The plantaris tendon in relation to the Achilles tendon in midportion Achilles tendinopathy

Studies on morphology, innervation and signalling substances

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“It always seems impossible until it’s done!!!”

(Nelson Mandela)

To my grandparents

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ABSTRACT

Midportion Achilles tendinopathy (tendinosis) is a troublesome painful condition, often characterised by pain, local swelling, tenderness and functional disability. Despite extensive research, the pathogenesis is poorly understood and treatment remains challenging. Features related to the peritendinous connective tissue can be of importance. Recently it has been suggested that the plantaris tendon might be involved in this condition. Furthermore, it has been hypothesised that tendon pain and the tendinosis-related tissue changes in tendinopathy might be mediated by signalling substances such as glutamate and acetylcholine. A clinical observation, not scientifically evaluated, has been that unilateral treatment for bilateral Achilles tendinosis can lead to an effect on the contralateral side.

The aim of this work was to examine the morphology and innervation patterns in the plantaris tendon and the peritendinous connective tissue in between the Achillles and plantaris tendons in midportion Achilles tendinopathy, and to evaluate if plantaris tendon removal has an effect on Achilles tendon structure. Another aim was to determine if unilateral treatment for Achilles tendinopathy targeting the peritendinous connective tissue can result in bilateral recovery. Furthermore the presence of non-neuronal cholinergic and glutamate systems was examined.

Sections of plantaris tendons with adjacent peritendinous connective tissue from patients with midportion Achilles tendinopathy were stained for morphology (H&E), and innervation patterns were evaluated using antibodies against general nerve marker (PGP9.5), sensory (CGRP) and sympathetic (TH) nerve fibres and Schwann cells (S-100β). Furthermore immunostainings against non-neuronal aceylcholine (ChAT) and glutamate signalling components (glutamate, VGluT2, NMDAR1) were performed. Plantaris tendon cells were cultured and also stained for glutamate signalling components, and were stimulated with glutamate and glutamate receptor agonist NMDA. Furthermore, Ultrasound Tissue Characterisation (UTC) was used to monitor the integrity of the Achilles tendon collagen structure after plantaris tendon removal.

Plantaris tendons exhibited tendinosis-like tissue patterns such as hypercellularity, collagen disorganisation and large numbers of blood vessels. The peritendinous connective tissue between the plantaris and Achilles tendons contained large numbers of fibroblasts and blood vessels and to some extent macrophages and mast cells. A marked innervation was found in the peritendinous connective tissue and there were also nerve fibres in the loose connective tissue spaces within the tendon tissue proper. Most nerve fibres were identified as sensory fibres. Some nerve fascicles in the peritendinous connective tissue showed absence of axons but homogenous reactions for Schwann cell marker. Tenocytes and cells in the peritendinous connective tissue expressed ChAT, glutamate, VGluT2 and NMDAR1. Tendon cells *in vitro* expressed VGluT2, NMDAR1 and glutamate. UTC showed significant improvement of Achilles tendon integrity 6 months after surgical plantaris tendon removal and scraping procedure. Eleven out of thirteen patients reported of a bilateral recovery after unilateral surgical treatment.

The results of this work show that plantaris tendons exhibit tendinosis-like tissue changes, internal innervation and features that suggest occurrence of glutamate and acetylcholine production and signalling. Plantaris removal improves Achilles tendon structure suggesting possible compressive/shearing interference between the Achilles and plantaris tendons in tendinopathy. The peritendinous connective tissue shows marked innervation, which thus might transmit pain when being compressed. The partial absence of axons indicates a possible nerve degeneration. On the whole, the study gives new evidence favouring that the plantaris tendon and the peritendinous connective tissue might be of importance for pain and the tendinopathy process in midportion Achilles tendinopathy.

ABBREVIATIONS

ACh	acetylcholine
BSA	bovine serum albumin
CD	colour Doppler
CD68	cluster of differentiation marker 68
cDNA	complementary DNA
CGRP	calcitonin gene-related peptide
ChAT	choline acetyltransferase
D-MEM	Dulbecco's modified eagle medium
ECM	extracellular matrix
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
GLS	glutaminase
GOT1	glutamic-oxaloacetic transaminase 1
HBSS	Hank’s balanced salt solution
H&E	haematoxylin and eosin
ICC	immunocytochemistry
IHC	immunohistochemistry
LDH	lactate dehydrogenase
mRNA	messenger ribonucleic acid
MRI	magnetic resonance imaging
MTJ	myotendinous junctions
NMDA	N-methyl-D-aspartate
OTJ	osteotendinous junction
PARP	poly (ADP)-ribose) polymerase
PBS	phosphate buffered saline
PGP9.5	protein gene product 9.5
PRP	platelet-rich-plasma
qPCR	real time quantitative polymerase chain reaction
SCX	scleraxis (encoding gene)
SP	substance P
TH	tyrosine hydroxylase
TRITC	tetramethylrhodamine isothiocyanate
US	ultrasound
UTC	ultrasound tissue characterisation
VGluT2	vesicular glutamate transporter 2

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I. **The plantaris tendon in association with mid-portion Achilles tendinosis – Tendinosis-like morphological features and presence of a non-neuronal cholinergic system**
Spang C, Alfredson H, Ferguson M, Roos B, Bagge J, and Forsgren S
Histology & Histopathology; **2013** 28(5): 623-632

