max stiffness was significantly lower in more-severe mice compared to WT regardless of exercise (Figure 44, B). Finally, less-severe mice were significantly lower in max stiffness than WT but higher than more-severe mice in the exercise group (P = 0.0382). Only WT had an effect of exercise on tendon max stiffness (P = 0.00675), but when max stiffness was normalized to tendon dimensions, there was no significant difference between genotypes and groups regarding max modulus (Figure 44, C). In addition, hysteresis remained unchanged between genotypes regardless of exercise (Figure 44, D). Finally, there was no significant difference between genotypes and groups in other parameters of tendon mechanics (data not shown).
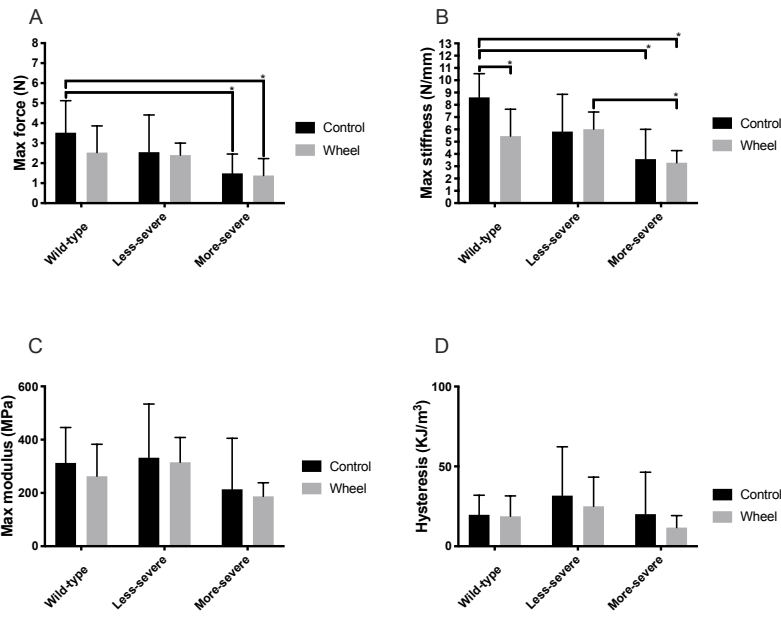
Figure 44 Mechanical data from genotypes of control and exercise groups. More-severe mice had significantly lower max force and max stiffness when compared to wild-type. This was independent of whether more-severe mice did exercise or not during the 30-day period. In control group: $N_{Wild-type}$=13, $N_{Less-severe}$=12, and $N_{More-severe}$=9. In exercise group: $N_{Wild-type}$=11, $N_{Less-severe}$=11, and $N_{More-severe}$=11.

Discussion

The present thesis demonstrates that a sequence of events occurs in early tendinopathy following onset of tendon soreness in patients. Overall, angiogenesis and some tissue anabolic/catabolic signaling were increased, and tendon A-P thickness increased before width, but with no accompanying change in tendon mechanical tissue properties. These findings suggest that tendinopathy pathogenesis represents a disturbed tissue homeostasis in response to repeated tendon overloading.

Study I

Clinical symptoms

The patients in our study did not deviate from the typical patient with tendinopathy presented in a health care setting since they experienced moderate pain (Figure **26**) related to the mid-portion of their tendon (Figure **27**)[45,69]. Symptoms in our patients seems to improve over time, and there was no difference in severity between groups except at day 44 (Figure **30**). Our study shows that increased training load in form of frequency (sessions per week) seems to be a risk factor for tendinopathy, but not volume or intensity (Figure **25**). Another study on runners that exercised with either a volume- or intensity-focused schedule with progression found no significant differences in risk between the two groups but that they did not look into training frequency[79]. They used schedules involving running 3 times per week during the entire 24-week intervention and it could be speculated that recovery-time between each run was achieved. A more intensive schedule with additional progression in frequency from 3 to 6 runs per week, leaving less recovery between each session, could have changed the outcome although such a design is highly questionable from an ethical viewpoint. Other studies have confirmed that adequate rest without any other intervention (e.g. HSR) does improve function, at least in lateral epicondylitis and gluteal tendinopathy[214,215]. Whether an optimal load does exist for tendinopathy is still controversial, although completely rest is also not recommended if a patient has developed AT or PT tendinopathy. Our study shows that pain was related to activity as expected (Figure **28** and Figure **29**). Interestingly, VISA scores from our patients seems to improve over time for AT tendinopathy (Figure **31**) and especially group T3 is declining in average NRS from day 41 to day 71 (Figure **30**). It

remains unclear whether our patients in this study consciously/unconsciously unload their tendons or if there is in fact a spontaneous healing process regardless of loading. Some studies have shown that patients change their tendon-load in training and/or daily physical activities (walking, standing, etc.) and therefore guard their injured leg[216–218]. Another study on mice found signs of tendinopathy in the contra-lateral leg after unilateral tenotomy and indicates that: 1) Patients with unilateral tendinopathy could have subclinical tendinopathy in their presumed healthy leg, and 2) Patients unload their injured leg and therefore overload their healthy leg[219]. Thus, improvement of function over time can be a combination of loading in both its intensity and direction. Our results also show an increase in symptoms from day 17 to day 44 in T3 (Figure **30**), indicating that symptoms may initially become more severe before finally declining. It seems that a pathological asymptomatic process may precede the stage where patients begin to feel soreness or pain in their Achilles tendon. This process increases the severity of symptoms in the early phase of tendinopathy but given enough time will subside which again improves function. However, not all patients spontaneously heal themselves back to completely normal function within short time and therefore this process could still be ongoing after several months.

Tendon morphology and vascularization

Our study shows that tendon cross-sectional area (CSA) measured on US was larger in patients when compared to their contra-lateral non-symptomatic side and healthy controls, which is consistent with findings in previous studies on chronic tendinopathy[220–223]. The increase in thickness was already present at T1 and thus, the increase in CSA during early tendinopathy was primarily due to increased thickness rather than increased width (Figure **32**). Interestingly, A-P thickness in our patients were smaller in T2 for AT tendinopathy while CSA was smaller in T2 for PT tendinopathy (Figure **33**). In our patients with PT tendinopathy, increased A-P thickness was only observed in T3. There is no certain explanation for the variation seen within groups of one particular type of tendinopathy and also across types of tendinopathy. However, sample sizes do differ with 155 and 45 for patients with AT and PT tendinopathy, respectively. Almost no US PD was present in healthy controls in our study, whereas the signal was significantly elevated in patients in all groups, indicating signs of pronounced hyper-vascularization (Figure **37**). In addition, the changes in US PD of our patients seemed to accompany the pattern of increasing tendon CSA that we observed, but TGF beta1 was only

elevated between T2 and T3, suggesting a slight tissue-anabolic stimulus in the later stage of tendinopathy (Figure **38**). Surprisingly, angiogenetic factors in our patients remained unchanged over time in tendinopathy. This indicates stimulation of angiogenesis in the very early phase of subclinical tendinopathy (Figure **39**). It might be that no further proliferation of vessels occurs 1-3 months into the development of tendinopathy. It is not possible to determine the role of angiogenesis, edema and new tendon tissue formation over the course of early tendinopathy from the present data. Variations of US PD in the groups are of particular interest, since such variations indicate angiogenesis but not necessarily hyper-vascularization. US PD has been shown to be influenced by prior activity level in other studies[74,146,224]. Our patients may have a potential to increase blood flow to their injured tendons on a much larger scale than healthy controls, but actual "snap-shot" measurements of US PD in this study may rather reflect training or daily activities in the days leading up to US during clinical examination. It remains speculative whether healthy controls possess the same degree of hyper-vascularization as patients when corrected for physical activity or if patients actually have *bona fide* neo-vascularization. Other studies have demonstrated hyper-vascularization and increased VEGF in competitive athletes despite no signs or symptoms of tendinopathy in the Achilles tendon following mechanical loading [173,224,225]. Clearly, these cross-sectional studies have limitations, but it nevertheless has a large sample size to characterize the pathological processes in early tendinopathy.

Matrix turnover and inflammation

Markers for tendinopathy in this study were chosen on the basis of known key regulators in tendon pathology regardless of outcome. Other studies have shown that chronic tendinopathy upregulate mRNA expression for anabolic tendon (collagen I and III) but also upregulate matrix degradation (TGF-beta and MMP factors)[137,155,226–228]. Also, other proteins such as versican, aggrecan and fibromodulin are increased in chronic tendinopathy[75,229]. Our study shows only discrete changes in very early tendinopathy on both anabolic and catabolic sides of signaling (Figure **38**). This is much in line with other studies on both healthy tendons where tissue renewal is minimal in AT after adolescent and tendinopathic AT after exercise[60,161]. In contrast, other studies have shown an increase in MMPs as a sign of extracellular matrix degradation, although not consistenly[128,155,203,230,231]. Also, we observed that fibronectin and tenascin C were

not significantly increased, thus not indicating tendon remodeling like other studies have found[232,233]. Other studies have shown that Decorin is one of the most abundant proteoglycans in tendons and also osmotically active[179,234,235]. Surprisingly, our biopsies did not show any increase in Decorin, suggesting that tendon swelling in early tendinopathy is caused by other factors than osmotically active proteoglycans attracting water. However, most studies have examined chronic tendinopathy which may limit the extent to which those results can be compared to ours on early tendinopathy. Other targets for mRNA included markers for cell stress, autophagy and inflammation, but these were not elevated in our study, suggesting a difference in changes during the very early phase versus chronic phase of tendinopathy (Figure **39**). Surprisingly, we saw no change in signs of inflammation (COX-1 and prostaglandin F receptor) even though these signs have been shown to be elevated in chronic tendinopathy in other studies[75,131,132,226,227,236] (Figure **39**). A study has shown that COX-2 is an inducible enzyme that increases quickly in response to inflammatory cytokines but subsides in an anti-inflammatory environment[237]. We speculate that inflammation in our patients may have been present before clinical symptoms and is therefore now diminished, reflecting the low levels of COX-2 we see. VEGF, a marker for angiogenesis, increases in tendinopathy and may even precede clinical symptoms as shown by other studies[173,225,238–240]. Interestingly, VEGF and other markers for angiogenesis were not elevated in our study, indicating no angiogenesis in early tendinopathy, at least not after clinical symptoms have appeared (Figure **39**). One possible explanation for our observations is inter-variable degrees of physical activity among our patients given that tendon loading can influence blood flow as well as tendinopathy[110]. Overall, the picture does not imply a strong inflammatory component in early tendinopathy among our patients. Thus, the discrete changes in early tendinopathy resemble processes appearing more in healthy tendons rather than in chronic tendinopathy as reported by other studies. Key regulators for resolution were not investigated in the present study, even though they might indicate low-grade inflammation[131,132]. The present study reported mRNA results normalized to results obtained to the contra-lateral asymptomatic tendon and may underestimate individuals with subclinical tendinopathy on the contra-lateral side. Thus, the process of ongoing subclinical tendinopathy on the contra-lateral leg might dilute any biological markers in patients with unilateral tendinopathy.

Nociception and tendinopathy

Our study shows that Substance-P was substantially increasing over time in early tendinopathy and is of special interest given the involvement of Substance-P in nerve-endings[241–243]. The higher levels of Substance-P we see in T3 compared to T1 suggests a gradual increase in nociceptive pathway stimulation in early tendinopathy (Figure **39**). This would then be interpreted by the patient, depending on the individual's perception of pain, as an increase in tendon soreness and/or pain. In fact, previous studies have shown elevated levels of Substance-P in chronic tendinopathy and that Substance P can be stimulated by loading[241,243]. In addition, other studies have shown that exogenous administration of Substance-P during tendon rupture healing does stimulate both angiogenesis and collagen organization, suggesting a coupling between the increased US PD and Substance-P seen in our study although speculative at this point[244,245]. It is noticeable that the present study shows both elevated levels of Substance-P and improved function in T3. Although one finding might contradict another there is a possible explanation. VISA does not measure pain sensation directly, and therefore neuro-modulative adaptations could take place in patients to cope with increased pain perception (Figure **39**). Thus, elevated local neurotransmitters for pain should be distinguished from actual pain perception, a process that occurs in the central nervous system. It could be that, preceding symptoms, there was an increase in angiogenesis accompanied by an increase in Substance-P. However, angiogenetic factors would later on decline when all the possible angiogenetic processes have taken place inside the tendon.

Mechanical properties

Tendon stiffness in our study shows that maximal force was unchanged relative to the contra-lateral leg, suggesting that tendinopathy does not interfere with mechanical properties in the early phase (Figure **40**). This is consistent with literature on chronic tendinopathy, although some studies did find decreased stiffness and modulus[221,246,247]. In contrast to other studies, the present study focused on early tendinopathy, and it is therefore possible that mechanical properties are changed at later time points (> 3 months). Another study have shown that unilateral tendinopathy predisposes the contra-lateral leg to tendinopathy as well, and therefore it is questionable whether a *bona fide* healthy contra-lateral leg does exist in

unilateral tendinopathic patients[248]. A meta-analysis comparing healthy tendons and symptomatic tendons showed increased stiffness in tendinopathy[249]. The present study did not measure mechanical properties on healthy controls, but it is still uncertain whether tendinopathy and/or a change of loading caused altered mechanical properties. Altered loading has been shown by another study to change tendon mechanics, and a period of unloading (caused by tendon pain) could induce changes in mechanical properties as the patient is guarding the tendinopathic leg[250]. Also, changes in direction of loading could influence tendon mechanics, as patients with AT have been shown by other studies to increase external hip movement and alter activation of neuromotor patterns in triceps surae during running[121,216]. Even short periods of immobilization have been shown to decrease tendon stiffness and thus, studies that find reduced tendon stiffness might in fact demonstrate the effect of reduced loading on the tendinopathic tendon rather than pathological changes associated with tendinopathy[251,252]. Tendon mechanics could be influenced by other factors such as enzymatic as well as non-enzymatic cross-links, but it is beyond the scope of the present study to measure these factors[253]. However, the fact that our patients shows no changes in tendon stiffness during the first 3 months of tendinopathy supports the notion that no major disruption of tendon tissue occurs to initiate tendinopathy. Degeneration of tendon tissue would influence load-bearing fibrils of tendon tissue and degrade them over time. Fibrils run throughout the entire tendon, and since loading is distributed homogenously, this would suggest the absence of a partial rupture or mechanical trauma in initiation of tendinopathy[49,50]. Thus, the present finding demonstrates that tendinopathic tendons maintain the microstructural pattern of an intact tendon with fibrils spanning the entire tendon length.

Limitations

There are numerous studies on chronic tendinopathy, but these studies are not directly comparable to the present study on early tendinopathy. Different types of injuries on different tendons can explain the heterogeneity seen among studies: Chronic vs. acute and subscapularis vs. AT[194]. Taking these observations into account might be like comparing "apples and oranges", because to our knowledge no one has conducted a similar study to the present one. Thus, the uniqueness of this study is therefore also a weakness in that it shares few similarities with previous studies. Gene expression is limited as seen by variation in the levels of mRNA. This

finding could be explained by two factors: 1) Tendinopathic events are region-specific within the tendon, or 2) The area of tendinopathy is very small compared to our needle biopsy. Other factors that might be relevant include physical activity (or lack thereof), training, type of sport, etiology, etc. The simple but yet subjective inclusion-criterion based on tendon soreness under three months has limitations. Firstly, it relies on the patient's history without any objective measurement. Secondly, pain perception is difficult to measure and varies among patients - what is sore or painful for one patient is not necessarily the same for another patient. However, lacking an objective system and given the resources available, this approach was probably the best choice to study early changes in tendinopathy. Future studies would ideally include a large group of asymptomatic runners and follow them through time with measurements of tendinopathy, although such studies would be labor-intensive. Thus, the current study only provides a brief "snap-shot" of events at each timepoint and therefore not enough information to conclude if these events are connected in the development of tendinopathy.

Conclusion

In summary, the present study demonstrates a sequence of events over time in early tendinopathy of humans regarding physiological and pathological changes. US PD and tendon thickness were increased in the first month of tendinopathy development, whereas no change was observed for mechanical properties. The discrete changes in anabolic signaling suggests that tendinopathy represents an altered tissue homeostasis caused by repeated mechanical overloading with inadequate adaptive response rather than initiation by a partial rupture of the tendon.

Study II

There were three crucible determinants of this study in order establish a potential animal model for tendinopathy. First, to determine whether more-severe MFS and less-severe MFS mice were able to voluntarily load their tendons on the running wheel despite musculoskeletal abnormalities. Second, if more-severe and less-severe mice were able to survive until the end of intervention (week 8). Third, whether more-severe and less-severe mice were able to load their tendons as much as wild-type mice. This study shows that loading of tendons in MFS mice did not differ from wild-type mice and thus demonstrate an isolated effect of genotype on tendon tissue. MFS affected size rather than quality of the tendon. However, the smaller tendons of MFS mice did not display any pathological changes when loaded with voluntary running.

Tendon morphology

Our study shows that more-severe mice had the smallest tendons, as both anterior-posterior thickness and diameter were smaller than WT mice (Figure **42**). Whether tendon thickness or diameter contributed most to the smaller CSA in more-severe mice is unknown. Interestingly, we observed that exercised mice seem to have smaller tendons than their sedentary counterparts regardless of genotype. Another study on haploinsufficient mice (Eln$^{+/-}$) with 50 % of normal elastin expression showed no significant difference in CSA of Achilles tendons when compared to wild-type[30]. They saw that collagen content was not affected, but fibril diameter distribution changed from fewer small (<50 nm) to larger (> 170 nm) fibrils compared to WT mice. We speculate that despite lower elastin content there might be sufficient compensatory mechanisms to keep tendon CSA within normal range. A possible explanation for the difference in fibrillin-1 and elastin concerns productivity. The present MFS animal model is based on 20 % production of fibrillin-1 while the animal model of the other study is a 50 % reduction in elastin. Compensatory mechanisms might be compromised if production of elastin was further lowered, but animal viability is decreased significantly when elastin levels are below 30 % of WT mice[254]. From picrosirius red there was no change in collagen orientation and thus, tendon quality is unchanged between genotypes (Figure **43**). In addition, we did not see any difference in collagen maturation between genotypes, and therefore it remains speculative whether any compensatory mechanisms did alter tendon ultrastructure. This finding contrasts with other

studies working with blood vessels from MFS patients as they have reported different findings in collagen alignment[255–257]. However, none of them looked at tendon tissue as it may differ from blood vessels.

Tendon mechanics

Two significant mechanical parameters are presented in this study: tendon mechanics at max force and max stiffness (Figure **44**). We observed that more-severe mice had weaker and less stiff tendons compared to WT mice regardless of exercise. However, no difference in mechanical strength was observed after values were normalized to tendon CSA, and thus, more-severe mice had weaker tendons, probably due to smaller tendons in general. Another study observed lower peak stress in ligaments after treatment with elastase, even though elastin makes up only 4 % of the dry weight it supports up to 30 % of tensile stress[24,258]. The discrepancy between their study and what we observed could be explained by differences between ligaments and tendons. Interestingly, we saw that tendons of more-severe mice were less stiff compared to WT mice, which may appear counter-intuitive since elastin is supposed to be compromised. Tendons of more-severe mice are expected to be stiffer because there is a more complete engagement of collagen fibers to tendon-loading, and this is also found in other studies on elastin haploinsufficient mice[30,259,260]. Also, we saw that more-severe mice seem to have longer tendons than WT mice which could play a potential role in collagen crimp[258] (data not shown). Other studies show that fascicles are capable of sliding relative to each other, and elastic fibers in IFM are believed to facilitate this sliding with minimal shear stress[159,160,261]. If so, compromised elastic fibers will increase shear stress as well[23]. This could explain why the tendons of more-severe mice in our study are less stiff since collagen was unable to crimp properly and therefore display features of being permanently elongated. Also, cross-linkings mediated by the enzyme lysyl oxidase have been shown by other studies to influence mechanical properties[262,263]. We found no differences across genotypes when stiffness was normalized to tendon CSA, which is similar to the observation of tendon strength. Thus, more-severe mice in our study had smaller tendons, and therefore weaker and less stiff tendons compared to WT mice.

Lastly, hysteresis in our study was unaffected by genotype and exercise, indicating that a secondary source of elastic recoil might be present[24] (Figure **44**). Our findings demonstrate

different effects of two distinguishable but yet adjacent molecules: Elastin primarily influences tendon mechanical properties, whereas fibrillin-1 is predominantly involved in tendon growth[31]. It was intriguing to find that, despite the smaller tendons, more-severe mice in this study were found to run the same distance and at the same velocity as WT mice. Thus, fibrillin-1 deficiency does not seem to influence the risk of developing tendinopathy, nor is it expected to elicit any symptoms since more-severe mice were able to withhold their training-load. Another possible explanation is the use of voluntary aerobic exercise instead of forced aerobic exercise. It remains questionable whether the protocol of voluntary aerobic exercise is sufficient to exceed a certain threshold to develop tendinopathy, even though this threshold might be even lower in more-severe mice. In fact, another study on MFS mice has shown an exercise optimum for mitigating aortic aneurysm corresponds to moderate effort[264]. The protocol of mechanical testing in the current study did not exceed 2,5 % tissue strain and might have been well within physiological ranges for both more-severe and WT mice. Thus, the lack of tendinopathy could also be due to insufficient loading and stress during voluntary aerobic exercise and mechanical testing, respectively. However, the present data demonstrate that fibrillin-1 is involved in tissue homeostasis rather than being directly linked to tissue pathology. A future study might implement the used animal model in a forced aerobic exercise regime.

Survival

The first finding concerns the relationship between cardiovascular function and exercise in MFS. As expected, we observed that more-severe mice had a worse survival rate compared to WT, and this is in accordance with the literature (Figure **41**). This study shows that voluntary aerobic exercise did not improve or worsen the median survival of more-severe mice.
In fact, we saw that exercise seems to increase survival rate of more-severe mice compared to their sedentary counterparts, although not significantly. It would be interesting to see the progression in survival rate after 3 or 4 months. Current recommendations on aerobic exercise in MFS patients have been limited to low intensity activities due to an increased risk of aortic dissection. However, the present study did not show this trend, and another similar study looking at Marfan syndrome mice during voluntary aerobic exercise has actually shown beneficial effects on aorta[265]. They investigated voluntary versus forced aerobic exercise of MFS mice and showed a biphasic response with optimum at moderate exercise for 2 - 5 km per day.

The protective effect of moderate exercise up to 55 % V_{O2max} that they observed was probably caused by inhibition of metalloproteinases and therefore reduced elastin fragmentation in aorta of MFS mice. However, aerobic exercise at high intensity did have diminishing returns on the desired protective effect on aorta. On average, MFS and WT mice in our study ran 3 - 4 km per day, which is much in line with what is seen in the literature[198,199,266] (Table 3). Although other studies have reported up to 23 km per day for young mice, some of them used a saucer-shaped wheel with low resistance, and the authors did not specify further details on resistance or velocity. Thus, difference in running distance is heavily influenced by strain of mice, age and experimental setup. However, whether mice always prefer to run at moderate effort is unclear, but the running velocity we observed seems to be well within the normal range. Another study confirmed that the body-weight of exercised MFS mice is the same as sedentary normal mice[265]. Thus, training load on tendons was assumed to be similar across all mice in this project, since there was no difference in running metrics as well. Any observable pathological changes in the tendon must therefore be an effect of genotype.

Limitations

The literature on MFS and tendons is scarce; most studies focus on blood vessels or use elastase treatment on animal models to induce changes in the tendon. Blood vessels and tendons are two distinct structures with different physiological demands. Arteries definitely contain more elastic fibers than tendons, and their 3D conformation is too complex to accommodate changes in blood pressure. The one study on elastin-deficient mice does share some resemblance to the present study albeit with a slightly different focus. Elastic fibers are elastin molecules surrounded by fibrillin-rich microfibrils, but elastic fibers are only a minor structural component of the tendon matrix[9]. The role of fibrillin-1 in tendons, besides being part of the same ECM macroaggregate as elastin, is still unknown. Thus, one limitation is that the content of elastin and fibrillin-1 in tendons was not measured.

Another limitation is related to the animal models in the present study. The median survival rate of more-severe mice is only 3 months, but they need to reach at least 3 weeks of maturity before any intervention. The survival rate decreases significantly if mice are removed from their mother too early. In addition, young mice are not physically mature enough to operate the running wheel. Thus, it could have been interesting to extend the exercise-period beyond 8

weeks. Tendon tissue in humans has a slow turnover, and therefore more running time might have introduced signs of tendinopathy in the animal model. Lastly, the animal model is not an inducible tissue-specific knockout, and there could be developmental compensatory mechanisms in the tendon that may impact the observations.