II. **Achilles tendinopathy – Do plantaris tendon removal and Achilles scraping improve tendon structure? A prospective study using Ultrasound Tissue Characterisation.**
Masci L, **Spang C**, van Schie H, and Alfredson H
BMJ Open Sport & Exercise Medicine; **2015** 1: e000005
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III. **Marked innervation but also signs of nerve degeneration in between the Achilles and plantaris tendons and presence of innervation within the plantaris tendon in midportion Achilles tendinopathy**
Spang C, Harandi VM, Alfredson H, and Forsgren S
Journal of Musculoskeletal and Neuronal Interactions; **2015** accepted, in press

IV. **Unilateral surgical treatment for patients with midportion Achilles tendinopathy may result in bilateral recovery**
Alfredson H, **Spang C**, and Forsgren S
British Journal of Sports Medicine; **2014** 48(19): 1421-1424
doi: 10.1136/bjsports-2012-091399

V. **Plantaris tendon tissue of tendinosis patients displays a glutamate signalling machinery that may influence tenocyte phenotype**
Spang C, Backman L, LeRoux S, Forsgren S, and Danielson P
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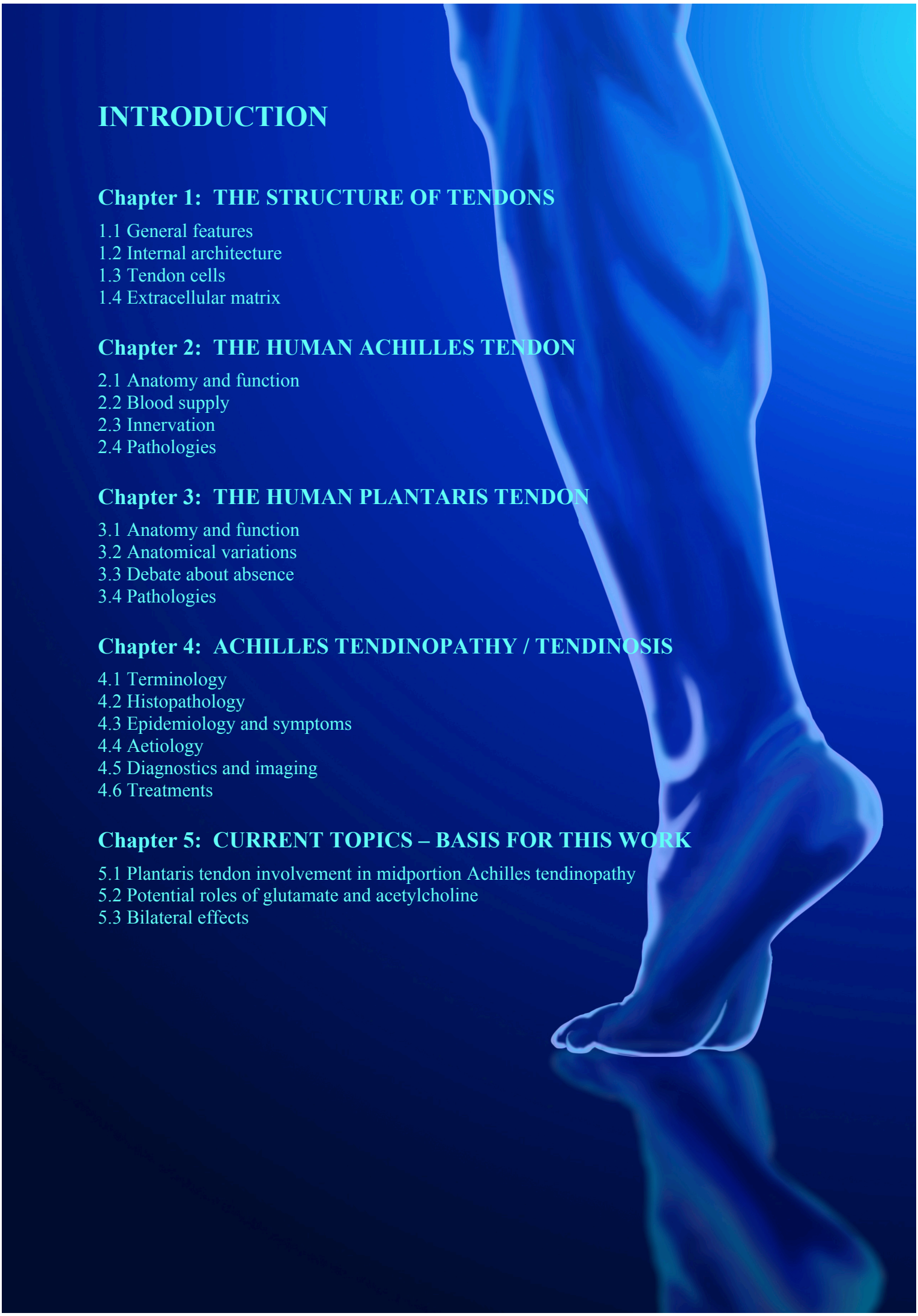

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Chapter 1: THE STRUCTURE OF TENDONS

1.1 GENERAL FEATURES

Tendons are dense and regularly arranged connective tissue structures located between skeletal muscles and bones, providing a functional link for force displacement (Karousou et al., 2010). They transmit the force created by the muscle to the corresponding bone and hereby make joint movement possible. The structure that connects the tendon to the muscle is called myotendinous junction (MTJ), and the area of union between tendon and bone is called the osteotendinous junction (OTJ).