Conclusion

In conclusion, fibrillin-1 deficiency does not influence survival of MFS mice on voluntary aerobic exercise, but it does decrease tendon dimensions. Tendon quality of more-severe mice was unaltered and therefore similar to WT mice.

There were no signs of developing tendinopathy or exacerbating aneurysm progression even though training-load was consistent across all genotypes. These findings do not support that fibrillin-1 deficient mice are an effective animal model for tendinopathy.

Concluding remarks and perspectives

Study I demonstrate that angiogenesis and thickness increase over time in early tendinopathy with discrete changes in anabolic signaling. Collagen fibers were intact as mechanical properties were unaltered in early tendinopathy, which lead to a focus on elastic fibers in study II. The results from study II do not support a direct role of elastic fibers in tendinopathy. Taken at face value, tendinopathy is neither seen as a process of inflammation nor a degenerative condition as these terms do not grasp the true complexity of tendinopathy. An alternative term such as "unstable equilibrium" fits more with the hypothesis generated by the findings of this study. The question still remain which events precede clinical symptoms of soreness, and therefore also the question which events tip the balance of tissue homeostasis into tendinopathy. A working hypothesis considers the fact that any stimulus will signal a tissue response, but whether adaptation occurs or not is depending on time and magnitude: "Stimulus + Recovery = Adaptation". Signaling molecules, histological and clinical findings must be seen in respect to their spatio-temporal distribution encompassing not only tendinopathy but also healthy tendons under load. Further, a clear distinction should be made between events that are not necessarily coupled, such as inflammation, anti-inflammation and resolution. Of utmost important is a consensus among researchers on definitions of such events and the time-factor of "early" in early tendinopathy as well as tendinopathy itself. Only by reaching common ground and establishing a base can research on tendinopathy grow. Future studies are encouraged to investigate whether inflammation precedes clinical symptoms or if inflammation is present at all with the correct definitions in mind. One such study might include following a large group of asymptomatic runners while they increase their training-load. Measurements with clinical tests, ultrasound, MRI and blood samples on a frequent basis (at least 3 times per week) would hopefully capture the very early discrete changes in developing tendinopathy. However, such studies require copious amounts of resources and time. Only by a reasonable deductive approach can the enigma be solved, ultimately appreciating the full biological spectrum from healthy tendon to tendinopathy. Thus, all researchers are encouraged to not go gentle into that good night[267].

References

1. Harcourt-Smith, W. E. H. & Aiello, L. C. Fossils, feet and the evolution of human bipedal locomotion. *Journal of Anatomy* (2004). doi:10.1111/j.0021-8782.2004.00296.x

2. Kannus, P. Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sport.* (2000). doi:10.1034/j.1600-0838.2000.010006312.x

3. KJAeR, M. Role of Extracellular Matrix in Adaptation of Tendon and Skeletal Muscle to Mechanical Loading. *Physiol. Rev.* (2004). doi:10.1152/physrev.00031.2003

4. Benjamin, M., Kaiser, E. & Milz, S. Structure-function relationships in tendons: A review. *Journal of Anatomy* (2008). doi:10.1111/j.1469-7580.2008.00864.x

5. Alfredson, H. & Lorentzon, R. Sclerosing polidocanol injections of small vessels to treat the chronic painful tendon. *Cardiovasc. Hematol. Agents Med. Chem.* (2007). doi:10.2174/187152507780363232

6. Shinaoka, A. *et al.* Architecture of the subendothelial elastic fibers of small blood vessels and variations in vascular type and size. *Microsc. Microanal.* (2013). doi:10.1017/S1431927612014341

7. Kim, J. *et al.* Crosslinked elastic fibers are necessary for low energy loss in the ascending aorta. *J. Biomech.* (2017). doi:10.1016/j.jbiomech.2017.07.011

8. Galatioto, J. *et al.* Cell type-specific contributions of the angiotensin II type 1a receptor to aorta homeostasis and aneurysmal disease-brief report. *Arterioscler. Thromb. Vasc. Biol.* (2018). doi:10.1161/ATVBAHA.117.310609

9. Alexander, R. M. Energy-saving mechanisms in walking and running. *J Exp Biol* (1991). doi:10.1016/j.fluid.2014.10.046

10. Ker, R. F. Mechanics of tendon, from an engineering perspective. *Int. J. Fatigue* (2007). doi:10.1016/j.ijfatigue.2006.09.020

11. Thorpe, C. T., Udeze, C. P., Birch, H. L., Clegg, P. D. & Screen, H. R. C. Specialization of tendon mechanical properties results from interfascicular differences. *J. R. Soc. Interface* (2012). doi:10.1098/rsif.2012.0362

12. Alexander, R. M. N. Elastic Mechanisms in the Locomotion of Vertebrates. *Netherlands J. Zool.* (1989). doi:10.1163/156854289X00200

13. Thorpe, C. T., Clegg, P. D. & Birch, H. L. A review of tendon injury: Why is the equine superficial digital flexor tendon most at risk? *Equine Veterinary Journal* (2010).

doi:10.2746/042516409X480395

14. Humphries, S. M., Lu, Y., Canty, E. G. & Kadler, K. E. Active negative control of collagen fibrillogenesis in vivo: Intracellular cleavage of the type I procollagen propeptides in tendon fibroblasts without intracellular fibrils. *J. Biol. Chem.* (2008). doi:10.1074/jbc.M708198200

15. Burjanadze, T. V. New analysis of the phylogenetic change of collagen thermostability. *Biopolymers* (2000). doi:10.1002/(SICI)1097-0282(200005)53:6<523::AID-BIP8>3.0.CO;2-7

16. Narayanan, A. S., Page, R. C., Kuzan, F. & Cooper, C. G. Elastin cross-linking in vitro. Studies on factors influencing the formation of desmosines by lysyl oxidase action on tropoelastin. *Biochem J* (1978). doi:10.1042/bj1730857

17. Dyksterhuis, L. B. & Weiss, A. S. Homology models for domains 21-23 of human tropoelastin shed light on lysine crosslinking. *Biochem. Biophys. Res. Commun.* (2010). doi:10.1016/j.bbrc.2010.05.013

18. Eyre, D. Cross-Linking in Collagen and Elastin. *Annu. Rev. Biochem.* (1984). doi:10.1146/annurev.biochem.53.1.717

19. Kostrominova, T. Y. & Brooks, S. V. Age-related changes in structure and extracellular matrix protein expression levels in rat tendons. *Age (Omaha).* (2013). doi:10.1007/s11357-013-9514-2

20. Thorpe, C. T. *et al.* The interfascicular matrix enables fascicle sliding and recovery in tendon, and behaves more elastically in energy storing tendons. *J. Mech. Behav. Biomed. Mater.* (2015). doi:10.1016/j.jmbbm.2015.04.009

21. Godinho, M. S. C., Thorpe, C. T., Greenwald, S. E. & Screen, H. R. C. Elastin is Localised to the Interfascicular Matrix of Energy Storing Tendons and Becomes Increasingly Disorganised With Ageing. *Sci. Rep.* (2017). doi:10.1038/s41598-017-09995-4

22. Thorpe, C. T., Udeze, C. P., Birch, H. L., Clegg, P. D. & Screen, H. R. C. Capacity for sliding between tendon fascicles decreases with ageing in injury prone equine tendons: A possible mechanism for age-related tendinopathy? *Eur. Cells Mater.* (2012). doi:10.22203/eCM.v025a04

23. Fang, F. & Lake, S. P. Multiscale mechanical integrity of human supraspinatus tendon in shear after elastin depletion. *J. Mech. Behav. Biomed. Mater.* (2016). doi:10.1016/j.jmbbm.2016.06.032

24. Henninger, H. B., Underwood, C. J., Romney, S. J., Davis, G. L. & Weiss, J. A. Effect of elastin digestion on the quasi-static tensile response of medial collateral ligament. *J. Orthop. Res.* (2013). doi:10.1002/jor.22352

25. Pang, X. *et al.* Three dimensional microstructural network of elastin, collagen, and cells in Achilles tendons. *J. Orthop. Res.* (2017). doi:10.1002/jor.23240

26. Wu, Y. T., Wu, P. T. & Jou, I. M. Peritendinous elastase treatment induces tendon degeneration in rats: A potential model of tendinopathy in vivo. *J. Orthop. Res.* (2016). doi:10.1002/jor.23030

27. Wu, Y. T., Su, W. R., Wu, P. T., Shen, P. C. & Jou, I. M. Degradation of elastic fiber and elevated elastase expression in long head of biceps tendinopathy. *J. Orthop. Res.* (2017). doi:10.1002/jor.23500

28. Thakkar, D., Grant, T. M., Hakimi, O. & Carr, A. J. Distribution and expression of type VI collagen and elastic fibers in human rotator cuff tendon tears. *Connect. Tissue Res.* (2014). doi:10.3109/03008207.2014.959119

29. Li, D. Y. *et al.* Elastin is an essential determinant of arterial morphogenesis. *Nature* (1998). doi:10.1038/30522

30. Eekhoff, J. D. *et al.* Functionally Distinct Tendons from Elastin Haploinsufficient Mice Exhibit Mild Stiffening and Tendon-Specific Structural Alteration. *J. Biomech. Eng.* (2017). doi:10.1115/1.4037932

31. Ramirez, F. & Pereira, L. The fibrillins. *International Journal of Biochemistry and Cell Biology* (1999). doi:10.1016/S1357-2725(98)00109-5

32. Ashworth, J. L., Kielty, C. M. & McLeod, D. Fibrillin and the eye. *Br. J. Ophthalmol.* (2000). doi:10.1136/bjo.84.11.1312

33. Kasamatsu, S. *et al.* Essential role of microfibrillar-associated protein 4 in human cutaneous homeostasis and in its photoprotection. *Sci. Rep.* (2011). doi:10.1038/srep00164

34. Malvankar, S. & Khan, W. S. Evolution of the Achilles tendon: The athlete's Achilles heel? *Foot* (2011). doi:10.1016/j.foot.2011.08.004

35. Fallon, J., Blevins, F. T., Vogel, K. & Trotter, J. Functional morphology of the supraspinatus tendon. *J. Orthop. Res.* (2002). doi:10.1016/S0736-0266(02)00032-2

36. Franchi, M. *et al.* Crimp morphology in relaxed and stretched rat Achilles tendon. *J. Anat.* (2007). doi:10.1111/j.1469-7580.2006.00666.x

37.	Gaut, L. *et al.* EGR1 regulates transcription downstream of mechanical signals during tendon formation and healing. *PLoS One* (2016). doi:10.1371/journal.pone.0166237

38.	Mandl, F. *Statistical Physics*. (John Wiley & Sons, 2008).

39.	Diamant, J., Keller, A., Baer, E., Litt, M. & Arridge, R. G. C. Collagen; Ultrastructure and Its Relation to Mechanical Properties as a Function of Ageing. *Proc. R. Soc. B Biol. Sci.* (1972). doi:10.1098/rspb.1972.0019

40.	Arnoczky, S. P., Lavagnino, M. & Egerbacher, M. The mechanobiological aetiopathogenesis of tendinopathy: Is it the over-stimulation or the under-stimulation of tendon cells? in *International Journal of Experimental Pathology* (2007). doi:10.1111/j.1365-2613.2007.00548.x

41.	Wang, J. H. C. Mechanobiology of tendon. *Journal of Biomechanics* (2006). doi:10.1016/j.jbiomech.2005.05.011

42.	Finni, T., Peltonen, J., Stenroth, L. & Cronin, N. J. On the hysteresis in the human Achilles tendon. *J. Appl. Physiol.* (2013). doi:10.1152/japplphysiol.01005.2012

43.	Van Oeveren, B. T., De Ruiter, C. J., Beek, P. J. & Van Dieën, J. H. Optimal stride frequencies in running at different speeds. *PLoS One* (2017). doi:10.1371/journal.pone.0184273

44.	M., G.-K., J., E., H., M. & N., M. Recurrence of Achilles tendon injuries in elite male football players is more common after early return to play: an 11-year follow-up of the UEFA Champions League injury study. *Br. J. Sports Med.* (2013). doi:10.1136/bjsports-2013-092271

45.	Habets, B., van den Broek, A. G., Huisstede, B. M. A., Backx, F. J. G. & van Cingel, R. E. H. Return to Sport in Athletes with Midportion Achilles Tendinopathy: A Qualitative Systematic Review Regarding Definitions and Criteria. *Sports Medicine* (2018). doi:10.1007/s40279-017-0833-9

46.	Lopes, A. D., Hespanhol, L. C., Yeung, S. S. & Costa, L. O. P. What are the main running-related musculoskeletal injuries? A systematic review. *Sports Medicine* (2012). doi:10.2165/11631170-000000000-00000

47.	Komi, P. V. Relevance of in vivo force measurements to human biomechanics. *J. Biomech.* (1990). doi:10.1016/0021-9290(90)90038-5

48.	Peter Magnusson, S., Aagaard, P., Rosager, S., Dyhre-Poulsen, P. & Kjaer, M. Load-displacement properties of the human triceps surae aponeurosis in vivo. *J. Physiol.*

(2001). doi:10.1111/j.1469-7793.2001.0277j.x

49. Svensson, R. B. *et al.* Evidence of structurally continuous collagen fibrils in tendons. *Acta Biomater.* (2017). doi:10.1016/j.actbio.2017.01.006

50. Rigozzi, S., Stemmer, A., Müller, R. & Snedeker, J. G. Mechanical response of individual collagen fibrils in loaded tendon as measured by atomic force microscopy. *J. Struct. Biol.* **176**, 9–15 (2011).

51. Leppilahti, J., Puranen, J. & Orava, S. Incidence of Achilles tendon rupture. *Acta Orthop. Scand.* (1996). doi:10.3109/17453679608994688

52. Gärdin, A. *et al.* T2∗ relaxation time in Achilles tendinosis and controls and its correlation with clinical score. *J. Magn. Reson. Imaging* (2016). doi:10.1002/jmri.25104

53. Thorpe, C. T. *et al.* Anatomical heterogeneity of tendon: Fascicular and interfascicular tendon compartments have distinct proteomic composition. *Sci. Rep.* (2016). doi:10.1038/srep20455

54. Thorpe, C. T. *et al.* Distribution of proteins within different compartments of tendon varies according to tendon type. *J. Anat.* (2016). doi:10.1111/joa.12485

55. Giannopoulos, A. *et al.* Cellular homeostatic tension and force transmission measured in human engineered tendon. *J. Biomech.* (2018). doi:10.1016/j.jbiomech.2018.07.032

56. Couppe, C. *et al.* Habitual loading results in tendon hypertrophy and increased stiffness of the human patellar tendon. *J. Appl. Physiol.* (2008). doi:10.1152/japplphysiol.90361.2008

57. Kannus, P., Józsa, L., Natri, A. & Järvinen, M. Effects of training, immobilization and remobilization on tendons. *Scand. J. Med. Sci. Sports* (2007). doi:10.1111/j.1600-0838.1997.tb00121.x

58. Heinemeier, K. M. & Kjaer, M. In vivo investigation of tendon responses to mechanical loading. *Journal of Musculoskeletal Neuronal Interactions* (2011).

59. Kjaer, M. & Heinemeier, K. M. Eccentric exercise: acute and chronic effects on healthy and diseased tendons. *J. Appl. Physiol.* (2014). doi:10.1152/japplphysiol.01044.2013

60. Heinemeier, K. M., Schjerling, P., Heinemeier, J., Magnusson, S. P. & Kjaer, M. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb14C. *FASEB J.* (2013). doi:10.1096/fj.12-225599

61. Epstein, D. The Sports Gene: Inside the Science of Extraordinary Athletic Performance. *Int. Sport Coach. J.* (2013). doi:10.1123/iscj.2014-0088

62. Darwin, C. *On the Origin of Species by Means of Natural Selection*. *D. Appleton and Company* (1859). doi:10.1126/science.146.3640.51-b

63. Ogbonmwan, I., Kumar, B. D. & Paton, B. New lower-limb gait biomechanical characteristics in individuals with Achilles tendinopathy: A systematic review update. *Gait Posture* (2018). doi:10.1016/j.gaitpost.2018.03.010

64. Bidell, M. R. & Lodise, T. P. Fluoroquinolone-Associated Tendinopathy: Does Levofloxacin Pose the Greatest Risk? *Pharmacotherapy* (2016). doi:10.1002/phar.1761

65. Hernandez, P. *et al.* Severe Burn-Induced Inflammation and Remodeling of Achilles Tendon in a Rat Model. *SHOCK* (2018). doi:10.1097/SHK.0000000000001037

66. Ribbans, W. J. & Collins, M. Pathology of the tendo Achillis: Do our genes contribute? *Bone Joint J.* (2013). doi:10.1302/0301-620X.95B3.30066

67. Abate, M., Schiavone, C., Salini, V. & Andia, I. Occurrence of tendon pathologies in metabolic disorders. *Rheumatology (United Kingdom)* (2013). doi:10.1093/rheumatology/kes395

68. Couppé, C. *et al.* Human Achilles tendon glycation and function in diabetes. *J. Appl. Physiol.* (2016). doi:10.1152/japplphysiol.00547.2015

69. Beyer, R. *et al.* Heavy slow resistance versus eccentric training as treatment for achilles tendinopathy: A randomized controlled trial. *Am. J. Sports Med.* (2015). doi:10.1177/0363546515584760

70. Alfredson, H., Pietilä, T., Jonsson, P. & Lorentzon, R. Heavy-load eccentric calf muscle training for the treatment of chronic achilles tendinosis. *Am. J. Sports Med.* (1998). doi:10.1177/03635465980260030301

71. Nørregaard, J., Larsen, C. C., Bieler, T. & Langberg, H. Eccentric exercise in treatment of Achilles tendinopathy. *Scand. J. Med. Sci. Sport.* (2007). doi:10.1111/j.1600-0838.2006.00545.x

72. Murtaugh, B. & Ihm, J. M. Eccentric training for the treatment of tendinopathies. *Curr. Sports Med. Rep.* (2013). doi:10.1249/JSR.0b013e3182933761

73. Woodley, B. L., Newsham-West, R. J. & Baxter, G. D. Chronic tendinopathy: Effectiveness of eccentric exercise. *British Journal of Sports Medicine* (2007). doi:10.1136/bjsm.2006.029769

74. Pingel, J. *et al.* The microvascular volume of the achilles tendon is increased in patients with tendinopathy at rest and after a 1-hour treadmill run. *Am. J. Sports Med.* (2013).

doi:10.1177/0363546513498988

75.   Heinemeier, K. M. *et al.* Effects of anti-inflammatory (NSAID) treatment on human tendinopathic tissue. *J. Appl. Physiol.* (2017). doi:10.1152/japplphysiol.00281.2017

76.   Farris, D. J., Trewartha, G. & Polly McGuigan, M. Could intra-tendinous hyperthermia during running explain chronic injury of the human Achilles tendon? *J. Biomech.* (2011). doi:10.1016/j.jbiomech.2010.12.015

77.   Sparta. SPARTA EVENTKALENDER. Available at: https://sparta.dk/run_page/.

78.   Ramskov, D. *et al.* Progression in Running Intensity or Running Volume and the Development of Specific Injuries in Recreational Runners: Run Clever, a Randomized Trial Using Competing Risks. *J. Orthop. Sport. Phys. Ther.* (2018). doi:10.2519/jospt.2018.8062

79.   Ramskov, D. *et al.* Run Clever - No difference in risk of injury when comparing progression in running volume and running intensity in recreational runners: A randomised trial. *BMJ Open Sport Exerc. Med.* (2018). doi:10.1136/bmjsem-2017-000333

80.   Malfait, F., Wenstrup, R. J. & De Paepe, A. Clinical and genetic aspects of Ehlers-Danlos syndrome, classic type. *Genetics in Medicine* (2010). doi:10.1097/GIM.0b013e3181eed412

81.   Nielsen, R. H. *et al.* Low tendon stiffness and abnormal ultrastructure distinguish classic Ehlers-Danlos syndrome from benign joint hypermobility syndrome in patients. *FASEB J.* (2014). doi:10.1096/fj.14-249656

82.   Nielsen, R. H. *et al.* Tendon protein synthesis rate in classic Ehlers-Danlos patients can be stimulated with insulin-like growth factor-I. *J. Appl. Physiol.* (2014). doi:10.1152/japplphysiol.00157.2014

83.   Symoens, S. *et al.* Comprehensive molecular analysis demonstrates type V collagen mutations in over 90% of patients with classic EDS and allows to refine diagnostic criteria. *Hum. Mutat.* (2012). doi:10.1002/humu.22137

84.   Franken, R. *et al.* Relationship between fibrillin-1 genotype and severity of cardiovascular involvement in Marfan syndrome. *Heart* (2017). doi:10.1136/heartjnl-2016-310631

85.   Dietz, H. C. *et al.* Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature* (1991). doi:10.1038/352337a0

86.   Pyeritz, R. E. The Marfan syndrome. *Am. Fam. Physician* (1986). doi:10.1016/0030-4220(63)90301-3

87.   Maron, B. J. *et al.* Recommendations for physical activity and recreational sports

participation for young patients with genetic cardiovascular diseases. *Circulation* **109**, 2807–2816 (2004).

88. Pelliccia, A. *et al.* Recommendations for competitive sports participation in athletes with cardiovascular disease. *Med. dello Sport* **58**, 157–191 (2005).

89. Marfan.com. Physical Activity Guidelines. (2017).

90. Atzeni, F. *et al.* Rheumatic manifestations in inflammatory bowel disease. *Autoimmunity Reviews* (2014). doi:10.1016/j.autrev.2013.06.006

91. Polachek, A., Li, S., Chandran, V. & Gladman, D. D. Clinical Enthesitis in a Prospective Longitudinal Psoriatic Arthritis Cohort: Incidence, Prevalence, Characteristics, and Outcome. *Arthritis Care Res.* (2017). doi:10.1002/acr.23174

92. Lui, P. P. Y. Tendinopathy in diabetes mellitus patients—Epidemiology, pathogenesis, and management. *Scandinavian Journal of Medicine and Science in Sports* (2017). doi:10.1111/sms.12824

93. Monnier, V. M., Kohn, R. R. & Cerami, A. Accelerated age-related browning of human collagen in diabetes mellitus. *Proc. Natl. Acad. Sci.* (1984). doi:10.1073/pnas.81.2.583

94. Skovgaard, D. *et al.* An advanced glycation endproduct (AGE)-rich diet promotes accumulation of AGEs in Achilles tendon. *Physiol. Rep.* (2017). doi:10.14814/phy2.13215

95. Raabe, M. E. & Chaudhari, A. M. W. Biomechanical consequences of running with deep core muscle weakness. *J. Biomech.* (2018). doi:10.1016/j.jbiomech.2017.11.037

96. Becker, J., James, S., Wayner, R., Osternig, L. & Chou, L. S. Biomechanical Factors Associated with Achilles Tendinopathy and Medial Tibial Stress Syndrome in Runners. *Am. J. Sports Med.* (2017). doi:10.1177/0363546517708193

97. Altman, A. R. & Davis, I. S. Barefoot running: Biomechanics and implications for running injuries. *Curr. Sports Med. Rep.* (2012). doi:10.1249/JSR.0b013e31826c9bb9

98. National Geoprahic. Breaking2. (2017).

99. Matthews, W., Ellis, R., Furness, J. & Hing, W. Classification of Tendon Matrix Change Using Ultrasound Imaging: A Systematic Review and Meta-analysis. *Ultrasound in Medicine and Biology* (2018). doi:10.1016/j.ultrasmedbio.2018.05.022

100. Maffulli, N., Khan, K. M. & Puddu, G. Overuse tendon conditions: Time to change a confusing terminology. *Arthroscopy* (1998). doi:10.1016/S0749-8063(98)70021-0

101. Singh, A., Calafi, A., Diefenbach, C., Kreulen, C. & Giza, E. Noninsertional Tendinopathy of the Achilles. *Foot and Ankle Clinics* (2017). doi:10.1016/j.fcl.2017.07.006

102. McAuliffe, S., McCreesh, K., Culloty, F., Purtill, H. & O'Sullivan, K. Can ultrasound imaging predict the development of Achilles and patellar tendinopathy? A systematic review and meta-analysis. *British Journal of Sports Medicine* (2016). doi:10.1136/bjsports-2016-096288

103. De Marchi, A. *et al.* In Achilles tendinopathy, the neovascularization, detected by contrast-enhanced ultrasound (CEUS), is abundant but not related to symptoms. *Knee Surgery, Sport. Traumatol. Arthrosc.* (2018). doi:10.1007/s00167-017-4710-8

104. Risch, L., Cassel, M. & Mayer, F. Acute effect of running exercise on physiological Achilles tendon blood flow. *Scand. J. Med. Sci. Sport.* (2018). doi:10.1111/sms.12874

105. Fredberg, U. & Bolvig, L. Significance of ultrasonographically detected asymptomatic tendinosis in the patellar and Achilles tendons of elite soccer players: A longitudinal study. *Am. J. Sports Med.* (2002). doi:10.1177/03635465020300040701

106. De Jonge, S. *et al.* Relationship between neovascularization and clinical severity in Achilles tendinopathy in 556 paired measurements. *Scand. J. Med. Sci. Sport.* (2014). doi:10.1111/sms.12072

107. Boesen, M. I., Koenig, M. J., Torp-Pedersen, S., Bliddal, H. & Langberg, H. Tendinopathy and Doppler activity: The vascular response of the achilles tendon to exercise. *Scand. J. Med. Sci. Sport.* (2006). doi:10.1111/j.1600-0838.2005.00512.x

108. Wezenbeek, E. *et al.* The Role of the Vascular and Structural Response to Activity in the Development of Achilles Tendinopathy: A Prospective Study. *Am. J. Sports Med.* (2018). doi:10.1177/0363546517750854

109. Yoshihara, M. & Yoshihara, M. 'Necessary and sufficient' in biology is not necessarily necessary–confusions and erroneous conclusions resulting from misapplied logic in the field of biology, especially neuroscience. *Journal of Neurogenetics* (2018). doi:10.1080/01677063.2018.1468443

110. Riley, G. Tendinopathy - From basic science to treatment. *Nature Clinical Practice Rheumatology* (2008). doi:10.1038/ncprheum0700

111. Jewson, J. L. *et al.* Pain duration is associated with increased muscle sympathetic nerve activity in patients with Achilles tendinopathy. *Scand. J. Med. Sci. Sport.* (2017). doi:10.1111/sms.12820

112. Scholes, M. *et al.* Men with unilateral Achilles tendinopathy have impaired balance on the symptomatic side. *J. Sci. Med. Sport* (2018). doi:10.1016/j.jsams.2017.09.594

113. Bakkegaard, M., Johannsen, F. E., Højgaard, B. & Langberg, H. Ultrasonography as a prognostic and objective parameter in Achilles tendinopathy: A prospective observational study. *Eur. J. Radiol.* (2015). doi:10.1016/j.ejrad.2014.11.028

114. Zhang, Q. *et al.* Sonoelastography shows that Achilles tendons with insertional tendinopathy are harder than asymptomatic tendons. *Knee Surgery, Sport. Traumatol. Arthrosc.* (2017). doi:10.1007/s00167-016-4197-8

115. Ooi, C. C., Schneider, M. E., Malliaras, P., Chadwick, M. & Connell, D. A. Diagnostic Performance of Axial-Strain Sonoelastography in Confirming Clinically Diagnosed Achilles Tendinopathy: Comparison with B-Mode Ultrasound and Color Doppler Imaging. *Ultrasound Med. Biol.* (2015). doi:10.1016/j.ultrasmedbio.2014.08.019

116. Bayliss, A. J. *et al.* Achilles tendon material properties are greater in the jump leg of jumping athletes. *J. Musculoskelet. Neuronal Interact.* (2016).