Healthy tendons appear in bright white colour and have a fibro-elastic texture that indicates high resistance to exposure of mechanical loads (Kannus, 2000). Depending on the mechanical requirements in the body, the tendons can vary in structure and matrix composition, and how the OCT is formed (Weinreb et al., 2014). Muscles creating powerful and resistive forces have short but broad tendons whereas muscles for delicate functions have long and thin tendons (Kannus, 2000).

1.2 INTERNAL ARCHITECTURE

The core of the tendon, the so-called tendon tissue proper, basically consists of collagen and elastin embedded in a proteoglycan-water matrix, altogether called extracellular matrix (ECM) (Franchi et al., 2007). The dry weight of the tendon is approximately 30 % of the total weight, of which collagen accounts for 60-85 %, proteoglycans for 0.2-5 %, and elastin for 1-2 % (Jozsa & Kannus, 1997; Thorpe et al., 2013). All these components are produced by tenocytes and tenoblasts, elongated fibroblastic cells, which are located in between the collagen fibres (Riley, 2008). These cell types are further described below.

There are several collagen types present in tendon tissue. Collagen type I is the main component, but there are also type III and IV fibres. These different types form a matrix with hierarchical levels and increasing complexity. The elastin is thought to be involved in recovering the wavy configuration following tendon stretching (Butler et al., 1978).

The smallest unit of the hierarchical tendon structure is tropocollagen, a triple-helix polypeptide chain. Soluble tropocollagen molecules form cross-links resulting in insoluble collagen molecules, which then aggregate into microfibrils and eventually into collagen fibrils. Several of these collagen fibrils generate a collagen fibre, the basic unit of the tendon (figure 1) (Kannus, 2000; Ottani et al., 2002). Collagen fibres are mainly characterised by having a parallel alignment from end to end in the tendon. However, they can also run transversely/horizontally resulting in a complex three-dimensional structure (Jozsa et al., 1979).

The endotendon, a fine sheath of connective tissue, binds several collagen fibres together, which then form a primary fibre bundle (subfascicle), and a bunch of primary fibres forms a secondary fibre bundle (fascicle). A group of secondary fascicles creates a tertiary bundle and the tertiary bundles finally build up the whole tendon, which is surrounded by a fine connective tissue sheath called epitenon (figure 1) (Elliott, 1965). The number and diameter

of fascicles and subfascicles can vary from tendon to tendon and occasionally even within the same tendon (Jozsa & Kannus, 1997).

Via the connective tissues such as endotenon and epitenon the tendon tissue proper is supplied with vascular, lymphatic and nerve structures (Jozsa & Kannus, 1997). Many tendons are furthermore surrounded by a loose areolar connective tissue called paratenon. The paratenon functions as elastic sleeve and permits free movement against surrounding structures. Other tendons have a synovial sheath, which performs the same task (Kvist et al., 1987).

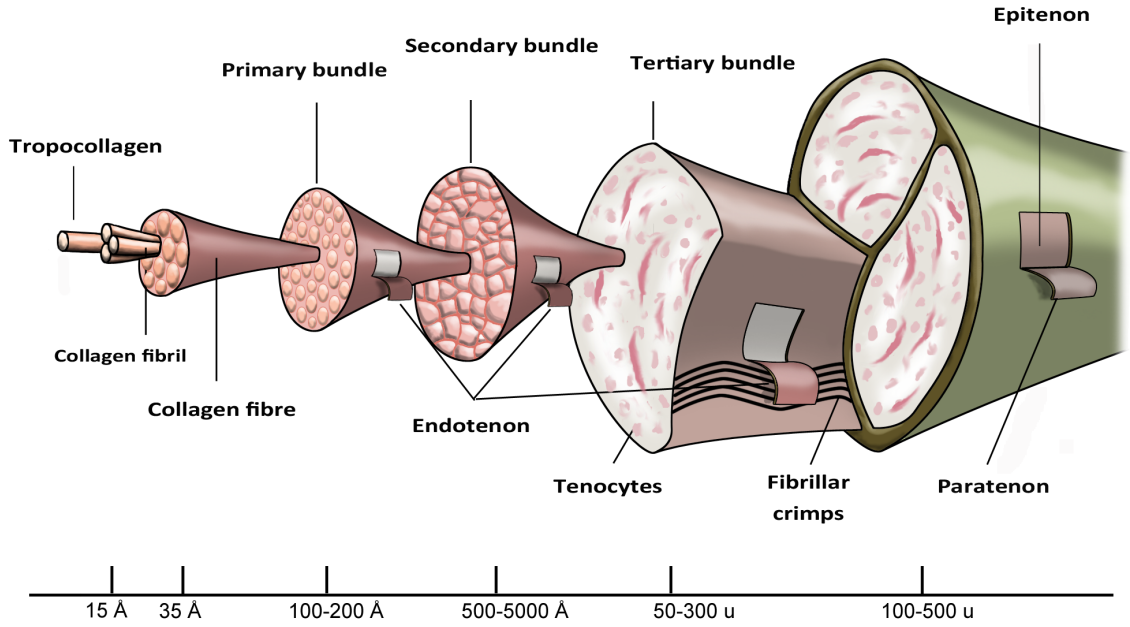


Figure 1: Internal architecture of tendons from collagen fibrils to the entire tendon.