117. Hutchison, A. M. *et al.* What is the best clinical test for achilles tendinopathy? *Foot Ankle Surg.* (2013). doi:10.1016/j.fas.2012.12.006

118. Lautenbacher, S., Peters, J. H., Heesen, M., Scheel, J. & Kunz, M. Age changes in pain perception: A systematic-review and meta-analysis of age effects on pain and tolerance thresholds. *Neuroscience and Biobehavioral Reviews* (2017). doi:10.1016/j.neubiorev.2017.01.039

119. Crofford, L. J. Chronic Pain: Where the Body Meets the Brain. *Trans. Am. Clin. Climatol. Assoc.* (2015). doi:10.1586/ern.09.20

120. Williamson, A. & Hoggart, B. Pain: A review of three commonly used pain rating scales. *Journal of Clinical Nursing* (2005). doi:10.1111/j.1365-2702.2005.01121.x

121. Wyndow, N., Cowan, S. M., Wrigley, T. V. & Crossley, K. M. Triceps surae activation is altered in male runners with Achilles tendinopathy. *J. Electromyogr. Kinesiol.* (2013). doi:10.1016/j.jelekin.2012.08.010

122. Paavola, M., Kannus, P., Paakkala, T., Pasanen, M. & Järvinen, M. Long-term prognosis of patients with achilles tendinopathy: An observational 8-year follow-up study. *Am. J. Sports Med.* (2000). doi:10.1177/03635465000280050301

123. Mikkelsen, U. R. *et al.* Life-long endurance exercise in humans: Circulating levels of inflammatory markers and leg muscle size. *Mech. Ageing Dev.* (2013). doi:10.1016/j.mad.2013.11.004

124. Khan, K. & Cook, J. The painful nonruptured tendon: Clinical aspects. *Clinics in Sports*

Medicine (2003). doi:10.1016/S0278-5919(03)00035-8

125. Uhthoff, H. K. & Sano, H. Pathology of failure of the rotator cuff tendon. *Orthopedic Clinics of North America* (1997). doi:10.1016/S0030-5898(05)70262-5

126. Cook, J. L., Rio, E., Purdam, C. R. & Docking, S. I. Revisiting the continuum model of tendon pathology: What is its merit in clinical practice and research? *British Journal of Sports Medicine* (2016). doi:10.1136/bjsports-2015-095422

127. Riley, G. The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology* (2004). doi:10.1093/rheumatology/keg448

128. Riley, G. P. *et al.* Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology. *Matrix Biol.* (2002). doi:10.1016/S0945-053X(01)00196-2

129. Riley, G. P. Gene expression and matrix turnover in overused and damaged tendons. in *Scandinavian Journal of Medicine and Science in Sports* (2005). doi:10.1111/j.1600-0838.2005.00456.x

130. Fredberg, U. *et al.* Ultrasonography as a tool for diagnosis, guidance of local steroid injection and, together with pressure algometry, monitoring of the treatment of athletes with chronic jumper's knee and Achilles tendinitis: A randomized, double-blind, placebo-controlled . *Scand. J. Rheumatol.* (2004). doi:10.1080/03009740310004126

131. Millar, N. L. *et al.* Inflammation is present in early human tendinopathy. *Am. J. Sports Med.* (2010). doi:10.1177/0363546510372613

132. Dakin, S. G. *et al.* Inflammation activation and resolution in human tendon disease. *Sci. Transl. Med.* (2015). doi:10.1126/scitranslmed.aac4269

133. Douglas, M. R., Morrison, K. E., Salmon, M. & Buckley, C. D. Why does inflammation persist: a dominant role for the stromal microenvironment? *Expert Rev. Mol. Med.* (2002). doi:10.1017/S1462399402005264

134. Buckley, C. D. *et al.* Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends in Immunology* (2001). doi:10.1016/S1471-4906(01)01863-4

135. Kragsnaes, M. S. *et al.* Immunohistochemical quantification by stereological technique of immune-competent cells in biopsies from achilles tendons in healthy and patients with chronic tendinopathy. *Ann. Rheum. Dis.* (2014). doi:10.1136/annrheumdis-2014-eular.1684

136. Dakin, S. G. *et al.* Chronic inflammation is a feature of Achilles tendinopathy and rupture. *Br. J. Sports Med.* (2018). doi:10.1136/bjsports-2017-098161

137. Pingel, J. *et al.* No inflammatory gene-expression response to acute exercise in human Achilles tendinopathy. *Eur. J. Appl. Physiol.* (2013). doi:10.1007/s00421-013-2638-3

138. Kjaer, M., Bayer, M. L., Eliasson, P. & Heinemeier, K. M. What is the impact of inflammation on the critical interplay between mechanical signaling and biochemical changes in tendon matrix? *J. Appl. Physiol.* (2013). doi:10.1152/japplphysiol.00120.2013

139. Dean, B. J. F., Gettings, P., Dakin, S. G. & Carr, A. J. Are inflammatory cells increased in painful human tendinopathy? A systematic review. *Br. J. Sports Med.* (2016). doi:10.1136/bjsports-2015-094754

140. Dakin, S. G., Dudhia, J. & Smith, R. K. W. Resolving an inflammatory concept: The importance of inflammation and resolution in tendinopathy. *Veterinary Immunology and Immunopathology* (2014). doi:10.1016/j.vetimm.2014.01.007

141. Buckley, C. D., Barone, F., Nayar, S., Bénézech, C. & Caamaño, J. Stromal Cells in Chronic Inflammation and Tertiary Lymphoid Organ Formation. *Annu. Rev. Immunol.* (2015). doi:10.1146/annurev-immunol-032713-120252

142. Duncan, W., Best, J., Golubitsky, M., Nijhout, H. F. & Reed, M. Homeostasis despite instability. *Math. Biosci.* (2018). doi:10.1016/j.mbs.2018.03.025

143. Danglot, B., Preux, P., Baudry, B. & Monperrus, M. Correctness attraction: a study of stability of software behavior under runtime perturbation. *Empir. Softw. Eng.* (2018). doi:10.1007/s10664-017-9571-8

144. Ackermann, P. W. Neuronal pathways in tendon healing. *Front. Biosci.* (2009). doi:10.2741/3593

145. Dean, B. J. F., Franklin, S. L. & Carr, A. J. The peripheral neuronal phenotype is important in the pathogenesis of painful human tendinopathy: A systematic review. *Clinical Orthopaedics and Related Research* (2013). doi:10.1007/s11999-013-3010-y

146. Roesch, H. J., Milanese, S., Osborne, B., Spurrier, D. J. & Thoirs, K. A. The acute effects of exercise on tendon dimensions and vascularity. An exploratory study using diagnostic ultrasound of the male Achilles tendon. *J. Sci. Med. Sport* (2018). doi:10.1016/j.jsams.2017.11.009

147. Jewson, J. L., Lambert, G. W., Storr, M. & Gaida, J. E. The Sympathetic Nervous System and Tendinopathy: A Systematic Review. *Sports Medicine* (2015). doi:10.1007/s40279-

014-0300-9

148. Sasaki, K. *et al.* Innervation pattern at the undersurface of the extensor carpi radialis brevis tendon in recalcitrant tennis elbow. *J. Orthop. Sci.* (2013). doi:10.1007/s00776-013-0406-1

149. Bjur, D., Danielson, P., Alfredson, H. & Forsgren, S. Immunohistochemical and in situ hybridization observations favor a local catecholamine production in the human achilles tendon. *Histol. Histopathol.* (2008). doi:10.14670/HH-23.197

150. Magnusson, S. P., Langberg, H. & Kjaer, M. The pathogenesis of tendinopathy: Balancing the response to loading. *Nature Reviews Rheumatology* (2010). doi:10.1038/nrrheum.2010.43

151. van der Ven, M. *et al.* Adding ultrasound to clinical examination reduced frequency of enthesitis in primary care psoriasis patients with musculoskeletal complaints. *Clin. Exp. Rheumatol.* (2016).

152. Magnusson, S. P. & Kjaer, M. The impact of loading, unloading, ageing and injury on the human tendon. *Journal of Physiology* (2018). doi:10.1113/JP275450

153. Miller, B. F. *et al.* Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J. Physiol.* (2005). doi:10.1113/jphysiol.2005.093690

154. Pingel, J. *et al.* 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: Evidence of tenocyte and matrix buckling. *J. Anat.* (2014). doi:10.1111/joa.12164

155. Pingel J, Fredberg U, Qvortrup K, Larsen JO, Schjerling P, Heinemeier K, Kjaer M, L. H. Local biochemical and morphological differences in human Achilles tendinopathy: a case control study. *BMC Musculoskelet. Disord.* (2012). doi:10.1186/1471-2474-13-53

156. Nakama, L. H., King, K. B., Abrahamsson, S. & Rempel, D. M. Evidence of tendon microtears due to cyclical loading in an in vivo tendinopathy model. *J. Orthop. Res.* (2005). doi:10.1016/j.orthres.2005.03.006

157. van Dijk, C. N., van Sterkenburg, M. N., Wiegerinck, J. I., Karlsson, J. & Maffulli, N. Terminology for Achilles tendon related disorders. *Knee Surgery, Sport. Traumatol. Arthrosc.* (2011). doi:10.1007/s00167-010-1374-z

158. Thorpe, C. T., Riley, G. P., Birch, H. L., Clegg, P. D. & Screen, H. R. C. Fascicles from energy-storing tendons show an age-specific response to cyclic fatigue loading. *J. R. Soc.*

Interface (2014). doi:10.1098/rsif.2013.1058

159.   Ritty, T. M., Ditsios, K. & Starcher, B. C. Distribution of the elastic fiber and associated proteins in flexor tendon reflects function. *Anat. Rec.* (2002). doi:10.1002/ar.10175

160.   Smith, K. D. *et al.* The organisation of elastin and fibrillins 1 and 2 in the cruciate ligament complex. *J. Anat.* (2011). doi:10.1111/j.1469-7580.2011.01374.x

161.   Heinemeier, K. M. *et al.* Carbon-14 bomb pulse dating shows that tendinopathy is preceded by years of abnormally high collagen turnover. *FASEB J.* (2018). doi:10.1096/fj.201701569R

162.   Thorpe, C. T. *et al.* Distribution of proteins within different compartments of tendon varies according to tendon type. *J. Anat.* (2016). doi:10.1111/joa.12485

163.   Karlsson, J., Kalebo, P., Goksor, L. A., Thomee, R. & Sward, L. Partial rupture of the patellar ligament. *Am. J. Sports Med.* (1992). doi:10.1177/036354659202000404

164.   Almekinders, L. C. & Temple, J. D. Etiology, diagnosis, and treatment of tendonitis: An analysis of the literature. *Medicine and Science in Sports and Exercise* (1998). doi:10.1097/00005768-199808000-00001

165.   Spiesz, E. M. *et al.* Tendon extracellular matrix damage, degradation and inflammation in response to in vitro overload exercise. *J. Orthop. Res.* (2015). doi:10.1002/jor.22879

166.   Serhan, C. N. *et al.* Resolution of inflammation: state of the art, definitions and terms. *FASEB J.* (2007). doi:10.1096/fj.06-7227rev

167.   Yasui, Y. *et al.* The Risk of Achilles Tendon Rupture in the Patients with Achilles Tendinopathy: Healthcare Database Analysis in the United States. in *BioMed Research International* (2017). doi:10.1155/2017/7021862

168.   Mehdizadeh, A., Gardiner, B. S., Lavagnino, M. & Smith, D. W. Predicting tenocyte expression profiles and average molecular concentrations in Achilles tendon ECM from tissue strain and fiber damage. *Biomech. Model. Mechanobiol.* (2017). doi:10.1007/s10237-017-0890-x

169.   Dakin, S. G. *et al.* Persistent stromal fibroblast activation is present in chronic tendinopathy. *Arthritis Res. Ther.* (2017). doi:10.1186/s13075-016-1218-4

170.   Yancopoulos, G. D. *et al.* Vascular-specific growth factors and blood vessel formation. *Nature* (2000). doi:10.1038/35025215

171.   Ferrara, N., Gerber, H. P. & LeCouter, J. The biology of VEGF and its receptors. *Nature Medicine* (2003). doi:10.1038/nm0603-669

172. Turner, M. D., Nedjai, B., Hurst, T. & Pennington, D. J. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta - Molecular Cell Research* (2014). doi:10.1016/j.bbamcr.2014.05.014

173. Pufe, T., Petersen, W., Tillmann, B. & Mentlein, R. The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch.* (2001). doi:10.1007/s004280100422

174. Pufe, T., Petersen, W. J., Mentlein, R. & Tillmann, B. N. The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease. in *Scandinavian Journal of Medicine and Science in Sports* (2005). doi:10.1111/j.1600-0838.2005.00465.x

175. Scott, A., Lian, Ø., Bahr, R., Hart, D. A. & Duronio, V. VEGF expression in patellar tendinopathy: A preliminary study. in *Clinical Orthopaedics and Related Research* (2008). doi:10.1007/s11999-008-0272-x

176. Merritt, E. K. *et al.* Heightened muscle inflammation susceptibility may impair regenerative capacity in aging humans. *J. Appl. Physiol.* (2013). doi:10.1152/japplphysiol.00019.2013

177. Langberg, H., Olesen, J. L., Gemmer, C. & Kjær, M. Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans. *J. Physiol.* (2002). doi:10.1113/jphysiol.2002.019141

178. Öhberg, L. & Alfredson, H. Effects on neovascularisation behind the good results with eccentric training in chronic mid-portion Achilles tendinosis? *Knee Surgery, Sport. Traumatol. Arthrosc.* (2004). doi:10.1007/s00167-004-0494-8

179. Corps, A. N. *et al.* Increased expression of aggrecan and biglycan mRNA in Achilles tendinopathy. *Rheumatology* (2006). doi:10.1093/rheumatology/kei152

180. Fu, S. C., Chan, K. M. & Rolf, C. G. Increased deposition of sulfated glycosaminoglycans in human patellar tendinopathy. *Clin. J. Sport Med.* (2007). doi:10.1097/JSM.0b013e318037998f

181. Parkinson, J., Samiric, T., Ilic, M. Z., Cook, J. & Handley, C. J. Involvement of proteoglycans in tendinopathy. *Journal of Musculoskeletal Neuronal Interactions* (2011).

182. Greene, D. A. & Winegrad, A. I. In vitro studies of the substrates for energy production and the effects of insulin on glucose utilization in the neural components of peripheral nerve. *Diabetes* (1979). doi:10.2337/diab.28.10.878

183. Rechthand, E., Smith, Q. R. & Rapoport, S. I. Facilitated Transport of Glucose from Blood

into Peripheral Nerve. *J. Neurochem.* (1985). doi:10.1111/j.1471-4159.1985.tb04087.x

184. Stecker, M. M. & Stevenson, M. Effect of glucose concentration on peripheral nerve and its response to anoxia. *Muscle and Nerve* (2014). doi:10.1002/mus.23917

185. Magnani, P. *et al.* Glucose transporters in rat peripheral nerve: Paranodal expression of GLUT1 and GLUT3. *Metabolism.* (1996). doi:10.1016/S0026-0495(96)90174-2

186. Muona, P., Sollberg, S., Peltonen, J. & Uitto, J. Glucose transporters of rat peripheral nerve: Differential expression of GLUT1 gene by Schwann cells and perineurial cells in vivo and in vitro. *Diabetes* (1992). doi:10.2337/diab.41.12.1587

187. Danielson, P., Andersson, G., Alfredson, H. & Forsgren, S. Extensive expression of markers for acetylcholine synthesis and of M2 receptors in tenocytes in therapy-resistant chronic painful patellar tendon tendinosis - a pilot study. *Life Sci.* (2007). doi:10.1016/j.lfs.2007.01.005

188. Ackermann, P. W., Li, J., Finn, A., Ahmed, M. & Kreicbergs, A. Autonomic innervation of tendons, ligaments and joint capsules. A morphologic and quantitative study in the rat. *J. Orthop. Res.* (2001). doi:10.1016/S0736-0266(00)90029-9

189. Ackermann, P. W., Salo, P. & Hart, D. A. Tendon innervation. *Adv. Exp. Med. Biol.* (2016). doi:10.1007/978-3-319-33943-6_4

190. Boesen, M. I., Boesen, A., Koenig, M. J., Bliddal, H. & Torp-Pedersen, S. Ultrasonographic Investigation of the Achilles Tendon in Elite Badminton Players Using Color Doppler. *Am. J. Sports Med.* (2006). doi:10.1177/0363546506290188

191. Maudlin, T. Physics, philosophy, and the nature of reality. *Ann. N. Y. Acad. Sci.* (2015). doi:10.1111/nyas.12877

192. Strippoli, P. *et al.* Uncertainty principle of genetic information in a living cell. *Theoretical Biology and Medical Modelling* (2005). doi:10.1186/1742-4682-2-40

193. Bass, E. Tendinopathy: Why the difference between tendinitis and tendinosis matters. *Int. J. Ther. Massage Bodyw. Res. Educ. Pract.* (2012). doi:10.3822/ijtmb.v5i1.153

194. Smith, R. *et al.* Advances in the understanding of tendinopathies: A report on the Second Havemeyer Workshop on equine tendon disease. *Equine Veterinary Journal* (2014). doi:10.1111/evj.12128

195. Lui, P. P. Y., Maffulli, N., Rolf, C. & Smith, R. K. W. What are the validated animal models for tendinopathy? *Scandinavian Journal of Medicine and Science in Sports* (2011). doi:10.1111/j.1600-0838.2010.01164.x

196. Olesen, A. T. *et al.* Muscle-specific changes in length-force characteristics of the calf muscles in the spastic Han-Wistar rat. *J. Appl. Physiol.* (2014). doi:10.1152/japplphysiol.00587.2014

197. Jacobs, B. Y., Kloefkorn, H. E. & Allen, K. D. Gait analysis methods for rodent models of osteoarthritis. *Curr. Pain Headache Rep.* (2014). doi:10.1007/s11916-014-0456-x

198. De Bono, J. P. Novel quantitative phenotypes of exercise training in mouse models. *AJP Regul. Integr. Comp. Physiol.* (2005). doi:10.1152/ajpregu.00694.2005

199. Creer, D. J., Romberg, C., Saksida, L. M., van Praag, H. & Bussey, T. J. Running enhances spatial pattern separation in mice. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.0911725107

200. Bangsbo, J. & Larsen, H. *Running & Science - in an Interdisciplinary Perspective*. (Munksgaard, 2000).

201. Bailey, J., Mata, T. & Mercer, J. A. Is the Relationship Between Stride Length, Frequency, and Velocity Influenced by Running on a Treadmill or Overground? *Int. J. Exerc. Sci.* (2017).

202. Deuis, J. R., Dvorakova, L. S. & Vetter, I. Methods Used to Evaluate Pain Behaviors in Rodents. *Front. Mol. Neurosci.* (2017). doi:10.3389/fnmol.2017.00284

203. Jones, G. C. *et al.* Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human Achilles tendon. *Arthritis Rheum.* (2006). doi:10.1002/art.21672

204. Seitz, A. L., McClure, P. W., Finucane, S., Boardman, N. D. & Michener, L. A. Mechanisms of rotator cuff tendinopathy: Intrinsic, extrinsic, or both? *Clinical Biomechanics* (2011). doi:10.1016/j.clinbiomech.2010.08.001

205. Couppe, C. *et al.* Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J. Appl. Physiol.* (2009). doi:10.1152/japplphysiol.00291.2009

206. Hansen, M. *et al.* Impact of oral contraceptive use and menstrual phases on patellar tendon morphology, biochemical composition, and biomechanical properties in female athletes. *J. Appl. Physiol.* (2013). doi:10.1152/japplphysiol.01255.2012

207. Maganaris, C. N. Tendon conditioning: artefact or property? *Proc. R. Soc. B Biol. Sci.* (2003). doi:10.1098/rsbl.2003.0004

208. Pereira, L. *et al.* Targetting of the gene encoding fibrillin-1 recapitulates the vascular aspect of marfan syndrome. *Nature Genetics* (1997). doi:10.1038/ng1097-218

209. Pereira, L. *et al.* Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1. *Proc. Natl. Acad. Sci. USA* (1999). doi:10.1073/pnas.96.7.3819

210. Cikach, F. S. *et al.* Massive aggrecan and versican accumulation in thoracic aortic aneurysm and dissection. *JCI Insight* (2018). doi:10.1172/jci.insight.97167

211. Kiernan, J. A. Sirius Red Staining Protocol for Collagen. Available at: http://www.ihcworld.com/_protocols/special_stains/sirius_red.htm.

212. Junqueira, L. C. U., Bignolas, G. & Brentani, R. R. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* (1979). doi:10.1007/BF01002772

213. Puchtler, H., Waldrop, F. S. & Valentine, L. S. Polarization Microscopic Studies of Connective Tissue Stained with Picro-Sirius Red FBA. *Beitr. Pathol.* (1973). doi:10.1016/S0005-8165(73)80016-2

214. Smidt, N. *et al.* Corticosteroid injections, physiotherapy, or a wait-and-see policy for lateral epicondylitis: A randomised controlled trial. *Lancet* (2002). doi:10.1016/S0140-6736(02)07811-X

215. Mellor, R. *et al.* Education plus exercise versus corticosteroid injection use versus a wait and see approach on global outcome and pain from gluteal tendinopathy: Prospective, single blinded, randomised clinical trial. *Br. J. Sports Med.* (2018). doi:10.1136/bjsports-2018-k1662rep

216. Creaby, M. W., Honeywill, C., Franettovich Smith, M. M., Schache, A. G. & Crossley, K. M. Hip Biomechanics Are Altered in Male Runners with Achilles Tendinopathy. *Med. Sci. Sports Exerc.* (2017). doi:10.1249/MSS.0000000000001126

217. Kim, S. & Yu, J. H. Changes of gait parameters and lower limb dynamics in recreational runners with achilles tendinopathy. *J. Sport. Sci. Med.* (2015).