1.3 TENDON CELLS

The structural components of tendons are produced by primary tendon cells, the so-called tenocytes and tenoblasts. They belong to a subpopulation of fibroblasts and constitute about 90-95 % of the cellular elements in the tendons (Riley, 2008). The remaining 5-10 % are chondrocytes at the osteotendinous junction, synovial cells in the tendon sheaths, and vascular cells like endothelial and smooth muscle cells (Jozsa & Kannus, 1997).

Tenoblasts are rounded/cylindrical in shape, have a high metabolic rate and do predominantly occur in younger ages. During maturation they become more elongated and spindle-shaped. Eventually they differentiate into tenocytes, which have a lower metabolic activity (Jozsa & Kannus, 1997). In normal tendons, the number of tenocytes is relatively sparse. The situation can, however, vary between different tendons in the body and between different locations along the tendon (Riley, 2008).

Tendon cells respond to mechanical stress and produce and release several components of the ECM. They are connected via gap junctions. Through these interconnections the cells form a three-dimensional network surrounding the collagen structures and providing the possibility for cell-to-cell interactions (McNeilly et al., 1996; Karousou et al., 2010).

1.4 EXTRACELLULAR MATRIX

As described above, tendons are predominantly composed of collagen. This collagen-rich hierarchical structure (see above) results in a tissue with high tensile strength. Every level of this hierarchy is interspersed with small amounts of non-collagenous matrix. Apart from elastic fibres, which are only scarcely present in human tendons, this matrix is basically composed of ground substance and anorganic substances (Thorpe et al., 2013).

The tendinous ground substance surrounds the collagen and consists of proteoglycans, glycosaminoglycans (GAGs), structural glycoproteins, and many other small molecules. The water-binding capacity of the macromolecules improves the biomechanical properties against shear and compressive forces. Furthermore, these molecules stabilise the whole collagenous system and are important for maintaining the ionic homeostasis and collagen fibrillogenesis (O'Brien, 1997; Thorpe et al., 2013).

Anorganic components account for less than 0.2 % of the tendon dry mass. Calcium has been found to be the most frequently present anorganic component in tendons, but also magnesium, manganese, cadmium, cobalt, copper, zinc, nickel, lithium, lead, fluoride, phosphor and silicon have been found. In general, it is assumed that anorganic components are involved in growth, development, and metabolism of musculoskeletal structures (Kannus, 2000).

The composition of the ECM can vary between tendons with different functions. This applies especially to pure positional tendons versus tendons with additional functions such as energy storing (Thorpe et al., 2013). Age-related changes are supposed to increase the risk of tendon injuries, especially in those with energy-storing function (Knobloch et al., 2008; Thorpe et al., 2013).

Chapter 2: THE HUMAN ACHILLES TENDON

2.1 ANATOMY AND FUNCTION

The Achilles tendon, also known as the calcaneal tendon, is the thickest and strongest tendon in the human body. It is a conjoined tendon of the distal parts of the gastrocnemius and soleus muscles, the main plantar flexors of the ankle (figure 2) (Doral et al., 2010). The most proximal part of the Achilles tendon arises from the two heads of the gastrocnemius muscle and forms a flat and broad aponeurosis. After half of its length, the tendon receives fibres from the soleus muscle onto its anterior/ventral surface (O'Brian, 2005). In the distal direction, the tendon becomes narrow and rounded until approximately 4 cm from its insertion onto the calcaneal bone. Thereafter, before inserting into a rough area of the lower part of the posterior surface of this bone, the tendon flattens, expands and becomes cartilaginous (O'Brian, 2005). The fibres of the gastrocnemius and soleus muscles can vary in their orientation, the degree of contribution to the Achilles tendon, and the extent of their fusion (Cummins et al., 1946).

Along their way to the insertion, the tendon fibres spiral up to 90° (Edama et al., 2014). Thus, the gastrocnemius component is found mainly in the lateral and posterior part of the Achilles tendon and the soleus part is mainly found on the medial and anterior area (O'Brian, 2005). This lateral rotation of the tendon assists in the supination of the ankle but is also assumed to create increased internal stress on the tendon's midportion at 2-6 cm above the calcaneal insertion (Jozsa & Kannus, 1997; O'Brian, 2005). This is the area that will be the focus of this work.