218. S., S.-L., K., C., T., W. & M., C. Effect of foot orthoses on ankle kinematics and kinetics in male runners with Achilles tendinopathy. *J. Sci. Med. Sport* (2012). doi:10.1016/j.jsams.2012.11.240

219. Lin, X. *et al.* Characterization of a Novel Calcific Achilles Tendinopathy Model in Mice: Contralateral Tendinopathy Induced by Unilateral Tenotomy. *Calcif. Tissue Int.* (2018). doi:10.1007/s00223-018-0465-6

220. Shaikh, Z. *et al.* Achilles tendinopathy in club runners. *Int. J. Sports Med.* (2012). doi:10.1055/s-0031-1299701

221. Arya, S. & Kulig, K. Tendinopathy alters mechanical and material properties of the Achilles tendon. *J. Appl. Physiol.* (2010). doi:10.1152/japplphysiol.00259.2009

222. Archambault, J. M. *et al.* Can sonography predict the outcome in patients with achillodynia? *J. Clin. Ultrasound* (1998). doi:10.1002/(SICI)1097-0096(199809)26:7<335::AID-JCU1>3.0.CO;2-A

223. Nadeau, M. J., Desrochers, A., Lamontagne, M., Larivière, C. & Gagnon, D. H. Quantitative ultrasound imaging of Achilles tendon integrity in symptomatic and asymptomatic individuals: Reliability and minimal detectable change. *J. Foot Ankle Res.* (2016). doi:10.1186/s13047-016-0164-3

224. Hullfish, T. J., Hagan, K. L., Casey, E. & Baxter, J. R. Achilles tendon structure differs between competitive distance runners and nonrunners despite no clinical signs or symptoms of midsubstance tendinopathy. *J. Appl. Physiol.* (2018). doi:10.1152/japplphysiol.00012.2018

225. Zheng, W., Seftor, E. A., Meininger, C. J., Hendrix, M. J. & Tomanek, R. J. Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-beta. *Am. J. Physiol. Heart Circ. Physiol.* (2001). doi:10.1152/ajpheart.2001.280.2.H909

226. Fu, S. C. *et al.* Increased expression of transforming growth factor-β1 in patellar tendinosis. *Clin. Orthop. Relat. Res.* (2002). doi:10.1097/00003086-200207000-00022

227. Fenwick, S. A. *et al.* Expression of transforming growth factor-beta isoforms and their receptors in chronic tendinosis. *J. Anat.* (2001). doi:10.1017/S0021878201007889

228. Heinemeier, K., Langberg, H., Olesen, J. L. & Kjaer, M. Role of TGF-β_1 in relation to exercise-induced type I collagen synthesis in human tendinous tissue. *J. Appl. Physiol.* (2003). doi:10.1152/japplphysiol.00403.2003

229. Parkinson, J. *et al.* Change in proteoglycan metabolism is a characteristic of human patellar tendinopathy. *Arthritis Rheum.* (2010). doi:10.1002/art.27587

230. De Mos, M. *et al.* Achilles tendinosis: Changes in biochemical composition and collagen turnover rate. *Am. J. Sports Med.* (2007). doi:10.1177/0363546507301885

231. Thorpe, C. T. *et al.* Tendon overload results in alterations in cell shape and increased markers of inflammation and matrix degradation. *Scand. J. Med. Sci. Sport.* (2015). doi:10.1111/sms.12333

232. Tillander, B., Franzén, L. & Norlin, R. Fibronectin, MMP-1 and histologic changes in rotator cuff disease. *J. Orthop. Res.* (2002). doi:10.1016/S0736-0266(02)00057-8

233. Riley, G. P., Harrall, R. L., Cawston, T. E., Hazleman, B. L. & Mackie, E. J. Tenascin-C and human tendon degeneration. *Am. J. Pathol.* (1996).

234. Vogel, K. G. & Heinegard, D. Characterization of proteoglycans from adult bovine tendon. *J. Biol. Chem.* (1985). doi:260, 9298-9306

235. Danielson, K. G. *et al.* Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J. Cell Biol.* (1997). doi:10.1083/jcb.136.3.729

236. Rolf, C. G., Fu, B. S. C., Pau, A., Wang, W. & Chan, B. Increased cell proliferation and associated expression of PDGFRβ causing hypercellularity in patellar tendinosis. *Rheumatology* (2001). doi:10.1093/rheumatology/40.3.256

237. Wu, K. K. Cyclooxygenase 2 induction: Molecular mechanism and pathophysiologic roles. *Journal of Laboratory and Clinical Medicine* (1996). doi:10.1016/S0022-2143(96)90023-2

238. Gerber, H. P. *et al.* VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.* (1999). doi:10.1038/9467

239. Legerlotz, K., Jones, E. R., Screen, H. R. C. & Riley, G. P. Increased expression of IL-6 family members in tendon pathology. *Rheumatol. (United Kingdom)* (2012). doi:10.1093/rheumatology/kes002

240. Alfredson, H., Lorentzon, M., Bäckman, S., Bäckman, A. & Lerner, U. H. cDNA-arrays and real-time quantitative PCR techniques in the investigation of chronic achilles tendinosis. *J. Orthop. Res.* (2003). doi:10.1016/S0736-0266(03)00107-4

241. Backman, L. J., Andersson, G., Wennstig, G., Forsgren, S. & Danielson, P. Endogenous substance P production in the Achilles tendon increases with loading in an in vivo model of tendinopathy - peptidergic elevation preceding tendinosis-like tissue changes. *J. Musculoskelet. Neuronal Interact.* (2011).

242. Schubert, T. E. O., Weidler, C., Lerch, K., Hofstädter, F. & Straub, R. H. Achilles tendinosis is associated with sprouting of substance P positive nerve fibres. *Ann. Rheum. Dis.* (2005). doi:10.1136/ard.2004.029876

243. Gotoh, M., Hamada, K., Yamakawa, H., Inoue, A. & Fukuda, H. Increased substance P in subacromial bursa and shoulder pain in rotator cuff diseases. *J. Orthop. Res.* (1998). doi:10.1002/jor.1100160515

244. Ackermann, P. W., Li., J., Lundeberg, T. & Kreicbergs, A. Neuronal plasticity in relation to nociception and healing of rat achilles tendon. *J. Orthop. Res.* (2003). doi:10.1016/S0736-0266(02)00207-3

245. Burssens, P. *et al.* Exogenously administered substance P and neutral endopeptidase inhibitors stimulate fibroblast proliferation, angiogenesis and collagen organization during Achilles tendon healing. *Foot Ankle Int.* (2005). doi:10.1177/107110070502601008

246. Child, S., Bryant, A. L., Clark, R. A. & Crossley, K. M. Mechanical properties of the achilles tendon aponeurosis are altered in athletes with achilles tendinopathy. *Am. J. Sports Med.* (2010). doi:10.1177/0363546510366234

247. Kongsgaard, M. *et al.* Fibril Morphology and Tendon Mechanical Properties in Patellar Tendinopathy. *Am. J. Sports Med.* (2010). doi:10.1177/0363546509350915

248. Fahlström, M., Lorentzon, R. & Alfredson, H. Painful conditions in the Achilles tendon region: A common problem in middle-aged competitive badminton players. *Knee Surgery, Sport. Traumatol. Arthrosc.* (2002). doi:10.1007/s00167-001-0255-x

249. Obst, S. J. *et al.* Are the Mechanical or Material Properties of the Achilles and Patellar Tendons Altered in Tendinopathy? A Systematic Review with Meta-analysis. *Sports Medicine* (2018). doi:10.1007/s40279-018-0956-7

250. Werkhausen, A. *et al.* Effect of training-induced changes in Achilles tendon stiffness on muscle-tendon behavior during landing. *Front. Physiol.* (2018). doi:10.3389/fphys.2018.00794

251. de Boer, M. D., Maganaris, C. N., Seynnes, O. R., Rennie, M. J. & Narici, M. V. Time course of muscular, neural and tendinous adaptations to 23 day unilateral lower-limb suspension in young men. *J. Physiol.* (2007). doi:10.1113/jphysiol.2007.135392

252. Couppé, C. *et al.* The effects of immobilization on the mechanical properties of the patellar tendon in younger and older men. *Clin. Biomech.* (2012). doi:10.1016/j.clinbiomech.2012.06.003

253. Herchenhan, A. *et al.* Lysyl oxidase activity is required for ordered collagen fibrillogenesis by tendon cells. *J. Biol. Chem.* (2015). doi:10.1074/jbc.M115.641670

254. Hirano, E., Knutsen, R. H., Sugitani, H., Ciliberto, C. H. & Mecham, R. P. Functional rescue of elastin insufficiency in mice by the human elastin gene: Implications for mouse models of human disease. *Circ. Res.* (2007). doi:10.1161/CIRCRESAHA.107.153510

255. Chow, M. J., Turcotte, R., Lin, C. P. & Zhang, Y. Arterial extracellular matrix: A mechanobiological study of the contributions and interactions of elastin and collagen. *Biophys. J.* (2014). doi:10.1016/j.bpj.2014.05.014

256. Lindeman, J. H. N. *et al.* Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in Marfan syndrome. *Proc. Natl. Acad. Sci.* (2010). doi:10.1073/pnas.0910312107

257. Recchia, D., Sharkey, A. M., Bosner, M. S., Kouchoukos, N. T. & Wickline, S. A. Sensitive detection of abnormal aortic architecture in Marfan syndrome with high-frequency ultrasonic tissue characterization. *Circulation* (1995). doi:10.1161/01.CIR.91.4.1036

258. Henninger, H. B., Valdez, W. R., Scott, S. A. & Weiss, J. A. Elastin governs the mechanical response of medial collateral ligament under shear and transverse tensile loading. *Acta Biomater.* (2015). doi:10.1016/j.actbio.2015.07.011

259. Li, D. Y. *et al.* Novel arterial pathology in mice and humans hemizygous for elastin. *J. Clin. Invest.* (1998). doi:10.1172/JCI4487

260. Carta, L. *et al.* Discrete contributions of elastic fiber components to arterial development and mechanical compliance. *Arterioscler. Thromb. Vasc. Biol.* (2009). doi:10.1161/ATVBAHA.109.193227

261. Haraldsson, B. T. *et al.* Lateral force transmission between human tendon fascicles. *Matrix Biol.* (2008). doi:10.1016/j.matbio.2007.09.001

262. Eleswarapu, S. V., Responte, D. J. & Athanasiou, K. A. Tensile properties, collagen content, and crosslinks in connective tissues of the immature knee joint. *PLoS One* (2011). doi:10.1371/journal.pone.0026178

263. Makris, E. A., Responte, D. J., Paschos, N. K., Hu, J. C. & Athanasiou, K. A. Developing functional musculoskeletal tissues through hypoxia and lysyl oxidase-induced collagen cross-linking. *Proc. Natl. Acad. Sci.* (2014). doi:10.1073/pnas.1414271111

264. Gibson, C. *et al.* Mild aerobic exercise blocks elastin fiber fragmentation and aortic dilatation in a mouse model of Marfan syndrome associated aortic aneurysm. *J. Appl. Physiol.* (2017). doi:10.1152/japplphysiol.00132.2017

265. Gibson, C. *et al.* Mild aerobic exercise blocks elastin fiber fragmentation and aortic dilatation in a mouse model of Marfan syndrome associated aortic aneurysm. *J. Appl. Physiol.* **123**, 147–160 (2017).

266. Allen, D. L. *et al.* Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. *J. Appl. Physiol.* (2001). doi:10.1530/eje.0.1330361

267. Thomas Dylan. https://poets.org/poem/do-not-go-gentle-good-night.

Paper 1 - Study I

RESEARCH ARTICLE

THE FASEB JOURNAL

Early development of tendinopathy in humans: Sequence of pathological changes in structure and tissue turnover signaling

Peter H. T. Tran[1,2] | Nikolaj M. Malmgaard-Clausen[1,2] | Rikke S. Puggaard[1,2] | René B. Svensson[1,2] | Janus D. Nybing[4] | Philip Hansen[4] | Peter Schjerling[1,2] | Amanda H. Zinglersen[1,2] | Christian Couppé[1,3] | Mikael Boesen[4] | S. Peter Magnusson[1,3] | Michael Kjaer[1,2]

[1]Institute of Sports Medicine Copenhagen, Department of Orthopaedic Surgery M, Bispebjerg Hospital, Copenhagen, Denmark

[2]Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

[3]Department of Physical & Occupational Therapy, Bispebjerg Hospital, Copenhagen, Denmark

[4]Department of Radiology, Bispebjerg-Frederiksberg Hospital, Frederiksberg, Denmark

Correspondence

Peter H. T. Tran, Institute of Sports Medicine Copenhagen, Department of Orthopaedic Surgery M, Bispebjerg Hospital, Building 8, Nielsine Nielsens vej 11, 2400 Copenhagen NV, Denmark.
Email: TranenPeter@gmail.com

Funding information

Lundbeck Foundation, Grant/Award Number: R198-2015-207; Nordea Foundation; Augustinus Foundation, Grant/Award Number: 17-3727; Novo Nordisk Foundation, Grant/Award Number: NNF16-0C0022846; Ministry of Culture, Grant/Award Number: FPK-2016-0069; The Danish Rheumatism Association, Grant/Award Number: R150-A4500

Abstract

Overloading of tendon tissue with resulting chronic pain (tendinopathy) is a common disorder in occupational-, leisure- and sports-activity, but its pathogenesis remains poorly understood. To investigate the very early phase of tendinopathy, Achilles and patellar tendons were investigated in 200 physically active patients and 50 healthy control persons. Patients were divided into three groups: symptoms for 0-1 months (T1), 1-2 months (T2) or 2-3 months (T3). Tendinopathic Achilles tendon cross-sectional area determined by ultrasonography (US) was ~25% larger than in healthy control persons. Both Achilles and patellar anterior-posterior diameter were elevated in tendinopathy, and only later in Achilles was the width increased. Increased tendon size was accompanied by an increase in hypervascularization (US Doppler flow) without any change in mRNA for angiogenic factors. From patellar biopsies taken bilaterally, mRNA for most growth factors and tendon components remained unchanged (except for TGF-beta1 and substance-P) in early tendinopathy. Tendon stiffness remained unaltered over the first three months of tendinopathy and was similar to the asymptomatic contra-lateral tendon. In conclusion, this suggests that tendinopathy pathogenesis represents a disturbed tissue homeostasis with fluid accumulation.

Abbreviations: ANGPTL4, Angiopoietin Like 4; A-P thickness, anterior-poster thickness; ATF3, Activating Transcription Factor 3; ATF4, Activating Transcription Factor 4; ATG12, Autophagy Related 12; BECN1, Beclin1; BMI, body mass index; C, contra-lateral asymptomatic leg; COL1A1, Collagen type I Alpha 1 chain; COL3A1, Collagen type III Alpha 1 chain; CSA, cross-sectional area; CTGF, Connective Tissue Growth Factor; DCN, Decorin; EGR1, Early Growth Response protein 1; FN1, Fibronectin; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HBB, Hemoglobin subunit beta; HC, healthy control persons; HSPA1A, HSP70; IGF1/IGF1-Ea, Insulin-like Growth Factor 1; ITGB1, Integrin beta-1; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta; MMP2, Matrix Metalloproteinase 2; MMP3, Matrix Metalloproteinase 3; MRI, magnetic resonance imaging; mRNA, Messenger Ribonucleic Acid; NRS, Numeric Rating Scale; PDGFRA, Platelet-Derived Growth Factor Receptor A; PTGFR, Prostaglandin F Receptor; PTGS1/COX1, Cyclooxygenase 1; RPLP0, Ribosomal Protein Lateral Stalk Subunit P0; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SEM, standard error of mean; T all, all tendinopathic patients; T1, 0-1 months; T2, 1-2 months; T3, 2-3 months; TAC1, Substance P; TGFb1, Transforming Growth Factor beta 1; TGFb2, Transforming Growth Factor beta 2; TGFb3, Transforming Growth Factor beta 3; TNC, TenascinC; TNMD, Tenomodulin; US, ultrasound; VEGFA, Vascular Endothelial Growth Factor A; VISA-A/P, The Victorian Institute of Sports Assessment-Achilles/Patellar.

The disturbance is likely induced by repeated mechanical overloading rather than a partial rupture of the tendon.

KEYWORDS

clinical, inflammation, mRNA, tendinopathy, ultrasound

1 | INTRODUCTION

Overloading of tendon tissue with resulting chronic pain (tendinopathy) is a common disorder in occupational-, leisure- and sports-activity. Tendinopathy can severely impair performance and occupational capacity.[1] Disease mechanisms have been studied for decades although tendon tissue turnover dynamics and pathogenesis of tendinopathy still remains poorly understood. Therefore, the treatment of chronic tendinopathy remains a challenge despite extensive research efforts.[2-4] Chronic tendinopathy is associated with pathological imaging findings, eg by ultrasonography (US) and magnetic resonance imaging (MRI). These findings include increased tendon size, hyper-vascularization, and signs of increased water content secondary to increased amounts of ground substance.[5-7] However, the sequence of these changes from the early phase of tendinopathy to chronicity is currently unknown. It has been suggested that tendinopathy is initiated by a partial rupture of the tendon despite that it has been difficult to document any imaging signs of tendon discontinuity in the human tendon[8] and only 10% of patients that suffer a complete tendon rupture have any pre-existing tendinopathy.[9] Furthermore, the cellular signaling of matrix regulating proteins is very different in early tendinopathy compared to tendon rupture.[10] It is likely that early development of tendinopathy is not initiated by a partial rupture and therefore not cause any changes in mechanical properties. The role of inflammation and its time course in tendinopathy has been addressed primarily in non-weight bearing shoulder tendons, suggesting that inflammatory mRNA biomarkers and cell infiltration can be demonstrated in the early phase of tendinopathy.[11,12] However, in chronic prolonged tendinopathy it has been difficult to detect any signs of inflammation [13,14] and anti-inflammatory medical treatment has not yielded successful clinical outcomes.[15] Thus, it is plausible that inflammation is primarily present in the early phase of tendinopathy where anti-inflammatory medication may play a role in the treatment strategy. The presence of hyper-vascularization is typically associated with chronic tendinopathy but how early this develops remains unclear.[16,17]

In the present study, we examined the early phase of unilateral tendinopathy in the Achilles and patellar tendon in sports active individuals with initial signs of disease (pain and soreness) during physical activity. In a cross-sectional design, we investigated patients who had symptoms for either 0-1, 1-2 or 2-3 months. Patients were investigated for tendon morphology (US, MRI), mechanical properties (US) and tendon vascularization (US). Furthermore, patients were investigated for biomarkers of inflammation, pain, autophagy and regulation of structural proteins from tendon biopsy samples quantitative reverse transcription polymerase chain reaction (RT-qPCR). All these findings were correlated with disease duration. Measures on the contra-lateral asymptomatic tendon of the patients and the tendon of healthy asymptomatic training-matched individuals were used as controls.

We hypothesized that in the early development of tendinopathy there would be a detectable incongruity between matrix protein anabolic and catabolic responses, leading to increased matrix protein signaling, increased tissue degradation signaling. This causes an upregulation of inflammatory activity and angiogenesis. We further hypothesized that this would be followed by an accumulation of connective tissue and edema mediating proteins. Also, we hypothesized that partial rupture does not initiate early development of tendinopathy and that passive mechanical properties of the tendon will remain unchanged.

2 | MATERIALS AND METHODS

2.1 | Design

Sports-active patients with palpatory tendon soreness and exercise-related pain present for less than three months in either their Achilles or patellar tendon were included along with 50 healthy controls with no prior symptoms in their tendons (Table 1). In a cross-sectional study design, we categorized symptom-longevity into the following: 0-1 month (T1), 1-2 months (T2) or 2-3 months (T3) (Figure 1). The current study was approved by the local Ethical Committee of the Capital Region Copenhagen (H-1-6019-857) and is in compliance with the Helsinki Declaration. In addition, this study was approved by The Danish Data Protection Agency (BFH-2016-019, I-Suite nr: 04519) and was registered on ClinicalTrials.gov (BBH-128, NCT02797925). Informed consent from participants was obtained after the nature and possible consequences of the study were explained. Patients with tendon pain and

TABLE 1 Overview of tendinopathic patients and healthy control persons

	Tendinopathic patients (n = 200)		Healthy control persons (n = 50)
	Tendinopathic tendon	**Contra-lateral healthy tendon**	**Healthy tendon**
Achilles tendon (n = 155)			
US	n = 155[a] (T1: 42, T2: 52 & T3: 61)	n = 118[a] (T1: 32, T2: 41 & T3: 45)	n = 35
MRI	n = 123 (T1: 31, T2: 41 & T3: 51)		
Patellar tendon (n = 45)			
US	n = 45[a] (T1: 14, T2: 17 & T3: 14)	n = 34[a] (T1: 11, T2: 12 & T3: 11)	n = 15
MRI	n = 34 (T1: 9, T2: 13 & T3: 12)		
Mechanical test	n = 39 (T1: 11, T2: 16 & T3: 12)	n = 28[a] (T1: 8, T2: 11 & T3: 9)	
Biopsy	n = 39 (T1: 11, T2: 16 & T3: 12)	n = 29[a] (T1: 9, T2: 11 & T3: 9)	

Note: In total 250 participants were included in the study. Patients with Achilles or patellar tendinopathy underwent US and MRI investigations. In addition, patients with patellar tendinopathy had tendon biopsy sampled and mechanical properties examined. Healthy control persons underwent examination of their tendons bilaterally.

Abbreviations: MRI, magnetic resonance imaging; US, ultrasound.

[a]In 37 patients with Achilles tendinopathy (10 in month one, 11 in month two and 16 in month three) and in 11 patients with patellar tendinopathy (3 in month one, 5 in month two and 3 in month three) bilateral symptoms were present, thus excluding the use of the contra-lateral tendon as healthy control persons.
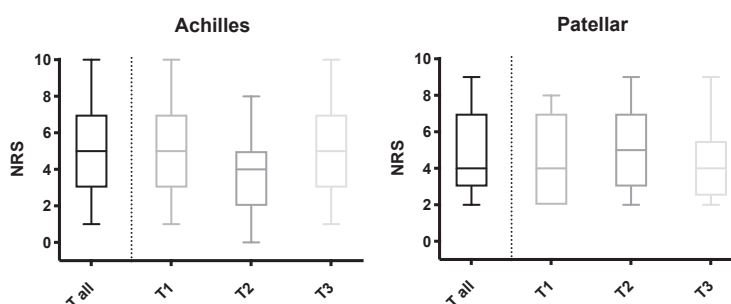
FIGURE 1 Subjective tendon pain score for Achilles (n = 145) and patellar patients (n = 43). Numeric Rating Scale (NRS) for pain was recorded for each patient (12 patients did not report NRS upon inclusion) during their first visit at the outpatient clinic. Severity of symptoms did not differ between the three groups ($P > .05$). T all: All tendinopathic patients. T1, T2 and T3: Tendinopathic with symptoms for 0-1, 1-2 and 2-3 months, respectively. Results are median (horizontal line), quartiles (upper and lower line in the box) and minimum/maximum value (the error bars)

soreness were divided into three groups based on the duration of their symptoms: 0-1 (T1), 1-2 months (T2) or 2-3 months (T3) (Figure 1). Patients were not allowed to have had any previous treatment (eg anti-inflammatory medication, glucocorticoid injection) in the affected tendon and were not allowed to have had any overuse injury previously in the affected tendon. The inclusion of participants was done from the out-patient sports clinic at Bispebjerg Hospital.

2.2 | Clinical evaluation and ultrasonographic measurements

Initially, a standardized clinical examination was carried out at the clinic. Patients were asked about their training history, symptoms and potential earlier treatments. In addition to basic information including medical history, family history, any ongoing infections, medication and profession, patients also filled out 3 questionnaires. One contained questions regarding physical

activity and injuries during their lifetime. The two other scores were a functional score questionnaire (VISA-Achilles and VISA-Patellar, respectively) and a Numeric Rating Scale (NRS) on pain in relation to activity. Both questionnaires have been previously evaluated for tendinopathy.[18,19] In the NRS questionnaire, patients were asked to score their average symptoms in relation to training according to NRS rating from 1-10 (most pain). Average symptoms were interpreted as an overall score during training. Height and weight were measured, and clinical examination involved inspection and palpation of Achilles or patellar tendons. Achilles tendinopathy patients were placed in prone position with both ankles hanging freely for ultrasound-scanning while patients with patellar tendinopathy were placed in supine position with their knees at 90°-flexion. The area with most soreness was marked and measured during clinical examination and was used as a guideline for ultrasound-scans. Signs of hyper-vascularization was found using power Doppler within the area of interest. The area with most power Doppler signal was determined by the investigator and was video-recorded in both longitudinal and transverse sections for at least 3 heart-cycles. Other signs of tendinopathy on ultrasound were based on tendon morphology: Dimensions, fibrillary structure, hyper- or hypo-echoic areas. Ultrasound-scanning was also performed on the contra-lateral non-symptomatic tendon in patients as well as bilaterally on healthy tendons in control participants. In each individual patient, tendon dimensions and Doppler recordings on the healthy tendon were determined at the same anatomical level as on the tendinopathic side. In healthy controls, the anatomical level was set to mid-portion of the tendon measured by a ruler. The diagnosis of Achilles or patellar tendinopathy was based on self-reported tendon-related pain with activity (lasting <3 months) and palpatory pain of the respective tendon (12.9% of Achilles patients (20/155) had insertional pain). Ultrasound-recordings on tendon dimensions and power Doppler were manually measured by the investigator in a blinded fashion using a plugin for ImageJ (FIJI, macOS 10.14.4).