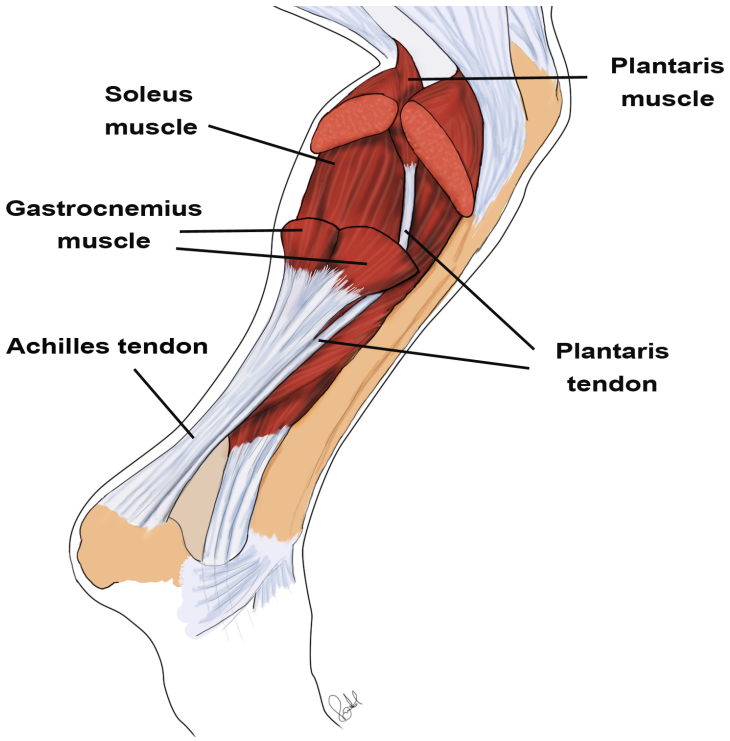


Figure 2: Anatomy of the triceps surae muscle (gastrocnemius and soleus muscle) and the plantaris muscle with their correspodning tendons. (Adapted from study III, figure 7)

On the dorsal, lateral and medial sides the Achilles tendon is surrounded by the paratenon (figure 3). It functions as an elastic sleeve that allows free movement of the tendon against surrounding tissues (Kvist et al., 1987; Jozsa & Kannus, 1997). The characteristics of the ventral aspect of the surrounding tissues are variously defined in the literature. Nevertheless, it is established that the Achilles tendon is attached ventrally to a fatty areolar tissue with rich vascularisation – a description not characteristic for the paratenon (Kvist et al., 1987). Thus, this tissue is often called "loose paratendinous connective tissue". Since it is located outside the tendon tissue proper it is often referred to as "peritendinous connective tissue".

Ventrally to the Achilles tendon, the so-called "Kager's fat pad" is located (figure 3). It is basically a mass of tissue including primarily adipose cells together with small bundles of elastic fibres and type I collagen (Shaw et al., 2007). Its boundaries are the ventral Achilles tendon, the calcaneus bone and the posterior border of the flexor hallucis longus muscle.

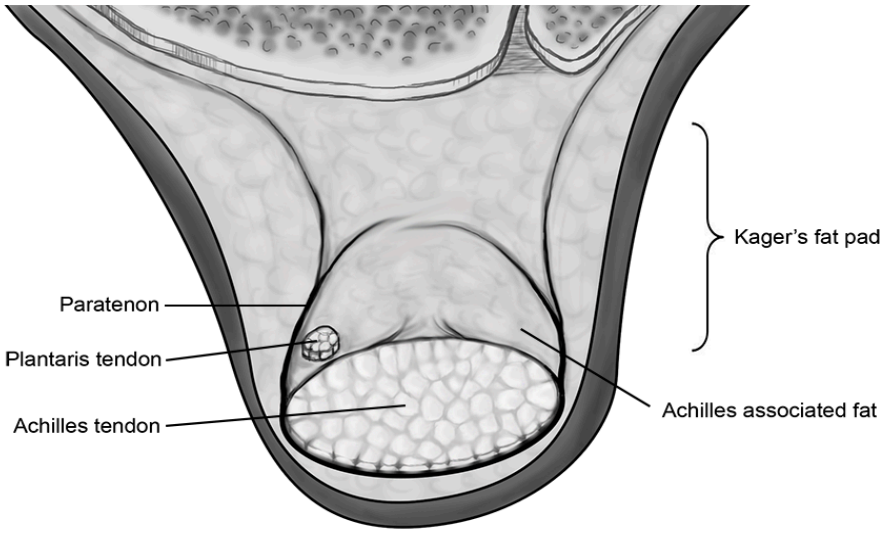


Figure 3: Transverse section of the midportion of the Achilles tendon.

2.2 BLOOD SUPPLY

Generally the blood supply of tendons is divided into three parts, related to the various tendon regions: The musculo-tendinous junction, the course of the tendon and the tendon-bone (osteotendinous) junction (O'Brien, 2005).

In the case of the Achilles tendon, some vessels derive from the perimysium of the triceps surae muscle continuing between the tendon fascicles, whilst some penetrate from the osteotendinous junction (Carr & Norris, 1989; O'Brien, 2005). The main supply, however, derives from the paratendinous network of blood vessels, which originates from the anterior and posterior tibial arteries, and to some extent from the peroneal arteries (Lagergren & Lindholm, 1959; Chen et al., 2009). Branches from these arteries run in parallel to the long axis of the tendon before penetrating transversely into the connective tissue sheaths

surrounding the tendon. Finally, they enter the tendon tissue proper via coursing into the endotenon (O'Brien, 2005).

Several studies have reported that the middle part of the Achilles tendon (the "midportion") is the least vascularised area of the tendon at all ages (Lagergren & Lindholm, 1959; Carr & Norris, 1989; Ahmed et al., 1998; Zantop et al., 2003). It has thus been assumed that this feature makes this part of the tendon particularly prone to injuries (Theobald et al., 2005).

2.3 INNERVATION

Tendons in general have a quite low degree of innervation. Under normal conditions, nerve fibres do not enter the tendon tissue proper, but terminate as nerve endings in the different surrounding connective tissues (paratenon, epitenon, endotenon) (Ackermann, 2013). There are, however, differences between different tendons in the body. The innervation mainly originates from neighbouring muscular, cutaneous, and peritendinous nerve trunks (Stilwell, 1957).

The Achilles tendon's innervation is sparse as compared to that of smaller tendons, such as finger flexors. It receives nerve fibres from different sources, including nerves innervating the triceps surae muscle but also cutaneous branches of the sural nerve. The main part of innervation is found in the paratenon, to some extent penetrating the epitenon, and running into the endotenon (Stilwell, 1957). Nevertheless, there are normally very few nerve fibres in the endotenon spaces.

Nerve fibres found in tendons are mainly unmyelinated Aγ-, Aδ-, B-, and C- nerve fibres mediating mechanoception, nociception, and vasomotor modulation (Ackermann, 2013). In addition to these classical features it is known that the peripheral nervous system can also participate in the regulation of several efferent actions, such as cell proliferation, expression of cytokines and growth factors, inflammation and immune responses. This is a so-called "efferent" role of afferent nociceptive nerve fibres (Bayliss, 1901). The homoeostatic regulation of a healthy tendon is highly dependent on the balance of neuromediator modulation (Ackermann, 2013). Recently, it has been shown that nerve fibres in the Achilles tendon express sensory, sympathetic and excitatory neuromediators (Alfredson et al., 2001; Bjur et al., 2005; Ackermann, 2013). The sympathetic nerve fibres are found perivascularly and to some extent as constituents in nerve fascicles – the location primarily being the paratenon.

2.4 PATHOLOGIES

As the largest tendon in the body, the Achilles tendon is exposed to a lot of repetitive strain from physical activities, such as running and jumping, but also to acceleration and deceleration. As a consequence, the tendon is susceptible to injuries like ruptures and degenerative changes (Asplund & Best, 2013).

The incidence of Achilles tendon ruptures has increased immensely in the last decades. Rupture mainly occurs in sedentary individuals that are involved in occasional physical activity, mostly in their fourth or fifth decade of life (Schepsis et al., 2002; Longo et al.,

2013). The incidence is seven injuries per 100 000 individuals in the general population and twelve per 100 000 among competitive athletes (Hess, 2010).

Other types of Achilles tendon pathologies are so-called degenerative disorders commonly called tendinopathies. The most common degenerative pathology is midportion Achilles tendinopathy (55-65 %) followed by tendinopathy in the insertional part (20-25 %) (Asplund & Best, 2013). Midportion Achilles tendinopathy will be further described in the following chapters and is the main topic of this work.

Chapter 3: THE HUMAN PLANTARIS TENDON

3.1 ANATOMY AND FUNCTION

The human plantaris muscle/tendon unit normally originates from the postero-superior aspect of the lateral femoral condyle and inserts into the calcaneal bone (figure 2). The plantaris consists of a small and short muscle belly, about 1.5 x 10 cm^2 in size, and a long tendon, varying in size and form between individuals (Spina, 2007; Dar et al., 2013). Due to its slender shape the tendon is also sometimes called 'freshmen's nerve' or 'fool's nerve' as it can be easily interpreted as a nerve (Spina, 2007).

The plantaris is a two-joint muscle, meaning its action can influence both joints involved. It contributes to ankle flexion if the foot is free, or to bending the knee if the foot is fixed. The plantaris muscle is very active when plantar flexion occurs in full knee extension. When flexion of the knee increases, the amplitude of activity falls progressively, due to mechanical insufficiency. Moderate plantaris activity during stair climbing and level walking suggests that the plantaris muscle assists the function of the knee in the loading situations (Basmajan & de Luca, 1985; Dar et al., 2013).

Due to the high density of muscle spindles within the muscle, the plantaris is thought to be an organ of proprioceptive function for the larger, more powerful plantarflexors such as the triceps surae muscle (Spina, 2007).

3.2 ANATOMICAL VARIATIONS

Cadaver studies, and examinations using imaging tools, have reported a huge variety concerning plantaris muscle/tendon anatomy. In a study from Nayak et al. (2010) on a population of native Indians three different types of origins were found: One is the lateral supracondylar ridge of the femur, capsule of knee joint and lateral head of gastrocnemius; another is the capsule of the knee joint and lateral head of gastrocnemius; the third is the lateral supracondylar ridge, capsule of knee joint, lateral head of gastrocnemius and fibrillar collateral ligament. In a case report by Kalniev et al. (2014) an additional plantaris muscle was observed originating from the soleus muscle. In another individual, a plantaris muscle with two separate heads, both with belly and a long tendon, was found (Sawant et al., 2012).