2.3 | Magnetic resonance imaging

Magnetic resonance imaging-scans of participants were performed on a 3-T MRI Siemens Verio scanner with axial and sagittal T1-weighted turbo spin echo sequence (TE: 17; TR: 500; matrix: 512 × 512; FOV: 150 mm; Slice thickness: 3 mm). Achilles-tendinopathy patients were scanned in supine with their affected leg in a foot coil maintaining the ankle joint in neutral position. Patellar tendinopathy patients were in supine position with a supportive pillow placed under their knee to keep a slightly flexed position. Participants were instructed to refrain from strenuous physical activities on the day of MRI scanning. Tendon dimensions were measured in an open source PACS system (Horos, Version 2.4.1, Geneva, Switzerland). Specific slice position

in transverse plane was chosen for measurement of thickness based on measured point of maximum soreness from clinical examination. Cross-sectional area (CSA), width and thickness were manually measured by the investigator in a blinded fashion.

2.4 | Patellar biopsy sampling and mRNA analysis

In patients with patellar-related tendinopathy, a tendon biopsy was obtained in both the symptomatic tendon and on the contra-lateral non-symptomatic tendon. Patellar tendon biopsies have previously been performed in our lab.[20] As previously, biopsies were taken on both legs while the patient was lying in a supine position with knees flexed 45 degrees. Using sterile procedures patients were locally anesthetized with 2-3 mL 1% lidocaine-injection (Lidokain Mylan, 1 mg/mL, Mylan, Oslo, Norway) subcutaneously at the proximal origin of the patellar tendon. A medio-lateral skin incision of 10 mm was cut to insert the 14G semi-automated biopsy instrument (Bard Magnum, Bard biopsy systems, USA). The biopsy needle was angled at ~45 degrees relative to the tendon in proximal to distal direction before insertion and was aimed to obtain tissue in the symptomatic area of the tendon. Biopsies were obtained after clinical examination and MR-scans. Biopsy samples were typically 10-20 mg of tendon tissue Biopsies were dissected to free tendon tissue from fat and subcutaneous tissue. The tendon biopsy was snap-frozen in liquid nitrogen and stored at −80°C for later biochemical analyses. Biopsies were analyzed for mRNA expression by using routine-protocols previously described.[21] Briefly, tendon biopsies (~10 mg) were cut into 0.5-mm slices and then weighed, all at −20°C. The tendon tissue slices were homogenized in 1 mL TriReagent (Molecular Research Center, Cincinnati, OH) using stainless-steel beads and a bead mixer before Bromo-chloropropane separated the sample into an aqueous and organic phase. Glycogen was then added to the aqueous phase to facilitate RNA precipitation. The RNA was precipitated with isopropanol and washed with 75% ethanol before the pellet was dissolved in RNase-free water. The RNA concentration was determined with the RiboGreen assay (R-11490, Molecular Probes). Finally, 125 ng extracted tendon RNA was converted into cDNA using reverse transcriptase (OmniScript, Qiagen) and 0.5 μL loaded on real-time PCR with primers for specific targets (Table 2). The Ct values were related to a standard curve made with known concentrations of DNA oligos (Ultramer™ oligos, Integrated DNA Technologies, Inc, Leuven, Belgium) with a DNA sequence corresponding to the sequence of the expected PCR product. Based on these Ct values, and accounting for the PCR efficiency, the relative difference between unknown samples was determined. The specificity of the

TABLE 2 Primers for real-time RT-PCR. The official gene names are shown in parenthesis, when common names are used

Target	Genbank ID	Sense	Anti-sense
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGTCTTGGTGGTTTT
COL3A1	NM_000090.3	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC
TenascinC (TNC)	NM_002160.3	CAGCCAAGATCCAGGCACTCAA	GTCCTTGGGGAAGGGGTACAGG
Decorin (DCN)	NM_001920.5	GGTGGGCTGGCAGAGCATAAGT	TGTCCAGGTGGGCAGAAGTCA
Fibronectin (FN1)	NM_212482.3	TTTGCTCCTGCACATGCTTT	TAGTGCCTTCGGGACTGGGTTC
Tenomodulin (TNMD)	NM_022144.3	GCGCCAGACAAGCAAGTGAAGA	TCTCATCCAGCATGGGGTCAAA
MMP2	NM_004530.5	CCGCCTTTAACTGGAGCAAAAACA	TTGGGGAAGCCAGGATCCATTT
MMP3	NM_002422.4	GATCCTGCTTTGTCCTTTGATGCTGT	CTGAGGGATTTGCGCCAAAAGTG
TGFb1	NM_000660.6	GAGGTCACCCGCGTGCTAATG	CACGGGTTCAGGTACCGCTTCT
TGFb2	NM_003238.4	CCCAAAAGCCAGAGTGCCTGAA	ATGTAGCGCTGGGTTGGAGATG
TGFb3	NM_003239.4	TGCCAAAGAGATCCATAAATTCGACA	CCTTAGAGGTAATTCCTTTGGGGCA
IGF1-Ea (IGF1)	NM_000618.3	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
CTGF	NM_001901.3	TGCGAAGCTGACCTGGAAGAGA	GCCGTCGGTACATACTCCACAGAA
ITGB1	NM_002211.4	GGATATTACTCAGATCCAACCACAGCA	TCAATGGGATAGTCTTCAGCTCTCTTG
EGR1	NM_001964.3	GCAGCCCTACGAGCACCTGACC	AACTGGTCTCCACCAGCACCTTC
PDGFRA	NM_006206.6	AAGTTGCCCGAGGAATGGAGTTTTT	AGCCAGATCACGGTGGACACAA
SubstP (TAC1)	NM_013996.2	TGGTACGACAGCGACCAGATCAA	TCTCTGCAGAAGATGCTCAAAGGG
HSP70 (HSPA1A)	NM_005345.5	GTGGCTGGACGCCAACACCTT	TTACACACCTGCTCCAGCTCCTTC
ATF3	NM_001674.4	GCTGCAAAGTGCCGAAACAAGA	TGAGCCTTCAGTTCAGCATTCACA
ATF4	NM_001675.4	GGTCAGTCCCTCCAACAACAGCA	TCCAAGTCGAACTCCTTCAAATCCA
COX-1 (PTGS1)	NM_000962.3	GGTTTGGCATGAAACCCTACACCT	CCTCCAACTCTGCTGCCATCT
PTGFR	NM_000959.4	TGTATTTGTTGGAGCCCATTTCTGGTT	GGAGAGCAAAAAGTGTTGTTTCACAGG
VEGFA	NM_001025366.2	ATGACGAGGGCCTGGAGTGTGT	CTCCTATGTGCTGGCCTTGGTG
ANGPTL4	NM_139314.3	GGGACAAGAACTGCGCCAAGA	ACTGGCCGTTGAGGTTGGAATG
ATG12	NM_004707.4	CCAGACCAAGAAGTTGGAACTCTCTAT	GCAAGTTGATTTTCTTTGTGGTTC
Beclin1 (BECN1)	NM_003766.4	CGCAGCTGGATAAGCTGAAGAAACC	CGACCCAGCCTGAAGTTATTGATTG
MAP1LC3B	NM_022818.5	CAGATCCCTGCACCATGCCGT	TTGGTTGGATGCTGCTCTCGAAT
HBB	NM_000518.5	CTCAAGGGCACCTTTGCCACAC	ATGGGCCAGCACACAGACCAG

PCR products was confirmed by comparing the melt curves for the unknown samples with melt curves of the DNA oligos. Expressed values are normalized to the internal control, RPLP0, an mRNA that codes for the large ribosomal protein P0. GAPDH mRNA was then used to validate RPLP0 and showed no significant difference in gene expression across groups (Figure 6).

2.5 │ Patellar tendon biomechanical properties

Patients with patellar tendinopathy had their tendons tested for mechanical properties with ultrasound isometric methodology. A detailed description of experimental setup and measurement has been previously described.[22,23] Patients were instructed to warm-up on a cycle ergometer (Monark, Sweden) for 10 minutes to precondition their tendons, making results more reproducible.[24] They were then seated on a custom-made chair with knee joints fixed in 90° angle and performed ramped isometric knee extensions over 8 seconds. On each leg, 4 maximal contractions were recorded with 1-2 minutes rest in between repetitions. Ultrasound imaging was performed on a Hitachi Hi Vision, Ascendus machine (Hitachi Medical Corporation) using a 100 mm long linear array B-mode transducer at 10 MHz. A force transducer was attached around the ankle and then triggered by the investigator to be synchronized with ultrasound recordings. Femur and tibia length were measured for estimating the tendon moment arm and knee extensor moment to determine tendon force.[25] A custom Matlab script (Matlab R2016b, The MathWorks Inc, USA) using a cross-correlation algorithm

was used to track the tendon insertions on patella and tibia.[26] Around 10-20 tracking nodes were placed on either apex patellar or tuberositas tibia and tracking was performed twice at each location. Tendon deformation was determined as the distance between the insertions and was then correlated with force measurements using a custom-made excel template to generate a force-deformation curve. Data were fitted to a second-order polynomial using Sigma Plot (Version 10.0, Systat Software, Germany) and stiffness at maximal force was determined on the fitted curve. Stiffness was then normalized to normal contra-lateral leg within each patient with unilateral symptoms.

The ultrasound quality was not always sufficient for tracking on all four recorded ramps, in which case the investigators selected ultrasound videos based on three criteria: (a) Full visualization of patellar origin from apex patellar to insertion on tuberositas tibiae, (b) Synchronized force and deformation data and (c) Natural movement of the tendon and bones to produce smooth curves with minimal noise.

2.6 | Statistics and sample size calculation

Based on previously published mean values and standard deviations for tendon size and stiffness, the estimated sample size was n = 55 tendons (vs healthy controls) in order to detect the outcome parameters between (or within the same individual comparing left to right side subjects of 10% (significance level of $P < .05$ and power of 0.80). Data was divided over patellar and Achilles tendons according to the duration of symptoms, ie T1, T2 and T3 with mean ± SEM (standard error of mean) indicated in graphs and text. Normal contra-lateral leg within the patients was pooled into group C while healthy control was pooled into group HC. MRI tendon dimensions were normalized to an average of T1. US tendon dimensions, tendon mechanics and mRNA-expression were normalized to the normal contra-lateral leg (C). Gene expressions were log-transformed before statistical analysis and represented on graphs with geometric means ± back-transformed SEM. A one-way ANOVA was performed between group T1, T2 and T3. If the one-way ANOVA was significant, individual differences between T1, T2 and T3 were tested with a post hoc test (Tukey). A paired t-test was performed between normal contra-lateral leg (C) and affected leg (T all) while an unpaired t-test was performed between healthy control persons (HC) and tendinopathic patients (T all). As Doppler-area for the healthy legs was often not present, comparisons to those were performed with non-parametric equivalents to the *t*-test, Mann-Whitney *U*-test and Wilcoxon. Differences were considered significant when $P < .05$. All statistical analyses were performed with Graphpad Prism 8.

3 | RESULTS

3.1 | Clinical symptoms

All the included 200 patients (age: 37.99 ± 0.75 years, BMI: 24.18 ± 0.20 kg/m², physical training experience: 10.15 ± 0.75 years) and the 50 healthy controls (age: 30.58 ± 1.09 years, BMI: 21.12 ± 0.39 kg/m², physical training experience: 7.25 ± 0.92 years) were sports-active. The intensity during training sessions was 15-16 on a Borg scale (7 to 20 (most intense)) in both groups. Tendinopathic patients (T all) had a training duration of 7.5 ± 0.3 h/wk, a frequency of 5.5 ± 0.2 sessions/week and an intensity of 15.5 ± 0.1 Borg scale points. The healthy controls (HC) had a training duration of 6.5 ± 0.4 h/wk, a frequency of 4.4 ± 0.2 sessions/week and an intensity of 15.3 ± 0.2 Borg scale points. Almost half (44%) of the patients were runners although a wide variety of other sports disciplines were represented. All three tendinopathic groups reported a similar pain level (NRS) (Figure 1) at the time of the visit to the outpatient clinic. The overall average VISA score was 70.3 ± 1.1 points (of 100), and the group average was similar in all three groups.

3.2 | Tendon morphology and hyper-vascularization

Tendon CSA (determined by US) in Achilles tendinopathy patients were overall (80.1 ± 2.7 mm²) (T all) larger than both the contra-lateral healthy tendon (67.3 ± 2.0 mm²) (C) and the tendon of healthy control individuals (63.1 ± 1.9 mm²) (HC). For the patellar tendinopathy group, CSA was larger in T all (97.9 ± 6.0 mm²) compared to C (80.5 ± 3.9 mm²) (Figures 2 and 3). There was no difference in CSA (determined by MRI) between the three groups (T1, T2, T3) for the Achilles nor for the patellar tendon (Figures 2 and 3). The anterior-posterior (A-P) thickness (determined by US) revealed that patients with early tendinopathy overall showed increased thickness compared to HC ($P < .05$) in both the Achilles and patellar tendon. Although there was no difference over time for A-P thickness for Achilles nor patellar tendons with US or MRI, the A-P thickness was already significantly higher than the contralateral tendon in the same individual early (T1) in Achilles tendon. In contrast, this occurred only late (T3) in the patellar tendon (Figures 2 and 3). The width of the tendon was unchanged (US and MRI) over time and was never elevated compared to C in patellar tendon (Figures 2 and 3). In contrast, the Achilles tendon overall width in tendinopathy (T all) was elevated compared to C and in the subgroups only in T3 a significant rise vs C was observed ($P < .05$). US determined Doppler flow of tendinopathic tendons (all three groups of both Achilles and patellar tendons pooled) was always larger than the ones observed in contra-lateral control tendons ($P < .05$, Figure 4).
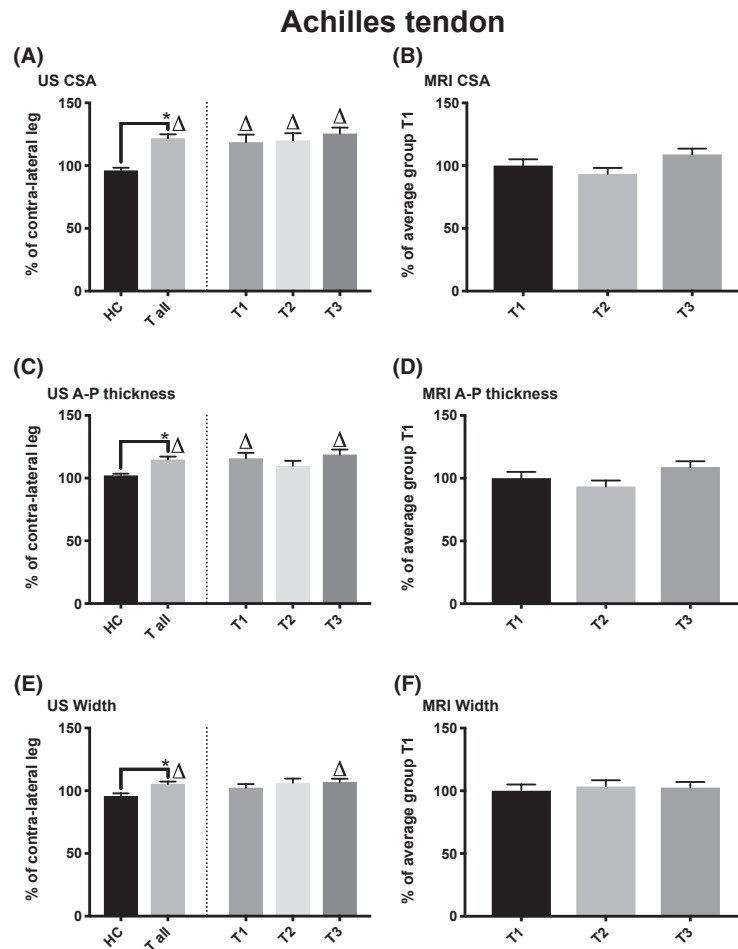
Achilles tendon

FIGURE 2 Tendon dimensions of Achilles tendinopathy patients with unilateral symptoms and healthy control persons (n = 35). Ultrasound-scanning (US) were performed on both legs (n = 155). The affected leg was then normalized to contra-lateral leg within each patient (A, C, E). Healthy controls (HC) were measured on the mid-portion of their tendon and then normalized to same anatomical level on their contra-lateral leg. Magnetic resonance imaging (MRI) was performed unilaterally on each patient (n = 123, bilateral symptoms included) and normalized to an average of T1 (n = 31; CSA: 89.28 mm^2, A-P Thickness: 6.76 mm & Width: 16.08 mm). CSA (A, B), A-P thickness (anterior-posterior thickness) (C, D) and width (E, F) were measured on transverse images corresponding to the tendon-area with most soreness. HC, Healthy control persons. *$P < .05$ versus HC and $^\Delta P < .05$ versus the contra-lateral asymptomatic leg in the same patient. Results are means ± SEM

3.3 | Gene expression

The mRNA analysis from the biopsies taken on patellar tendinopathy patients demonstrated that most growth factors and tendon components remained unchanged in early tendinopathy. Only mRNA for TGF-beta 1 was significantly elevated in T3 compared to T2 while that of MMP2 was significantly elevated in group T all compared to group C (Figure 5). Further, markers of cell stress, inflammation, autophagy and angiogenesis remained unchanged in all three groups (Figure 6). Finally, the nociceptive marker, substance P was significantly higher in group T3 compared to group T1 (Figure 6).

3.4 | Mechanical properties

Tendon stiffness at max force remained unchanged in the affected tendons compared to the contra-lateral healthy tendon in all groups (Figure 7).
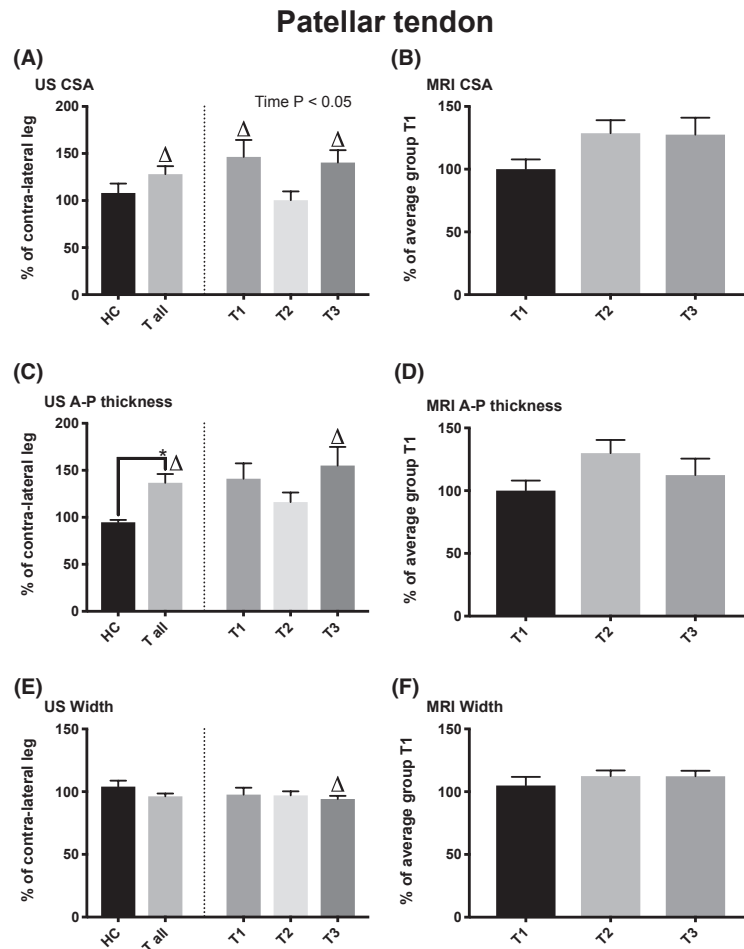
FIGURE 3 Tendon dimensions of patellar tendinopathy patients with unilateral symptoms and healthy control (HC) persons (n = 13). Ultrasound-scanning (US) were performed on both legs (n = 45). The affected leg was then normalized to contra-lateral leg within each patient. Magnetic resonance imaging (MRI) was performed unilaterally on each patient (n = 34, bilateral symptoms included) and normalized to an average of T1 (n = 9; CSA: 90.07 mm^2, A-P Thickness: 4.35 mm & Width: 24.52 mm). CSA (A, B), A-P thickness (anterior-posterior thickness, C, D) and width (E, F) were measured on transverse images corresponding to the tendon-area with most soreness. *P < .05 versus healthy controls and $^\Delta P$ < .05 versus the contra-lateral asymptomatic leg in the same patient. Results are means ± SEM

4 | DISCUSSION

To the best of our knowledge, this study demonstrates for the first time that already in the early phases of tendinopathy there is a gradual increase in angiogenesis, increased tendon size and rise in some tissue anabolic/catabolic signaling while tendon mechanical tissue properties remain unchanged. This suggests that tendinopathy pathogenesis represents disturbed tissue homeostasis in response to the repeated tendon overloading.

The US data demonstrate that patients with tendinopathy in the early phase (T1-3) have a larger tendon cross—sectional area compared to the contralateral asymptomatic side and compared to HC individuals, which is consistent with previous studies in chronic tendinopathy.[27] The data also show that even in early in tendinopathy the CSA is increased compared to both the asymptomatic contralateral tendon in the same patient as well as compared to dimensions of tendon in healthy control individuals tendon, which is primarily due to an increase in tendon thickness (Figures 2 and 3). For Achilles tendon already early in tendinopathy, A-P thickness was elevated compared to the contralateral healthy control tendon, whereas width
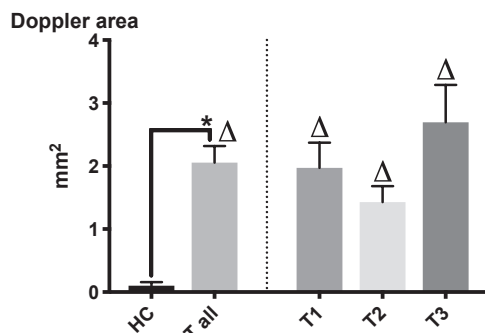
Doppler area

FIGURE 4 Ultrasound Doppler area on affected leg of patients (bilateral symptoms included, n = 200) and normal leg of healthy control persons (n = 48). Power Doppler was visualized from the tendon-area with most soreness in patients while it was from mid-portion of healthy control (HC) persons. Doppler area is expressed as absolute values in cm². Statistically significant differences were calculated using one-way ANOVA for T1, T2, and T3 with Tukey's post hoc test. Difference between T all and HC (and between T1, T2 and T3, respectively, with HC) was examined with a Mann-Whitney *U* test. A Wilcoxon was performed to compare Tall, T1, T2 and T3 to their contra-lateral leg of C1, C2 and C3, respectively. *$P < .05$ versus HC and $^\Delta P < .05$ versus the contra-lateral asymptomatic leg in the same patient. Results are means ± SEM

was only elevated at 2-3 months (Figure 2). In the patellar tendon, a change in A-P thickness could only be seen later in the tendinopathy development (Figure 2), while no elevation in width could be detected (Figure 3). In addition, the changes in Doppler determined flow seemed to accompany the increasing tendon size, which suggests that angiogenesis and hyper-vascularization explained a major part of the elevated tendon size the first 3 months of tendinopathy (Figure 4). The relative role between angiogenesis, edema and new tendon tissue formation over the course of early tendinopathy cannot be distinguished from the present data. However, it is noteworthy that the rise in TGF beta1 signaling was detected only between group T2 and T3 (three months after symptom onset), suggesting that at least some connective tissue anabolic stimulus appears to be upregulated in the later part of the early tendinopathy development phase. Surprisingly, no major changes were detected over time in tendinopathy for angiogenic factors such as VEGF or ANGPTL4 (Figure 6). This could indicate that stimulation of angiogenesis is initiated very early in the tendinopathy process and that no additional rise in angiogenic factors is required for vessel proliferation from 1-3 months into the development of tendinopathy. This fits with reports of tendon angiogenesis in the Achilles tendon in competitive distance runners compared to non-runners despite no signs or symptoms of tendinopathy, and the up-regulation of VEGF both tendon in response to mechanical

loading.[28-30] Clearly, the cross-sectional design of the study has inherent limitations, but by including a large sample size at each time point, the experimental setup allows for a detailed characterization of tendinopathy development in the early disease period.