Variations have also been observed for the insertional (tendon) part. Nayak and collaborators classified 3 types of insertions: The flexor retinaculum of the foot, directly to the calcaneal bone, and at various levels of the Achilles tendon (Nayak et al., 2010). In a large study on 750 lower limbs, Daseler & Anson (1943) found that 80% of plantaris tendons insert separately from the Achilles tendon. They defined four types of insertion: (1) insertion into the calcaneus, separately and anteriorly to the Achilles tendon, directly attaching to the superior border of calcaneus bone; (2) insertion into the medial aspect of the calcaneus bone adjacent to the Achilles tendon; (3) finishing of the tendon in a wide insertion medially at a terminal portion of the Achilles tendon and the adjacent calcaneus bone; and finally (4) attachment medially into the Achilles tendon before insertion. Van Sterkenburg and co-workers (2011a) have classified even nine different insertion sites. A firm attachment to the Achilles tendon was hereby found in 11 out of 107 examined cadavers (10%): Three onto the

Achilles tendon midportion, three with a retinaculum-like structure (holding the Achilles and plantaris tendons together) transversally constricting the Achilles and plantaris tendons 32-88 mm proximally to the insertion; two into the deep fascia; and two adhering onto the anteromedial side, and one onto the anterior side of the Achilles tendon by cords of solid tissue but inserting into calcaneus bone.

Despite the different definitions, and partly different findings in the studies mentioned, they all have in common that the plantaris tendon insertion is mostly found at the calcaneus bone, independently of the Achilles tendon. However, marked differences can be present and insertion into the Achilles tendon occurs in a subgroup of individuals (Harvey et al., 1983; Schlicht & Morrison, 1992; Dos Santos et al, 2009).

It has been hypothesised that these variations described above are the result of evolutionary processes. Since the human plantaris muscle does not have important functions as compared to the situation for several animals, it has been discussed that it might have become vestigial as the foot is evolved for long-distance walking. The muscle is useful for other primates for grasping with their feet (Sawant et al., 2012). In a book of Robert Wiedersheim (1893) on human anatomy and relevance to man's evolutionary history, the plantaris muscle was described as one of the human organs that became wholly or in part functionless in human evolution. Daseler & Anson (1943) suggested that the plantaris muscle was earlier attached to the plantar aponeurosis of the foot, but with the evolutionary processes of erect posture, the insertion of the muscle got shifted to a higher position possibly explaining the Achilles tendon insertion. In several animals, like the American bear, the plantaris muscle can be found to be attached to the plantar aponeuris.

A very interesting observation is that a plantaris tendon attachment onto the Achilles tendon is rarely seen bilaterally. This gives some evidence that parts of these variations might also develop during life, depending on how the two lower extremities are used (Van Sterkenburg et al., 2011a).

3.3 DEBATE ABOUT ABSENCE

Not only structural variations have been reported. Many studies have actually described an absence of the plantaris muscle/tendon in a subgroup of humans (4-21 %) (Danforth, 1924; Daseler & Anson, 1943; Harvey et al., 1983; Moss, 1988; Freeman et al., 2008; Nayak et al., 2010; Kose et al., 2014). Some authors suggest that a possible absence occurs bilaterally (Dos Santos et al., 2009; Mackay & McCulloch, 1990), whereas in a recent MRI study absence was also described for single legs (Kose et al., 2014).

Van Sterkenburg and co-workers (2011a) could not find any absence of the plantaris tendon in 107 cadavers examined, and discussed the importance of appropriate examination/dissecton methods for its identification. The difficulty to find the plantaris tendon, especially in distal insertion areas, might be due the adherence with the Achilles tendon because of their close relationships or to a structural connection between the two tendons. A problem in this regard is that only a few studies clearly indicated how the surgical examination/dissection was performed. Therefore it is not easy to draw the right conclusions from these studies. It is, nevertheless, very clear that the plantaris muscle/tendon unit does not follow a similar pattern in all humans.

3.4 PATHOLOGIES

Ruptures of the plantaris tendon often occur without specific trauma (Dar et al., 2013). It has been shown that most patients suffering from plantaris tendon rupture have no sensory or motoric deficits, and that healing takes place very quickly (Harmon et al., 2006). Patients can go back to full loading already 3 weeks after injury (Harmon et al., 2006). Ruptures mostly occur either at the insertional area or at the musculotendinous junction. Midportion ruptures are rare (Harmon et al., 2006). A very interesting finding is that the plantaris tendon is often intact in patients with Achilles tendon ruptures (Vanderhooft, 1997).

Rupture of the muscle part of the plantaris was first associated with the term “tennis leg” (Powell, 1883). The classical manifestation of a “tennis leg“ patient is in a middle-aged person who complains of sports-related acute pain in the middle portion of the calf, associated with a snapping sensation (Gilbert et al., 1996). Meanwhile it is quite clear that although isolated plantaris muscle/tendon ruptures can occur (Mennen, 1983; Allard et al., 1992; Helms et al., 1995; Mozena & Pearson, 2004), tennis leg is mainly a result of a rupture of the medial head of the gastrocnemius muscle at the musculotendinous juncion rather than a plantaris rupture (Delgado et al., 2002).

Chapter 4: ACHILLES TENDINOPATHY / TENDINOSIS

4.1 TERMINOLOGY

The term midportion Achilles ***tendinopathy*** has been defined as a clinical syndrome characterised by the tendon pain, impaired function, and swelling located at 2-7 cm from the insertion onto the calcaneus (Khan et al., 1999). This area is sometimes also described as the "main body of the Achilles tendon" (van Dijk et al., 2011). The clinical term tendinopathy does not necessarily imply the presence of structural tissue abnormalities (van Dijk et al., 2011). It is, however, known that the majority of symptomatic tendons exhibit structural abnormalities (Astrom & Rausing, 1995).