In long term chronic tendinopathy an upregulation of mRNA for both tendon components such as collagen I and III as well as for growth factors like TGF-beta and proteolytic MMP factors have been reported.[6] Further, in chronic tendinopathy the protein content of proteoglycans and proteins like versican, aggrecan and fibromodulin have been shown to be increased.[15,31] In the present study of early tendinopathy, there were much more discrete changes, and only TGF-beta increased in the third month of the tendinopathy phase whereas the other regulators or components of tendon tissue and their turnover were unchanged during early tendinopathy development (Figure 5). We targeted our mRNA approach and included markers for cell stress, autophagy, and inflammation, but could not detect any changes in the early phase of tendinopathy for these markers either (Figure 6). Collectively these observations indicate that there is only an upregulation of major tissue modifying factors in the chronic phase of tendinopathy. It was somewhat surprising that we did not observe any signs of changes in mRNA of cyclooxygenase-1 (COX-1) or prostaglandin F receptor. Although this finding corroborates other reports on chronic tendinopathic tissue, we expected some inflammatory signs in the early phase of tendinopathy.[11,12,15] We did observe an increase in TGF-beta as an indicator of some inflammation, but the overall picture does not imply that early tendinopathy has a strong inflammatory component. We have not investigated regulators of inflammation and resolution, and it cannot be excluded that more discrete indicators of inflammation may have played a role in early tendinopathy.[11,12] It should be noted that mRNA only provides an indication of cell production of a given substance and therefore does not directly correspond to the amount of protein present in the tissue. However, because we investigated the very early stage of tendinopathy, we deemed it justified to give mRNA measurements a priority in order to detect potential early responses. In the present study, we have reported the mRNA results normalized to results obtained on the contra-lateral asymptomatic tendon, which may underestimate the difference between healthy and diseased tendon, as we cannot entirely exclude that processes are already ongoing in the asymptomatic contra-lateral tendon related to subclinical tendinopathy.

The rise in substance-P mRNA over time in all three groups of early tendinopathy patients is interesting for several reasons. First, since substance-P is a nociceptive stimulator and since all three tendinopathic groups presented with the same pain level whereas there is a significantly higher expression in T3 vs T1, it could be suggested that a gradual increase in nociceptive

Tendon matrix proteins and turnover
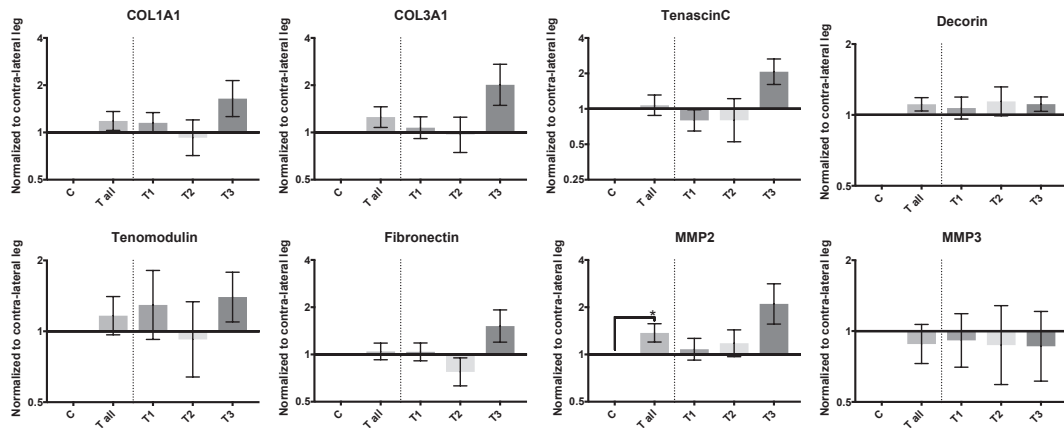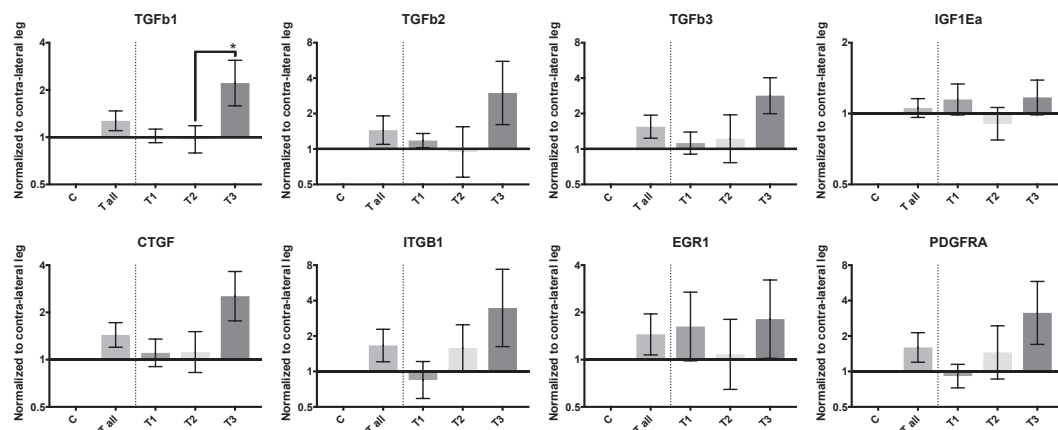

Tendon growth factors

FIGURE 5 mRNA-expression in patellar biopsies for tendon turnover and growth. Values are normalized to normal contra-lateral leg within each patient (n = 29, no patients with bilateral symptoms included). C, Normal contra-lateral leg within each patient; COL1A1, Collagen type I Alpha 1 chain; COL3A1, Collagen type 3 Alpha 1 chain; CTGF, Connective Tissue Growth Factor; EGR1, Early Growth Response protein 1; IGF, Insulin-like Growth Factor; ITGB1, Integrin beta-1; MMP, Matrix Metalloproteinase; PDGFRA, Platelet-Derived Growth Factor Receptor A; TGFb, Transforming Growth Factor beta. *$P < .05$ between indicated groups. Results are geometric means ± back-transformed SEM

pathway stimulation over this period could take place presently. Secondly, in addition to the nociceptive role that substance-P has, earlier studies have shown that substance-P is present in human tendinopathy and that it can be stimulated by loading of the tendon.[32,33] Interestingly it has recently been demonstrated that exogenous administration of substance P during tendon rupture healing stimulate both angiogenesis and collagen organization, which suggests that the rise in vascularity and rise in TGF-beta could be coupled to a rise in substance P expression and synthesis albeit speculative at this point.[34]

There were no changes in tendon stiffness compared to the contralateral leg during the first three months of tendinopathy (Figure 7). This supports the notion mechanical properties are unaltered in the early phase of tendinopathy. In chronic tendinopathy (>3 months duration) some studies have demonstrated a decrease in tendon mechanical properties of the tendinopathic tendon[35,36] but not all.[20,37] The present study focused on early tendinopathy, and therefore it cannot be excluded that tendon mechanical properties may be altered at a later stage. However, the fact that there was
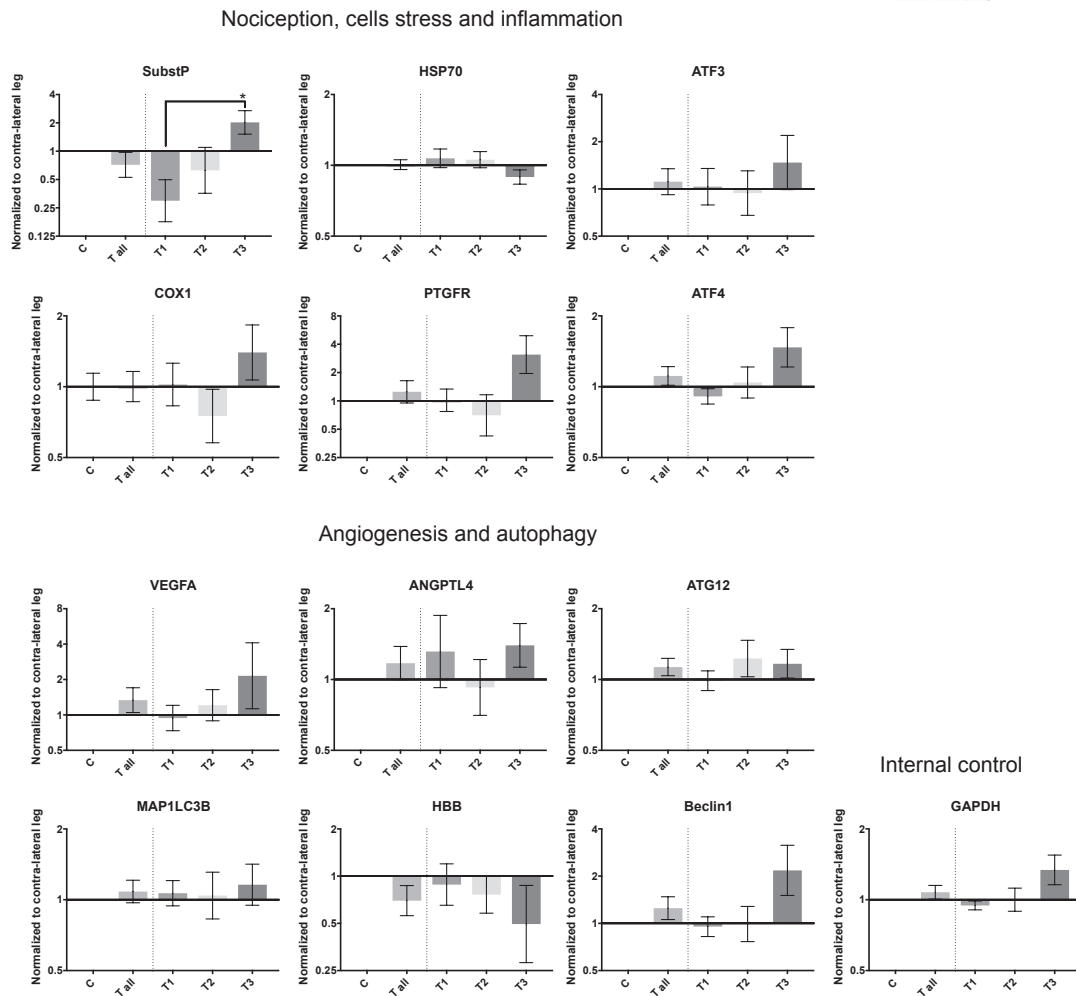
FIGURE 6 mRNA-expression in patellar biopsies for tendon inflammation. Values are normalized to normal contra-lateral leg (n = 29, no patients with bilateral symptoms included). ANGPTL4, Angiopoietin Like 4; ATF3, Activating Transcription Factor 3; ATG12, Autophagy Related 12; COX-1, Cyclooxygenase 1; HBB, Hemoglobin subunit beta; HSP70, 70 kilodalton heat shock proteins; MAP1LC3B, Microtubule Associated Protein 1 Light Chain 3 Beta; PTGFR, Prostaglandin F Receptor; SubstP, Substance P; VEGFA, Vascular Endothelial Growth Factor A. *$P < .05$ between indicated groups. Results are geometric means ± back-transformed SEM

no change in tendon stiffness in the first 3 months of tendinopathy disputes the notion that the tendinopathy per se relates to mechanical properties of the tendon. It is noteworthy that even short periods of immobilization leads to a decrease in tendon stiffness.[23] Therefore it cannot be ruled out that previous investigations showing a reduction in tendon stiffness with long-term chronic tendinopathy, in fact, represent an effect of reduced training and a relative unloading of the tendinopathic tendon rather than pathological changes associated with tendinopathy per se.[38,39] The fact that we did not observe any changes in normalized tendon stiffness with

the development of tendinopathy supports the notion that no major disruption of the tendon occurs to initiate the tendinopathy. This is partly supported by the lack of a clearly detectable inflammatory response, which we would expect to be prominent if tendinopathy was initiated by a mechanical trauma. Further, our findings support the assumption that a tendinopathic tendon maintains the microstructural pattern of an intact tendon with fibrils spanning the entire tendon length and that loading is therefore distributed homogenously in the tendon.[40,41] There is however, an increased CSA which theoretically would influence the calculation of tendon modulus.
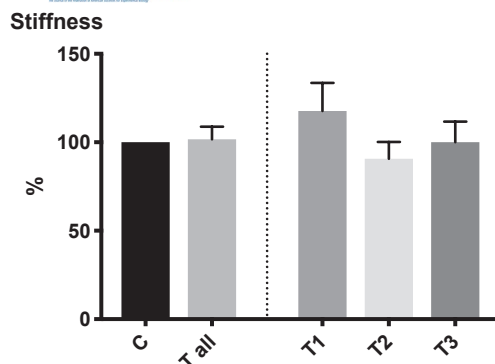
Stiffness

FIGURE 7 Mechanical stiffness of patellar tendons at maximal force. For each patient, stiffness on the affected side was relative to the unaffected contra-lateral leg (C) (no bilateral patients included). All of the affected legs are grouped in T all (n = 28). Patients were additionally grouped in T1 (n = 8), T2 (n = 11) and T3 (n = 9) according to symptom duration. Stiffness is expressed as a normalized value. Results are means ± SEM

However, the increased CSA represents vessels and water content, and these components are unlikely to be tensile bearing. Clearly, tendon mechanical properties can be influenced by other factors than the fibril structures, and enzymatic as well as non-enzymatic cross-links have been shown to influence tendon stiffness.[42] It was beyond the scope of the present study to measure these factors. The observations of the present study support our hypothesis that tendinopathy pathogenesis is not driven by a partial rupture of the tendon or structural changes that result in altered mechanical properties. It should be noted that the patients in this study were slightly older than the healthy controls. This was an unintended outcome because we used the tendinopathetic patient's preferred training partners as a healthy control subject. Although aging does change tendon properties, the difference between groups in the present study was small and unlikely to explain the obtained differences.[43] Furthermore, many of the comparisons were made within-subject and thus was unaffected by age.

In summary, the present study provides important insights into the sequence of pathophysiological changes in structure and tissue turnover signaling during the early phase of tendinopathy development in humans. We observed a gradual increase in angiogenesis, tendon size and tissue anabolic signaling over the first months of tendinopathy development whereas no change was observed for passive mechanical tissue properties. Altogether, these findings suggests that the pathogenesis of tendinopathy represents an altered tissue homeostasis by repeated mechanical overloading rather than representing a repair response to a partial rupture of the tendon.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Conception: P.H.T.T., P.M., M.B., and M.K.; research design: P.H.T.T., J.D.N., P.H., C.C., M.B., P.M., and M.K.; data acquisition/analysis: P.H.T.T., N.M.M., R.S.P., R.B.S., J.D.N., A.H.Z., P.H., P.S., and M.K.; resource assistance: N.M.M., R.S.P., and P.S.; writing, drafting, and editing: P.H.T.T., N.M.M., R.S.P., R.B.S., J.D.N., P.H., P.S., C.C., A.H.Z., M.B., P.M., and M.K.; project administration: M.K.

DATA AVAILABILITY STATEMENT
All data associated with this study are present in the paper.

REFERENCES
1. Lopes AD, Hespanhol LC, Yeung SS, Costa LOP. What are the main running-related musculoskeletal injuries? A systematic review. *Sport Med*. 2012;42(10):891-905.
2. Magnusson SP, Kjaer M. The impact of loading, unloading, ageing and injury on the human tendon. *J Physiol*. 2018;597(5):1283-1298.
3. Magnusson SP, Langberg H, Kjaer M. The pathogenesis of tendinopathy: balancing the response to loading. *Nat Rev Rheumatol*. 2010;6(5):262-268.
4. Miller BF, Olesen JL, Hansen M, et al. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol*. 2005;567(3):1021-1033.
5. Pingel J, Lu Y, Starborg T, et al. 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: evidence of tenocyte and matrix buckling. *J Anat*. 2014;224(5):548-555.
6. Pingel J, Fredberg U, Qvortrup K, et al. Local biochemical and morphological differences in human Achilles tendinopathy: a case control study. *BMC Musculoskelet Disord*. 2012;13:13-54.
7. De Mos M, Van El B, Degroot J, et al. Achilles tendinosis: changes in biochemical composition and collagen turnover rate. *Am J Sports Med*. 2007;35(9):1549-1556.
8. Gärdin A, Rasinski P, Berglund J, Shalabi A, Schulte H, Brismar TB. T2∗ relaxation time in Achilles tendinosis and controls and its correlation with clinical score. *J Magn Reson Imaging*. 2016;43(6):1417-1422.
9. Leppilahti J, Puranen J, Orava S. Incidence of Achilles tendon rupture. *Acta Orthop Scand*. 1996;67(3):277-279.
10. Jones GC, Corps AN, Pennington CJ, et al. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human Achilles tendon. *Arthritis Rheum*. 2006;54(3):832-842.

11. Millar NL, Hueber AJ, Reilly JH, et al. Inflammation is present in early human tendinopathy. *Am J Sports Med*. 2010;38(10):2085-2091.

12. Dakin SG, Martinez FO, Yapp C, et al. Inflammation activation and resolution in human tendon disease. *Sci Transl Med*. 2015;7(311):311ra173.

13. Pingel J, Fredberg U, Mikkelsen LR, et al. No inflammatory gene-expression response to acute exercise in human Achilles tendinopathy. *Eur J Appl Physiol*. 2013;113(8):2101-2109.

14. Kjaer M, Bayer ML, Eliasson P, Heinemeier KM. What is the impact of inflammation on the critical interplay between mechanical signaling and biochemical changes in tendon matrix? *J Appl Physiol*. 2013;115(6):879-883.

15. Heinemeier KM, Øhlenschlæger TF, Mikkelsen UR, et al. Effects of anti-inflammatory (NSAID) treatment on human tendinopathic tissue. *J Appl Physiol*. 2017;123(5):1397-1405.

16. Öhberg L, Lorentzon R, Alfredson H. Neovascularisation in Achilles tendons with painful tendinosis but not in normal tendons: an ultrasonographic investigation. *Knee Surg Sport Traumatol Arthrosc*. 2001;9(4):233-238.

17. Kundert H-P, Metzdorf A, Hodler J, et al. Achilles tendons: clinical relevance of neovascularization diagnosed with power doppler US. *Radiology*. 2003;227(2):556-560.

18. Robinson JM, Cook JL, Purdam C, et al. The VISA-A questionnaire: a valid and reliable index of the clinical severity of Achilles tendinopathy. *Br J Sports Med*. 2001;35(5):335-341.

19. Iversen JV, Bartels EM, Jørgensen JE, et al. Danish VISA-A questionnaire with validation and reliability testing for Danish-speaking Achilles tendinopathy patients. *Scand J Med Sci Sport*. 2016;26(12):1423-1427.

20. Kongsgaard M, Qvortrup K, Larsen J, et al. Fibril morphology and tendon mechanical properties in patellar tendinopathy: effects of heavy slow resistance training. *Am J Sports Med*. 2010;38(4):749-756.

21. Heinemeier KM, Lorentzen MP, Jensen JK, et al. Local trauma in human patellar tendon leads to widespread changes in the tendon gene expression. *J Appl Physiol*. 2016;120(9):1000-1010.

22. Hansen P, Bojsen-Moller J, Aagaard P, Kjaer M, Magnusson SP. Mechanical properties of the human patellar tendon, in vivo. *Clin Biomech*. 2006;21(1):54-58.

23. Boesen AP, Dideriksen K, Couppe C, et al. Effect of growth hormone on aging connective tissue in muscle and tendon: gene expression, morphology, and function following immobilization and rehabilitation. *J Appl Physiol*. 2014;116(2):192-203.

24. Maganaris CN. Tendon conditioning: artefact or property? *Proc R Soc B Biol Sci*. 2003;270:39-42.

25. Visser JJ, Hoogkamer JE, Bobbert MF, Huijing PA. Length and moment arm of human leg muscles as a function of knee and hip-joint angles. *Eur J Appl Physiol Occup Physiol* 1990;61(5-6):453-460.

26. Beyer R, Agergaard A-S, Magnusson SP, Svensson RB. Speckle tracking in healthy and surgically repaired human Achilles tendons at different knee angles-A validation using implanted tantalum beads. *Transl Sport Med*. 2018;1(2):79-88.

27. Nadeau MJ, Desrochers A, Lamontagne M, Larivière C, Gagnon DH. Quantitative ultrasound imaging of Achilles tendon integrity in symptomatic and asymptomatic individuals: reliability and minimal detectable change. *J Foot Ankle Res*. 2016;9:9-30.

28. Hullfish TJ, Hagan KL, Casey E, Baxter JR. Achilles tendon structure differs between competitive distance runners and nonrunners despite no clinical signs or symptoms of midsubstance tendinopathy. *J Appl Physiol*. 2018;125(2):453-458.

29. Zheng W, Seftor EA, Meininger CJ, Hendrix MJ, Tomanek RJ. Mechanisms of coronary angiogenesis in response to stretch: role of VEGF and TGF-beta. *Am J Physiol Heart Circ Physiol*. 2001;280(2):H909-H917

30. Pufe T, Petersen W, Tillmann B, Mentlein R. The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch*. 2001;439(4):579-585.

31. Parkinson J, Samiric T, Ilic MZ, Cook J, Feller JA, Handley CJ. Change in proteoglycan metabolism is a characteristic of human patellar tendinopathy. *Arthritis Rheum*. 2010;62(10):3028-3035.

32. Backman LJ, Andersson G, Wennstig G, Forsgren S, Danielson P. Endogenous substance P production in the Achilles tendon increases with loading in an in vivo model of tendinopathy-peptidergic elevation preceding tendinosis-like tissue changes. *J Musculoskelet Neuronal Interact*. 2011;11(2):133-140.

33. Gotoh M, Hamada K, Yamakawa H, Inoue A, Fukuda H. Increased substance P in subacromial bursa and shoulder pain in rotator cuff diseases. *J Orthop Res*. 1998;16(5):618-621.

34. Ackermann PW, Li J, Lundeberg T, Kreicbergs A. Neuronal plasticity in relation to nociception and healing of rat achilles tendon. *J Orthop Res*. 2003;21(3):432-441.

35. Arya S, Kulig K. Tendinopathy alters mechanical and material properties of the Achilles tendon. *J Appl Physiol*. 2010;108(3):670-675.

36. Child S, Bryant AL, Clark RA, Crossley KM. Mechanical properties of the achilles tendon aponeurosis are altered in athletes with achilles tendinopathy. *Am J Sports Med*. 2010;38(9):1885-1893.

37. Couppé C, Kongsgaard M, Aagaard P, et al. Differences in tendon properties in elite badminton players with or without patellar tendinopathy. *Scand J Med Sci Sport*. 2013;23(2):e89-e95.

38. de Boer MD, Maganaris CN, Seynnes OR, Rennie MJ, Narici MV. Time course of muscular, neural and tendinous adaptations to 23 day unilateral lower-limb suspension in young men. *J Physiol*. 2007;583(3):1079-1091.

39. Couppé C, Suetta C, Kongsgaard M, et al. The effects of immobilization on the mechanical properties of the patellar tendon in younger and older men. *Clin Biomech* 2012;27(9):949-954.

40. Svensson RB, Herchenhan A, Starborg T, et al. Evidence of structurally continuous collagen fibrils in tendons. *Acta Biomater*. 2017;50:293-301.

41. Rigozzi S, Stemmer A, Müller R, Snedeker JG. Mechanical response of individual collagen fibrils in loaded tendon as measured by atomic force microscopy. *J Struct Biol*. 2011;176:9-15.

42. Herchenhan A, Uhlenbrock F, Eliasson P, et al. Lysyl oxidase activity is required for ordered collagen fibrillogenesis by tendon cells. *J Biol Chem*. 2015;290(26):16440-16450.

43. Svensson RB, Heinemeier KM, Couppé C, Kjaer M, Magnusson SP. Effect of aging and exercise on the tendon. *J Appl Physiol*. 2016;121(6):1353-1362.

Paper 2 - Study II

Physiological Reports

ORIGINAL RESEARCH

The influence of fibrillin-1 and physical activity upon tendon tissue morphology and mechanical properties in mice

Peter H. T. Tran[1,2,3] iD, Tanja Skrba[1,2,3], Elisabeth Wondimu[1], Giuseppina Galatioto[1], René Brüggebusch Svensson[2,3,4], Annesofie T. Olesen[2,3], Abigail L. Mackey[2,3,5] iD, S. Peter Magnusson[4], Francesco Ramirez[1] & Michael Kjaer[2,3]

1 Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York
2 Institute of Sports Medicine, Department of Orthopedic Surgery M, Bispebjerg Hospital, Copenhagen, Denmark
3 Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
4 Department of Physical and Occupational Therapy, Bispebjerg Hospital, Copenhagen, Denmark
5 Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Abstract

Fibrillin-1 mutations cause pathological changes in connective tissue that constitute the complex phenotype of Marfan syndrome. In this study, we used fibrillin-1 hypomorphic and haploinsufficient mice ($Fbn1^{mgr/mgR}$ and $Fbn1^{+/-}$ mice, respectively) to investigate the impact of fibrillin-1 deficiency alone or in combination with regular physical activity on tendon tissue morphology and mechanical properties. Morphological and biomechanical analyses revealed that $Fbn1^{mgr/mgR}$ but not $Fbn1^{+/-}$ mice displayed smaller tendons with physical properties that were unremarkable when normalized to tendon size. $Fbn1^{mgR/mgR}$ mice ($n = 43$) $Fbn1^{+/-}$ mice ($n = 27$) and wild-type mice (WT, $n = 25$) were randomly assigned to either control cage conditions ($n = 54$) or to a running on a running wheel for 4 weeks ($n = 41$). Both fibrillin-1-deficient mice ran voluntarily on the running wheel in a manner similar to WT mice (3–4 km/24 h). Regular exercise did not mitigate aneurysm progression in $Fbn1^{mgR/mgR}$ mice ($P < 0.05$) as evidenced by unmodified median survival. In spite of the smaller size, tendons of fibrillin-1-deficient mice subjected to regular exercise showed no evidence of overt histopathological changes or tissue overload. We therefore concluded that lack of optimal fibrillin-1 synthesis leads to a down regulation of integrated tendon formation, rather than to a loss of tendon quality, which also implies that fibrillin-1 deficiency in combination with exercise is not a suitable animal model for studying the development of tendon overuse (tendinopathy).

Introduction

Mutations in the extracellular matrix (ECM) protein fibrillin-1 cause pathological changes in the connective tissue and results in the pleiotropic manifestations of Marfan syndrome (MFS), which includes thoracic aortic aneurysm (TAA) (Ramirez et al., 2018). It is unknown to what extent tendon tissue is influenced by fibrillin-1 deficiency. While only 2% of tendon dry weight is composed of elastic fibers compared to 60–80% collagen fibers, fibrillin-1 might still play an important role for tendon adaptation to mechanical loading (Thorpe et al., 2012).

Despite the impressive tensile strength of tendons, over-loading of tendon tissue in humans in relation to occupational- or leisure activity represents a significant clinical and socioeconomic burden, but our knowledge regarding the pathogenesis of tendinopathy limited (Magnusson et al., 2010; Heinemeier et al., 2013; Pingel et al., 2014; Dakin et al., 2015). For example, we still do not know whether voluntarily running in MFS results in a beneficial physiological adaptation of tendon tissue or possibly in a tendinopathic phenotype due to tissue overloading. It is equally unclear what the impact of voluntarily running might be on cardiovascular disease in MFS. Contrary to the believe that increased blood pressure during voluntarily running would exacerbate TAA progression, it has recently been reported that moderate exercise actually decreased the rate of aneurysm growth in mice with a mild non-lethal form of MFS (Gibson et al., 2017; Thijssen et al., 2019). Unfortunately there are no data in the literature of controlled trials that have longitudinally evaluated how exercise influences TAA progression in MFS patients (Thijssen et al., 2019).

To address the aforementioned issues, we analyzed biomechanical and histological properties of tendons isolated from wild-type (WT) mice and mice with different degrees of fibrillin-1 deficiency (50 and 80% deficiency) that had been subjected to either control cage conditions or voluntary running. The results of our experiments demonstrated that fibrillin-1-deficient mice tolerated voluntarily running comparable to WT mice without a change in median survival or any sign of tendon histopathology or tissue overload.

Methods

Animals

All experiments used 3- to 4-week-old male fibrillin-1-deficient mice and sex-and age-matched WT littermates maintained on the C57BL/6J genetic background. Fibrillin-1-deficient mice included Fbn1$^{mgR/mgR}$ mice, which produce ~ 20% of normal fibrillin-1 and display early onset progressively severe MFS (median survival 3 months), and Fbn1$^{+/-}$ mice, which produce ~ 50% of normal fibrillin-1 and show no appreciable clinical signs of MFS and have a normal life span (Pereira et al., 1997; Carta et al., 2009). The Institutional Animal Care and Use Committees of the Icahn School of Medicine at Mount Sinai in New York City reviewed and approved all animal studies.

Experimental setup

The mice were randomized to either control (cage with no access to running wheel) or to wheel-running regimen (cage equipped with running wheel). The period of voluntarily running or sedentary control was from week 4 to week 8 when the mice were euthanized for tissue collection. Mice in the wheel-running group were single-housed with a custom-made running wheel and had voluntary access to the running wheel during both diurnal and nocturnal cycles. The running wheel consisted of a 0.11-m diameter wheel connected to sampling box and could rotate freely in the cage. Mice were housed in polycarbonate regular temperature cages (dimensions 257 mm × 483 mm × 152 mm) (PC10196-RT, Allentown) with wire bar lids in stainless steel (WBL 1019 MMB, Rim Rod Design, Allentown). The lid was lifted 10 mm to fit the running wheel and have it run freely. There was an internal resistance in the running wheel system of 1.45 g on average (min: 1.03 g and max: 2.00 g) and therefore all running wheels were calibrated to have a resistance of 2.00 g using lead weights in the sampling box. Wheel revolutions would rotate a plastic plate with four holes inside the sampling box, allowing diode light to pass through the holes and into a light receiver. Thus, it was possible to count every 45° degrees rotation as a light impulse (light–no light), eight impulses would equal one revolution. Data samples were automatically stored on local SD cards at 1 Hz and collected by the investigators at 9 AM every day when the mice were least active. The lights in the animal facility were turned off at 7 PM and back on at 7 AM to mimic a nocturnal lifestyle. All cages had bedding and *ad libitum* access to food and water. The temperature in the facility was fixed at 22°C. Due to a power outage seven mice had insufficient data for analysis and were omitted from the results. The remaining data were analyzed in Matlab (R2017a, Mathworks) to obtain distance and velocity. Average velocity was calculated as the number of revolutions if the wheel rotated more than a set threshold of 45° during 1 sec and divided by time passed until the wheel was hitting the threshold. The number of revolutions was converted to distance using the formula for circumference of a circle. All procedures and animal care were approved by The American Association for Laboratory Animal Science (IACUC).

Tissue sampling

After euthanizing at 8 weeks with carbon dioxide (100% at flow 3 L/min) the Achilles and flexor digitorum longus tendons of the forelimbs were dissected free of other tissues and the hindlimbs were collected with all tissue intact for histology. The flexor digitorum longus tendon was packed in gauze with PBS (phosphate-buffered saline) and stored at −80°C before biomechanical testing. The Achilles tendons for histology were formalin-fixed as whole limbs in 4% paraformaldehyde for 48 h at 4°C and

140

were placed in a vertical position with the ankle in neutral position and the femur in 45° to tibia (Cikach et al., 2018). Sections of 2 micrometer were cut using a microtome and then placed in a 37° Celsius water bath to be picked up by superfrost plus glass slides. An ice bucket with dry-ice was placed on top of the microtome to cool down the paraffin block during sectioning and cold forceps were used to handle the sections. Slides were left to dry overnight at 32°C. Each limb provided approximately two to four sections per slide and 30–35 slides in total. An estimated 50% of the sections were not usable for histochemistry either because they were shredded by the microtome because of calcified bone present in the sample or due to suboptimal transfer from microtome to the water bath. Necropsy was a standardized procedure for all mice and confirmed the diagnosis of aortic aneurysms in $Fbn1^{mgR/mgR}$ mice that was lost during the experiment.

Biomechanical testing

Each end of the flexor digitorum longus tendon was dried and glued to clamps, using commercial superglue. The middle part of the tendon was kept moist by wrapping it in a thin piece of gauze soaked in PBS. The clamps were part of a custom-made Deben mechanical rig (20N tensile stage, Petri dish version, Deben Ltd, Stuffolk, UK). Deben software was used to control movement of the clamps. The setup was then secured to actuators of the linear mechanical testing machine with the tendon sub-emerged in PBS. To remove any initial slack of the tendon before testing 0.1N was applied to the tendon and the length of the tendon was measured as distance from clamp to clamp. Testing protocol consisted of 6 preconditioning cycles, 10 testing cycles and then finished with a maximum test, pulling the clamps apart until the tendon broke. Precondition-phase was necessary since the tendons had been thawed from 80°C and this process could alter the fiber alignment. Thus, tendons would become slack again after precondition and had to be recalibrated with 0.1N. After recalibration the tendon length and width were measured. Tendons were tested at 2.5% strain and 4 mm/second (determined by pilot study) through all cycles. Sampling time was set to 10 Hz through the duration of tests. Specimens were visualized and recorded using an Olympus light microscopy with Leica camera attached. Force- and position data were obtained from the Deben Software during mechanical testing. Values were normalized with tendon dimensions to stress–strain graphs using excel. Mechanical properties, such as max force and stiffness, that are dependent on tissue dimensions and material properties, such as modulus and hysteresis, that are independent of tissue dimensions were analyzed.

Collagen alignment

Sections were stained with Picrosirius red (Direct red 80, CAT#365548, Sigma-Aldrich) to visualize collagen fibers using a standard protocol (Kiernan,2014; Puchtler et al., 1973; Junqueira et al., 1979). Briefly, sections were deparaffinated with xylene before hydration in decreasing ethanol solutions. Afterward, sections were stained in Picrosirius red working solution (1 mg Sirius red added to 1 L 1.3% (w/v) picric acid solution in diH_2O) at room temperature for 1 h and then washed two times in glacial acetic acid. Sections were rehydrated with ethanol before being mounted with Cytoseal® and coverslips. A bright-field microscope equipped for polarization microscopy (Axio Lab.A1, Zeiss, Germany) was used for visualization under 20x zoom (20x/0.45 Pol, N-Achroplan, 0.227 7 μm/pi, Zeiss, Germany): Red for mature collagen fibers and cyan for unmatured collagen fibers. The stage was manually rotated in incremental degrees of 10° from 0° to 180° to observe changes in color intensity and pictures were then taken for every 10° using a mounted 6Mpx camera (AxioCam 506, Zeiss, Germany). Pictures were taken with the software ZEN Lite (Zen Lite 2.3, Windows, Zeiss) with the following configurations: Camera acquisition ROI size 2048 and offset 35,280. Exposure set to 20,000 ms with 10 cycles (time series). Data analysis of the pictures was done in Fiji ImageJ2 (2.0.0-rc-69/1.52i, macOs) by measuring the color intensity of red and green. Fibers that were aligned under polarized light would display an intensive red light while fibers arranged 90° to the light would become dim. Rotating from 0° to 360° would vary the light intensity of the sample and therefore was plotted as a sinus-curve with 2π period. Thus, maximum light intensity is given a value of 1 or −1 on the y-axis while 0 is for minimum light intensity (dim). Given that every periodic π (180°) would give the same signal as 0°, π/2 would reveal maximum light intensity and the percentage of light intensity was measured as relative to 2π. This takes into account that absolute lack of light or dimness is not achievable because some fibers will always be aligned to a given angle. An absolute 100% light intensity is an expression of completely unreflective light passing through the sample and is neither possible due to some fibers being out of alignment.

Statistics

Mantel–Cox test (logrank test) was used to analyze the survival rate of mice in wheel-running and control group within genotypes. A one-way ANOVA was used to analyze running-data for effect of genotype on distance and velocity. For other data a two-way ANOVA was performed to determine any effects of genotype, groups, and their

interaction on tendon composition, dimensions, and mechanical properties. If the two-way ANOVA was significant, individual differences within genotype and groups were tested with a post hoc test (Tukey). Statistical analysis was performed in Prism (GraphPad, Prism 8.0.1, MacOs) and in all cases, a p-value less than 0.05 was considered significant. Data are presented as mean ± standard error of the mean unless indicated otherwise in text under figure or table.

Results

Voluntarily aerobic running and survival

There was no significant difference between genotypes for running distance and average running velocity (Table 1). Most of the running occurred between 10 PM and 9 AM, approximately three hours after the lights of the animal facility were switched off (data not shown). All groups with access to running wheel ran 3–4 km/24 h (Table 1). There was no significant change in the survival rate of $Fbn1^{mgR/mgR}$ mice relative to the same mutant animals under resting conditions (Fig. 1).

Tendon morphology and mechanical properties

There was a significant difference in baseline tendon dimensions between genotypes (Fig. 2). $Fbn1^{mgR/mgR}$ tendons exhibited a smaller cross-sectional area (CSA) due to a smaller width and a decreased anterior–posterior thickness compared to the tendons of WT mice ($P < 0.05$) (Fig. 2A–C). By contrast, the dimensions of $Fbn1^{+/-}$ tendons were comparable to those of WT mice ($P > 0.05$). In all three groups of mice, voluntarily running had appreciable impact on tendon morphology (Fig. 2). $Fbn1^{mgR/mgR}$ mice had the weakest tendons of all genotypes regardless of voluntarily running (Fig. 3). Tendon max force was statistically lower in $Fbn1^{mgR/mgR}$ compared to WT mice ($P < 0.05$) but was similar between $Fbn1^{mgR/mgR}$ - and $Fbn1^{+/-}$ mice (Fig. 3A). By contrast,

max force in less severe mice of both resting and wheel-running groups was not different than WT mice (Fig. 3A). The maximal stiffness was significantly lower in $Fbn1^{mgR/mgR}$ mice than WT mice (Fig. 3B) regardless of level of physical activity level. Finally, $Fbn1^{+/-}$ mice had lower max stiffness than WT mice but higher max stiffness than $Fbn1^{mgR/mgR}$ mice in the wheel running-group ($P < 0.05$)(Fig. 3B). An effect of voluntarily running upon tendon max stiffness was only observed in the WT group, as evidenced by decreased tendon stiffness ($P < 0.05$) (Fig. 3B). When stiffness was normalized to tendon dimensions max modulus values were similar between genotypes and wheel-running groups (Fig. 3C). In addition, hysteresis remained similar between genotypes and was not modified by voluntarily running (Fig. 3D). Finally, there was no significant difference between genotypes and groups in other parameters of tendon mechanics (data not shown). Immature collagen and fiber alignment analyzed with immunohistochemistry were not significantly different between any of the genotypes (Fig. 4). Also, there was no significant effect of voluntarily running on the quality of tendons in any of the groups, and even when genotypes were pooled together in either a sedentary control or in a running wheel-group no difference was observed between active and sedentary mice (Fig. 4A,B).

Discussion

Three are the major new findings of our study. First, substantial fibrillin1 deficiency affected the size rather than the quality of $Fbn1^{mgR/mgR}$ tendons. Second, smaller tendons did not display pathological changes when loaded with voluntary running. Third, voluntarily aerobic running did not improve (or worsen) the median survival of $Fbn1^{mgR/mgR}$ mice.

Elastic fibers are made of a central core of elastin surrounded by fibrillin-rich microfibrils (Heinemeier et al., 2013). Unlike in the aorta, elastic fibers are a minor structural component of the tendon matrix. In spite of being part of the same ECM macroaggregate, elastin and

Table 1. Running data over 30 days for mice on running-wheel.

Experimental groups	Number of mice	Distance (km)Mean ± SE	Average velocity (m/sec)Mean ± SE
Wild-type	11	116 ± 22	0.20 ± 0.02
Less-severe MFS	14	102 ± 15	0.21 ± 0.01
More-severe MFS	14	89 ± 13	0.22 ± 0.01

Distance (km) was calculated as total distance during the 30-day period. Average velocity (m/s) is a measured distance for each running-bout that resulted in a rotation of the wheel more than 45° during 1 sec divided by time until it no longer exceeded the threshold of 45° per second. SE, standard error of mean.
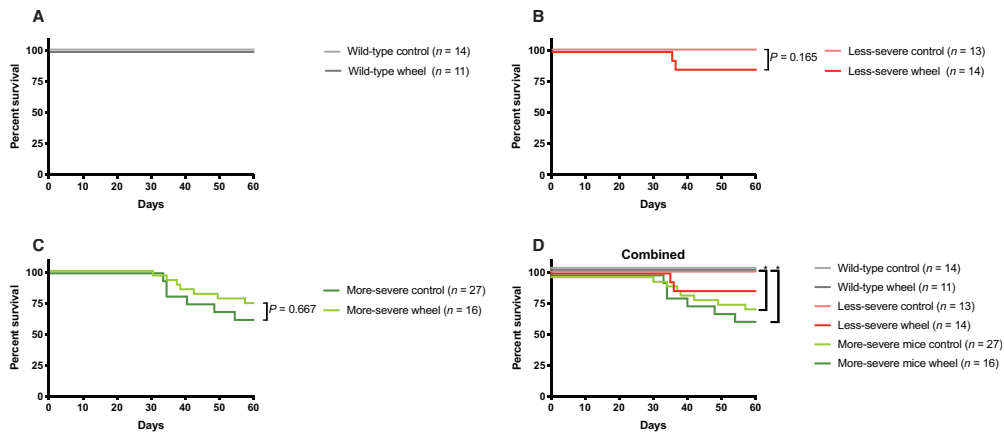
Figure 1. 30-day survival-curves for all three genotypes. (A) wild-type mice. (B) Less-severe MFS mice. (C) More-severe MFS mice.(D) All mice genotypes. There was no difference in survival between wheel running-group and controls in any of the groups. More-severe MFS mice had a median survival of 10 weeks regardless of wheel running and therefore showed significantly lower survival-rate when compared to wild-type. *$P<0.05$.

fibrillin-1 deficiency have very different effects of tendon physiology. In this study, $Fbn1^{mgR/mgR}$ mice displayed markedly smaller tendons than WT mice due to reduced anterior–posterior thickness and width diameter (Fig. 2), which was associated with unaltered modulus and collagen orientation. The haploinsufficient elastin mice ($Eln^{+/-}$ mice) on the other hand, showed no significant reduction in Achilles tendon size, but had an increased mechanical stiffness (Eekhoff et al., 2017). Interestingly, in the present study tendons from $Fbn1^{mgR/mgR}$ were less stiff than their healthy counterparts. Tendons from $Fbn1^{mgR/mgR}$ were expected to become stiff as there is

more complete engagement of collagen fibers to tendon loading, which has been shown in other studies on elastin haploinsufficiency mice (Li et al., 1998; Carta et al., 2009; Eekhoff et al., 2017). It has been shown that there is a significant increase in the linear stiffness in $Eln^{+/-}$ in the absence of a change in other mechanical parameters (Eekhoff et al., 2017). In the present study, it appeared that tendons from more-severe fibrillin-deficient ($Fbn1^{mgR/mgR}$) mice seem longer than wild type (data not shown). This suggests a role of elastin in collagen crimp with tendons becoming slacker if elastin is somehow compromised (Henninger et al., 2015). A potential
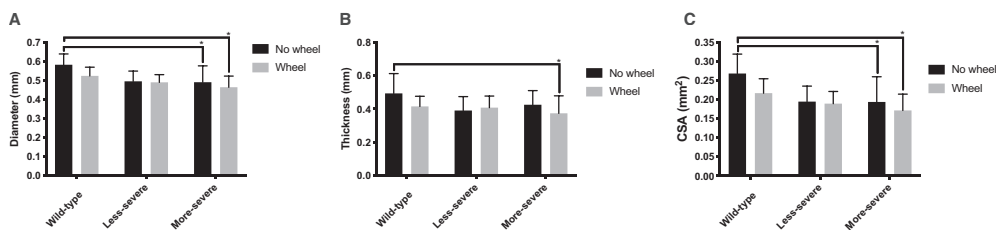
Figure 2. Tendon dimensions of mice in both wheel-running and control group. (A) Diameter (mm). (B) Thickness (mm). (C) Cross-sectional area (CSA, mm^2). Diameter, thickness, and cross-sectional are of more-severe MFS mice were significant less than that in wild-type. There was no significant difference of less-severe MFS mice when compared to other genotypes regardless of groups. *$P<0.05$. In control group: $N_{Wild-type}$ = 14, $N_{Less-severe}$ = 12, and $N_{More-severe}$ = 9. In wheel-running-group: $N_{Wild-type}$ = 11, $N_{Less-severe}$ = 11, and $N_{More-severe}$ = 10.
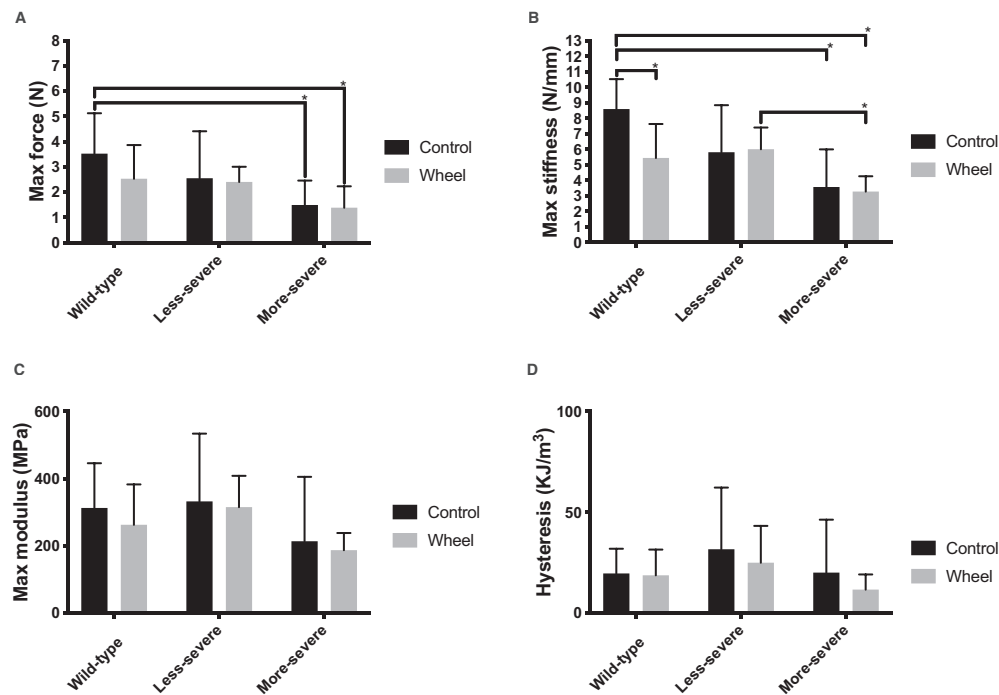
Figure 3. Mechanical data from genotypes of control and wheel-running group. (A) Maximal force (N). (B) Maximal stiffness (N/mm). (C) Maximal modulus (MPa). (D) Hysteresis (KJ/m^3). More-severe mice had significantly lower max force and max stiffness when compared to wild-type. $^*P$<0.05. In control group: $N_{Wild-type}$ = 13, $N_{Less-severe}$ = 12, and $N_{More-severe}$ = 9. In wheel-running group: $N_{Wild-type}$ = 11, $N_{Less-severe}$ = 11, and $N_{More-severe}$ = 11.

shifting of the stress–strain curve from right to left would minimize the physiological normal range of such tendons, thus also explains why maximum force was lowest in Fbn1$^{mgR/mgR}$. However, hysteresis was unaffected by genotype and exercise, indicating that a secondary source of elastic recoil might be present (Henninger et al., 2013). It seems that deficiency of elastin and fibrillin, respectively, does influence mechanical properties somewhat different and interact with each other when it comes to mechanical properties of the tendon (Henninger et al., 2015). Taken at face value, these findings suggest that elastin primarily influences the mechanical properties rather than the size of tendons, whereas fibrillin-1 is predominantly involved in optimizing tendon growth rather than influencing tissue mechanics (Fig. 3) (Ramirez and Pereira, 1999). The different responses of elastin or fibrillin-1-deficient tendons to voluntarily running induced loading is another example of the distinct roles that these elastic fiber components play a role in tendon physiology.

Pathological changes in tendon tissue were not observed after voluntary running in either mice producing half of the normal amount of fibrillin-1 (Fbn1$^{+/-}$ mice) or those making only 20% of it (Fbn1$^{mgR/mgR}$ mice). By contrast, degradation of elastic fibers has been associated with clinical signs of chronic tendinopathy (overuse-injuries) in Eln$^{+/-}$ mice (Wu et al., 2016; Wu et al., 2017). While the underlying mechanism remains unknown, differentiation of functions by the two major components of the elastic fibers has also been reported in the mouse arteries (Carta et al., 2009). The lack of pathological changes in tendon of fibrillin deficient mice indicates that the use of this mouse model in combination with voluntary running is not optimal for studying tendon overuse injury. While we cannot rule out that a higher mechanical work load than used in the present study could have caused pathological tendon changes, the fact that fibrillin-1-deficient mice had lower tendon diameter (but similar body weight), but ran a similar distance
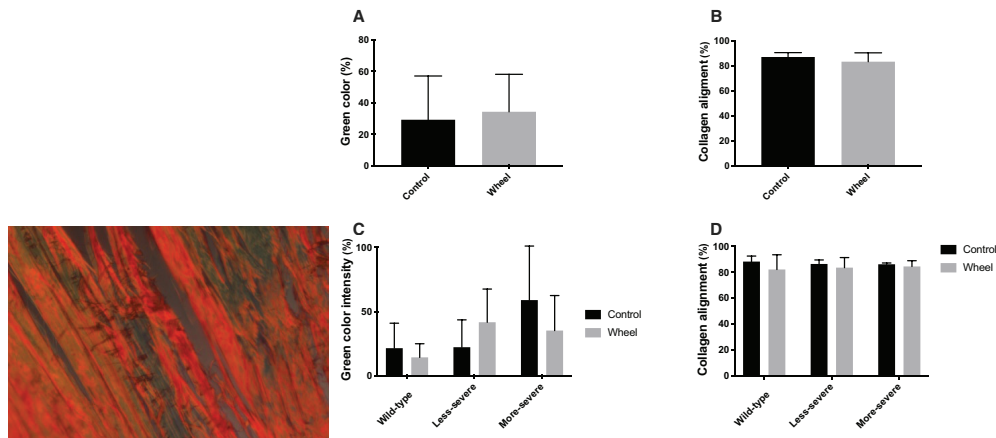
Figure 4. Results from Picrosirius staining of genotypes in control and wheel running-group. (Left panel image: example image). (A) Overall green color (%). (B) Overall collagen alignment (%). (C) Green color intensity (%). (D) Collagen alignment (%). Left panel image: example image. In control group: $N_{Wild-type}$ = 8, $N_{Less-severe}$ = 4, and $N_{More-severe}$ = 3. In wheel-running group: $N_{Wild-type}$ = 3, $N_{Less-severe}$ = 7, and $N_{More-severe}$ = 5.

as healthy mice exclude that these mutant animals could be used as an experimental model to study tendinopathy pathogenesis. In humans, tissue remodeling in tendons is limited, and more chronic physiological, or pathological adaptation of tendon required several months (Heinemeier et al., 2013). In the present study on mice, we chose the 60-day period to ensure that a significant number in the diseased group would still be alive.

Our last new finding concerns the relationship between cardiovascular function and wheel running in MFS. It was recently reported that mild aerobic wheel running (2–5 km/day) alleviated the degree of vessel degeneration in mice with a non-lethal form of MFS in which the ascending aorta dilates but does not rupture ($Fbn1^{C1039G/+}$ mice)(Gibson et al., 2017). However, we found no modifications in aneurysm progression to dissection and premature death when our mice with lethal MFS ($Fbn1^{mgR/mgR}$ mice) were subjected to a similar running regimen (3–4 km/day). Interestingly, a running intensity up to 65% V_{O2max} showed protective effects against elastin fiber fragmentation within the aortic wall which supports the notion that other factors than volume may determine optimum exercise regime. We have no clear explanation for the discrepancy between the two studies short of noting that median survival (and implicitly aneurysm dissection and rupture) is a more robust read out of running-induced modifications of arterial disease than elastic fiber fragmentation.

Limitations

Most studies on MFS have focused on blood vessels in animal knockout models and rarely on changes in tendon (Pereira et al., 1997; Li et al., 1998; Pereira et al., 1999; Galatioto et al., 2018; Ramirez et al., 2018). However, blood vessels and tendons are distinct structures with the former containing much more elastic fibers (Kannus, 2000; Shinaoka et al., 2013; Pang et al., 2017), and therefore a direct comparison to previous studies is difficult. Fibrillin-1 may play a role in elastogenesis, which has been shown in blood vessels (Carta et al., 2009) but never in loaded tendons according to our knowledge. Unfortunately, elastin content was not measured in tendons, which would have provided valuable information.

In the current study, the exercise period was 30 days, and it should be noted that the severe animal-model have an inferior survival rate due to aortic complications (Pereira et al., 1997; Pereira et al., 1999) with a median survival rate of only 90 days (Pereira et al., 1999). It is therefore possible that 30 days of voluntarily exercise was insufficient to see any pathological change in tendons.

In conclusion, our findings correlated fibrillin-1 deficiency with the development of smaller but morphologically and biomechanically normal tendons that can tolerate voluntarily running loading to the same extent as the WT counterparts. Furthermore, we found that mice with severe MFS tolerated voluntarily aerobic running

without developing tendinopathy or exacerbating aneurysm progression.

Conflict of Interest

None declared.

References

Carta, L., S. Smaldone, L. Zilberberg, D. Loch, H. C. Dietz, D. B. Rifkin, et al. 2009. p38 MAPK is an early determinant of promiscuous Smad2/3 signaling in the aortas of fibrillin-1 (Fbn1)-null mice. J. Biol. Chem. 284:5630–5636. https://doi.org/10.1074/jbc.M806962200.

Carta, L., J. E. Wagenseil, R. H. Knutsen, B. Mariko, G. Faury, E. C. Davis, et al. 2009. Discrete contributions of elastic fiber components to arterial development and mechanical compliance. Arterioscler. Thromb. Vasc. Biol. 29:2083–2089. https://doi.org/10.1161/ATVBAHA.109.193227.

Cikach, F. S., C. D. Koch, T. J. Mead, J. Galatioto, B. B. Willard, K. B. Emerton, et al. 2018. Massive aggrecan and versican accumulation in thoracic aortic aneurysm and dissection. JCI Insight 3(5):97167. https://doi.org/10.1172/jci.insight.97167.

Dakin, S. G., F. O. Martinez, C. Yapp, G. Wells, U. Oppermann, B. J. F. Dean, et al. 2015. Inflammation activation and resolution in human tendon disease. Sci. Transl. Med. 7(311):311ra173–311ra173. https://doi.org/10.1126/scitranslmed.aac4269.

Eekhoff, J. D., F. Fang, L. G. Kahan, M. G. Espinosa, A. J. Cocciolone, J. E. Wagenseil, et al. 2017. Functionally distinct tendons from elastin haploinsufficient mice exhibit mild stiffening and tendon-specific structural alteration. J. Biomech. Eng. 139. https://doi.org/10.1115/1.4037932.

Galatioto, J., C. I. Caescu, J. Hansen, J. R. Cook, I. Miramontes, R. Iyengar, et al. 2018. Cell type-specific contributions of the angiotensin II type 1a receptor to aorta homeostasis and aneurysmal disease-brief report. Arterioscler. Thromb. Vasc. Biol. 38:588–591. https://doi.org/10.1161/ATVBAHA.117.310609.

Gibson, C., C. Nielsen, R. Alex, K. Cooper, M. Farney, D. Gaufin, et al. 2017. Mild aerobic exercise blocks elastin fiber fragmentation and aortic dilatation in a mouse model of Marfan syndrome associated aortic aneurysm. J. Appl. Physiol. 123(1):147–160. https://doi.org/10.1152/japplphysiol.00132.2017.

Heinemeier, K. M., P. Schjerling, J. Heinemeier, S. P. Magnusson, and M. Kjaer. 2013. Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb14C. FASEB J. 27:2074-2079. https://doi.org/10.1096/fj.12-225599.

Henninger, H. B., C. J. Underwood, S. J. Romney, G. L. Davis, and J. A. Weiss. 2013. Effect of elastin digestion on the quasi-static tensile response of medial collateral ligament. J.

Orthop. Res. 31:1226–1233. https://doi.org/10.1002/jor.22352.

Henninger, H. B., W. R. Valdez, S. A. Scott, and J. A. Weiss. 2015. Elastin governs the mechanical response of medial collateral ligament under shear and transverse tensile loading. Acta Biomater. 25:304–312. https://doi.org/10.1016/j.actbio.2015.07.011.

Junqueira, L. C. U., G. Bignolas, and R. R. Brentani. 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem. J. 11:447–455. https://doi.org/10.1007/BF01002772.

Kannus, P. 2000. Structure of the tendon connective tissue. Scand. J. Med. Sci. Sport. 10:312–320. https://doi.org/10.1034/j.1600-0838.2000.010006312.x.

Kiernan, J. A.. 2014. Sirius Red Staining Protocol for Collagen [Online]. http://www.ihcworld.com/_protocols/special_stains/sirius_red.htm

Li, D. Y., G. Faury, D. G. Taylor, E. C. Davis, W. A. Boyle, R. P. Mecham, et al. 1998. Novel arterial pathology in mice and humans hemizygous for elastin. J. Clin. Invest. 102:1783–1787. https://doi.org/10.1172/JCI4487.

Magnusson, S. P., H. Langberg, and M. Kjaer. 2010. The pathogenesis of tendinopathy: Balancing the response to loading. Nat. Rev. Rheumatol. 6(5):262–268.

Pang, X., J. P. Wu, G. T. Allison, J. Xu, J. Rubenson, M. H. Zheng, et al. 2017. Three dimensional microstructural network of elastin, collagen, and cells in Achilles tendons. J. Orthop. Res. 35:1203–1214. https://doi.org/10.1002/jor.23240.

Pereira, L., K. Andrikopoulos, J. Tian, S. Y. Lee, D. R. Keene, R. Ono, et al. 1997. Targetting of the gene encoding fibrillin-1 recapitulates the vascular aspect of marfan syndrome. Nat. Genet. 17:218–222

Pereira, L., S. Y. Lee, B. Gayraud, K. Andrikopoulos, S. D. Shapiro, T. Bunton, et al. 1999. Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1. Proc. Natl. Acad. Sci. USA 96:3819–3823. https://doi.org/10.1073/pnas.96.7.3819.

Pingel, J., Y. Lu, T. Starborg, U. Fredberg, H. Langberg, A. Nedergaard, et al. 2014. 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: Evidence of tenocyte and matrix buckling. J. Anat. 224:548–555. https://doi.org/10.1111/joa.12164.

Puchtler, H., F. S. Waldrop, and L. S. Valentine.1973. Polarization microscopic studies of connective tissue stained with picro-sirius red FBA. Beitr. Pathol. 150: 174–187. https://doi.org/10.1016/S0005-8165(73)80016-2.

Ramirez, F., and L. Pereira. 1999. The fibrillins. Int. J. Biochem. Cell Biol. 31:255–259

Ramirez, F., C. Caescu, E. Wondimu, and J. Galatioto. 2018. Marfan syndrome; A connective tissue disease at the crossroads of mechanotransduction, TGFβ signaling and cell stemness. Matrix Biol. 71:82–89

146

Shinaoka, A., R. Momota, E. Shiratsuchi, M. Kosaka, K. Kumagishi, R. Nakahara, et al. 2013. Architecture of the subendothelial elastic fibers of small blood vessels and variations in vascular type and size. Microsc. Microanal. 19:406–414. https://doi.org/10.1017/S1431927612014341.

Thijssen, C. G. E., L. R. Bons, A. L. Gökalp, R. R. J. Van Kimmenade, M. M. Mokhles, A. Pelliccia, et al. 2019. Exercise and sports participation in patients with thoracic aortic disease: a review. Expert Rev. Cardiovasc. Ther. 17:251–266. https://doi.org/10.1080/14779072.2019.1585807.

Thorpe, C. T., C. P. Udeze, H. L. Birch, P. D. Clegg, and H. R. C. Screen. 2012. Specialization of tendon mechanical

properties results from interfascicular differences. J. R. Soc. Interface 9:3108–3117. https://doi.org/10.1098/rsif.2012.0362.

Wu, Y. T., P. T. Wu, and I. M. Jou.2016. Peritendinous elastase treatment induces tendon degeneration in rats: A potential model of tendinopathy in vivo. J. Orthop. Res. 34:471–477. https://doi.org/10.1002/jor.23030.

Wu, Y. T., W. R. Su, P. T. Wu, P. C. Shen, and I. M. Jou. 2017. Degradation of elastic fiber and elevated elastase expression in long head of biceps tendinopathy. J. Orthop. Res. 35:1919–1926. https://doi.org/10.1002/jor.23500.

Co-authorship declarations

G R A D U A T E S C H O O L O F H E A L T H A N D M E D I C A L S C I E N C E S
U N I V E R S I T Y O F C O P E N H A G E N

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

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

1. Declaration by	
Name of PhD student	Peter Hung Thanh Tran
E-mail	TranenPeter@gmail.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Mechanisms behind development of Tendinopathy: Early Structural, Inflammatory, Nociceptive and Clinical changes in recreational runners

2. The declaration applies to the following article	
Title of article	Early development of tendinopathy in humans: Sequence of pathological changes in structure and tissue turnover signaling

Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☒ Date: 24 May 2019	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

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

Latest update of the declaration: December 2018

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

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	26 may 2019	Peter Hung Thanh Tran	MD	*[signature]*
2.	26 may 2019	S. Peter Magnusson	Professor	*[signature]*
3.	26may 2019	Michael Kjær	Professor	*[signature]*
4.				
5.				
6.				
7.				
8.				
9.				
10.				

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 26 may 2019

Principal supervisor: *[signature]*

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.

Date: 26 may 2019

PhD student: *[signature]*

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

2

PHD-THESIS
DECLARATION OF CO-AUTHORSHIP

The declaration is for PhD students and must be completed for each conjointly authored article. Please note that if a manuscript or published paper has ten or less co-authors, all co-authors must sign the declaration of co-authorship. If it has more than ten co-authors, declarations of co-authorship from the corresponding author(s), the senior author and the principal supervisor (if relevant) are a minimum requirement.
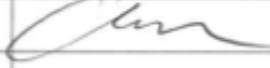
1. Declaration by	
Name of PhD student	Peter Hung Thanh Tran
E-mail	TranenPeter@gmail.com
Name of principal supervisor	Michael Kjær
Title of the PhD thesis	Mechanisms behind development of Tendinopathy: Early Structural, Inflammatory, Nociceptive and Clinical changes in recreational runners

2. The declaration applies to the following article	
Title of article	The influence of fibrillin-1 and physical activity upon tendon tissue morphology and mechanical properties in mice

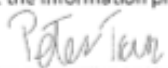| Article status	
Published ☐ Date:	Accepted for publication ☐ Date:
Manuscript submitted ☒ Date: 20 May 2019	Manuscript not submitted ☐
If the article is published or accepted for publication, please state the name of journal, year, volume, page and DOI (if you have the information).	

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

Latest update of the declaration: December 2018

150

4. Material from another thesis / dissertation[ii]

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

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	*Peter Tran*
2.	20 May 2019	Tanja Skrba	MD	*Tanja Skrba*
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup		

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson	PhD	*René B. Svensson*
6.	20 May 2019	Annesofie Thorup		
7.	20 May 2019	Abigail Mackey		
8.	20 May 2019	Peter S. Magnusson	Professor	
9.	20 May 2019	Francesco Ramirez	Professor	
10.	20 May 2019	Michael Kjær	Professor	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup	PROFESSOR	*signature*
7.	20 May 2019	Abigail Mackey	Associate Professor	*Abigail M*
8.	20 May 2019	Peter S. Magnusson	Professor	
9.	20 May 2019	Francesco Ramirez	Professor	
10.	20 May 2019	Michael Kjær	Professor	*signature*

6. Signature of the principal supervisor

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 29 May 2019
Principal supervisor: *signature*

7. Signature of the PhD student

I solemnly declare that the information provided in this declaration is accurate to the best of my knowledge.
Date: 29 Maj 2019
PhD student: *Peter Tran*

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

---

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

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu	PhD	*Elisabeth Wondimu*
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup		
7.	20 May	Abigail Mackey		

5. Signatures of the co-authors3

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto	PhD	*Giuseppina Galatioto*
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup		
7.	20 May 2019	Abigail Mackey		
8.	20 May 2019	Peter S. Magnusson	Professor	
9.	20 May 2019	Francesco Ramirez	Professor	
10.	20 May 2019	Michael Kjær	Professor	

5. Signatures of the co-authors3

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup Olesen		*signature*
7.	20 May 2019	Abigail Mackey		
8.	20 May 2019	Peter S. Magnusson	Professor	
9.	20 May 2019	Francesco Ramirez	Professor	
10.	20 May 2019	Michael Kjær	Professor	

5. Signatures of the co-authors[iii]

	Date	Name	Title	Signature
1.	20 May 2019	Peter Tran	MD	
2.	20 May 2019	Tanja Skrba	MD	
3.	20 May 2019	Elisabeth Wondimu		
4.	20 May 2019	Giuseppina Galatioto		
5.	20 May 2019	René B. Svensson		
6.	20 May 2019	Annesofie Thorup		
7.	20 May 2019	Abigail Mackey		
8.	20 May 2019	Peter S. Magnusson	Professor	
9.	20 May 2019	Francesco Ramirez	Professor	*signature*
10.	20 May 2019	Michael Kjær	Professor	

Vietnam-flygtning i Århus:

JEG UNDSLAP HELVEDET

– Jeg havde aldrig set sne før, så jeg gemte mig under dynen, fortæller Tran Phat.

Tekst: ARNE MØLLER
Foto: HANS BØLCHO

Træt og lykkelig, men sandsynligvis mærket for livet, sidder den 52-årige Tran Phat sammen med sin familie i en moderne lejlighed i Grøftehøjparken i Viby ved Århus. Mere end to års mareridt er slut for den vietnamesiske flygtning.

I små hold er hans familie flygtet fra Vietnam. En flugt fra vold, plyndringer og såkaldte opdragelseslejre.

– Jeg var engang en holden mand i Vietnam, siger Tran Phat. – Jeg havde tre forretninger, men siden kommunisterne overtog styret, har det for mig været et helvede at leve i mit eget land. Mine sønner har været i hæren og den ene i en opdragelseslejr, hvor han fra klokken fire om morgenen til langt ud på aftenen skulle opdyrke marker og rydde skove. De fik ingen løn, kun en skål ris.

Når jeg var inde i Saigon for at skaffe stoffer til mine forretninger, blev jeg ofte stoppet af soldaterne, og de stjal alt, hvad jeg havde købt. Mine forretninger blev beslaglagt, og jeg blev afkrævet tårnhøje skatter. Medicinen, der kom fra Europa, stjal de. De syge fik den aldrig, de måtte bare arbejde sig til døde.

Da jeg besluttede at flygte, havde jeg allerede hjulpet 52 til flugt. Vi var 225 flygtninge om bord i en båd, der maksimalt kunne rumme 100. Turen kostede 10.000 danske kroner og vare-

de over tre døgn. Den gik over Siam-bugten fra Saigon til Malaysia. Der var mange thailandske patruljebåde, og de thailandske politifolk er nærmest sørøvere.

Hvis de opdager en flygtninge-båd om natten, bliver den overfaldet, og de røver alt, myrder, voldtager og ødelægger. Men sørøverne var ikke det ene-

ste, vi frygtede, havet er også fuldt af farer.

Den morgen, vi fik landkending med Malaysia, var det hårdt vejr. Ikke langt fra vores båd lå en lille motorbåd på omkring seks meter, med over 30 om bord. Pludselig slog en bølge over båden, og alle blev skyllet over bord. Det var frygteligt at se, hvordan forældrene magtesløse så deres børn forsvinde i havet. Jeg så også en lille jolle fyldt med flygtninge. De havde kun to årer, de andre brugte hænderne til at ro med. Vietnameserne gør alt for at komme væk.

Vi nåede vel til Malaysia, og der opholdt jeg mig i en flygtningelejr i fire måneder, indtil jeg kunne komme videre til Danmark. Jeg var lykkelig for at komme væk fra Vietnam, selv om det er hårdt at opholde sig i

en flygtningelejr. Der var ikke mad nok, og mange var syge, men jeg holdt mig oppe i troen på at komme til Danmark og blive forenet med min familie, der var flygtet før mig.

Jeg græd af lykke, da jeg så min kone og mine børn. Men jeg var meget bange for de første dage, jeg var i Danmark. Det var meget koldt, og jeg har aldrig før set sne. Jeg skal ærligt indrømme, at jeg krøb ind under dynen og gemte mig. Sneen, troede jeg, var ris, der faldt ned fra himlen.

Nu skal jeg vænne mig til Danmark og lære jeres sprog. Så håber jeg, at jeg med tiden kan få et job, for jeg bryder mig ikke om at få penge foræret. Jeg tror på og beder til, at mit eget land Vietnam engang igen bliver et godt land, så mine børn og børnebørn kan vende tilbage.

Tran Phat måtte nøjes med at læse breve om, hvor dejlig hans første barnebarn var. Da han kom til Danmark, kunne han se, at det var rigtigt. Barnebarnet hedder Maria.
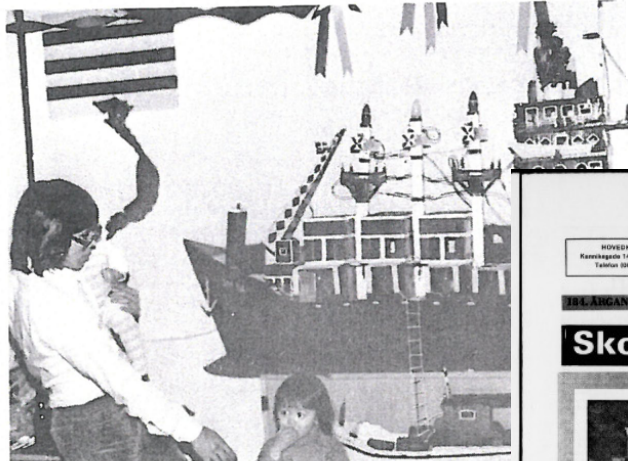
52-årig bedstefar genforenet med sin familie i Danmark efter dramatisk flugt

Billeder af de vietnamesiske flygtninge i synkefærdige gamle skuder var ... medierne verden over, og var med til at påvirke regeringen til at modtage b... ninge.

En lykkelig flygtning igen sammen med sin familie, kone, børn, svigerbørn og barnebørn.

...gemmer aldrig, at de er ...
selvom de ikke er »politiske« flygtninge, som f.eks. chilenerne. De tænker næppe nogensinde på at vende tilbage.

De har brudt alle broerne, og også de-

Tilsynet med udlændinge:

Flygtningenes første møde med Danmark

Vicepolitimester Henning Dahlfelt er ofte den første kontakt flygtningene får med Danmark. Den er ikke altid lige nem

den har givet alle, der har deltaget, et førstehånds kendskab til de problemer, som flygtninge kæmper med. Erfaringer og oplysninger er bragt med hjem til dem, der skal afgøre flygtningenes skæbne. Den medmenneskelige forståelse og respekten for flygtningen er øget.

Aarhuus Stiftstidende

184. ÅRGANG ★ NR. 54184 — Grundlagt år 1794 — ved kgl. privilegium — ONSDAG 26. OKTOBER 1977

Læssepapris: hverdage kr. 2,00
søn- og helligdage kr. 2,60
Nette-oplag 1. halvår 1977
72.445 hverdage, 88.780 søndage

Skovbakken som anpartsselskab. Sporten side 24

Vietnameserne da de steg ud af flyet i Tirstrup. Udover de ejendele, de bærer på, har de hver kun ganske få kilo bagage med sig.

(FOTO: Børge Venge)

En enlig fader, Vu Trong, med de seks børn, han med fra Vietnam. Moderen menes druknet på et senere flugt. Læs mere om denne familie side 12.

Vietnam-pige fødte under fly-rejse til Danmark

48 flygtninge til Århus i morges fra Thailand og Malaysia

48 vietnamesiske flygtninge kom i dag til Århus fra flygtningelejre i Thailand og Malaysia. De skulle egentlig have været ...

Så, men nu nåede ikke længere end til Indien, fordi en 17-årig pige fødte under mellemlandingen i Indien i nat.

De var to gravide kvinder i gruppen, da den startede fra Bangkok i går eftermiddag dansk tid. De havde under mellemlandingen i Indien i nat ...

fødslerne ikke var ventet foreløbig, og fødte flygtningene var ledsaget af den norske læge, Cleve Sichen, der har været stationeret i Malaysia og har medvirket ...

ved lægeundersøgelsen her.

– Pigen skulle ikke føde før 18. november, sagde ...

Vietnam-flygtning, der nu er i Århus, kommer fra en lejr i det sydlige Thailand og fra Vietnam i Østlige Thailand og Pulau Besar i det nordøstlige Malaysia.

Aarhuus Stiftstidendes udenrigs-redaktør, Aage Holm-Pedersen, har ledsaget Dansk Flygtningehjælps repræsentant, Helge Griffin, på hans rejse ud for at hente flygtningene til Danmark, og vi række artikler i de kommende dage vil fortælle om flugten fra Vietnam, om de vilkår, kommunisterne bidrejer gennem kommunistisk besætning, og som den umenneskelighed af mødre efter flugten.

De første artikler kan læses i dag på side 12.

FORTSÆTTES S...