The term ***tendinosis*** represents histopathological changes (see below) diagnosed via histological methods and/or imaging tools, such as ultrasound and colour Doppler (US+CD) and magnetic resonance imaging (MRI) (Khan et al., 1999; Alfredson et al., 2003). Individuals with tendinosis-related tissue changes can be asymptomatic (Docking et al., 2014).

Pathology related to the paratenon is called ***paratendinopathy or paratendinitis*** and is characterised by an acute or chronic inflammation and/or degeneration of the paratenon. It sometimes co-occurs with tendinosis in the tendon tissue proper (van Dijk et al., 2011).

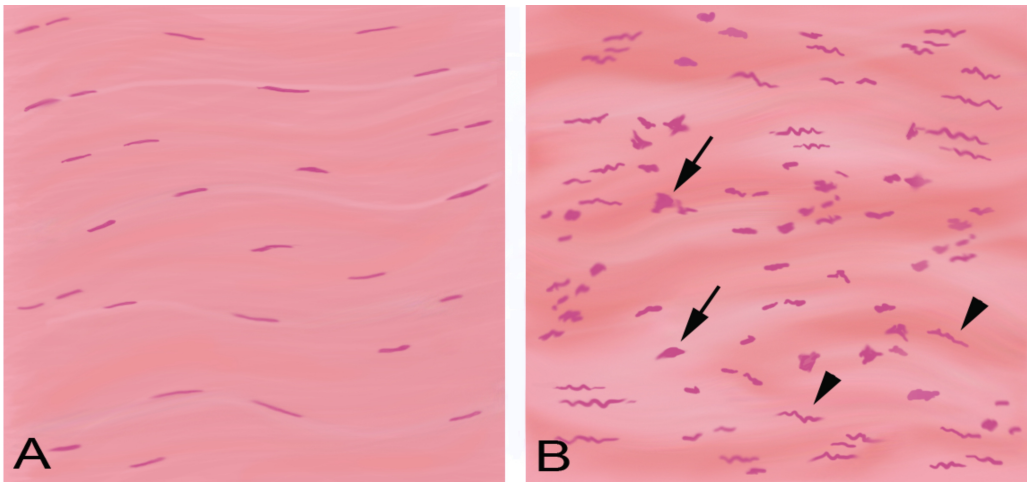


Figure 4: Histology of a normal tendon (A) and a tendon with tendinosis-like tissue changes (B). Arrows point on rounded tendon cells, arrow-heads indicate wavy-shaped cells.

4.2 HISTOPATHOLOGY (TENDINOSIS)

It is nowadays widely accepted that chronic tendinosis is a degenerative rather than an inflammatory condition (Maffulli et al., 1998; Khan et al., 2002). There are no signs of a classical inflammation inside the Achilles tendon, including the non-existence of inflammatory cells (Khan et al., 1999) and there are no elevated levels of prostaglandin E_2 (Alfredson et al., 1999). The main features of tendinosis-related tissue changes are

hypercellularity, increased vascularity and disorganised collagen fibre structure (figure 4) (Järvinen et al., 1997; Khan et al., 1999; Alfredson, 2005). The higher amount of cells is also combined with an increased number of cells that have rounded nuclei, indicating higher metabolism and cell turnover (Chuen et al., 2004). Furthermore, changes in the composition of the non-collagenous matrix, such as proteoglycans, have been observed (Parkinson et al., 2011).

4.3 EPIDEMIOLOGY AND SYMPTOMS

Achilles tendinopathy is often associated with strenuous physical activity such as running and jumping. It accounts for 55-65 % of all Achilles tendon disorders (Järvinen, et al., 2005). The occurrence of this condition is especially frequent in individuals who are active in middle- and long-distance running, orienteering, track and field, tennis, badminton, volleyball, and soccer (Johansson, 1986; Lysholm & Wiklander, 1987; Kvist, 1991, 1994; Fahlström et al., 2002a,b; Järvinen, et al., 2005). The annual incidence in top-level runners is 7-9 % (Johansson, 1986; Lysholm & Wiklander, 1987). In the general population the incidence is about 2 % (de Jonge et al, 2011a).

Achilles tendon disorders in general are more common in older than younger individuals (Kannus et al., 1989). Furthermore it has been shown that individuals with a quite low level of physical activity, or who even not at all are participating in physical activity on a regular basis, can be affected (Rolf & Movin, 1997).

Achilles tendinopathy normally develops with a gradual onset of pain and without any occurrence of trauma. Initially, patients often neglect the pain because the feeling of discomfort – pain and stiffness – disappears during physical activity. Later on, morning stiffness is common and symptoms also occur during loading. At this stage, local swelling is often seen (Cook et al., 2002; Kader et al., 2002).

4.4 AETIOLOGY

Despite extensive research the aetiology and pathogenesis of Achilles tendinopathy are poorly understood. It is, however, very likely that intrinsic and extrinsic factors interact in this condition (Khan & Maffulli, 1998).

Excessive load is considered to be the main stimulus for developing tendinopathy. However, there are also other extrinsic sports-related factors such as training errors, changes in training patterns, poor equipment, and environmental conditions that seem to have an impact (Kvist, 1991; Järvinen et al., 2005). Furthermore, it has been shown that some types of drugs like fluoroquinolone and corticosteroids can play a role as well (Järvinen et al., 2005).

Beside extrinsic factors there are several intrinsic aspects that can predispose for tendinopathy. Generally age, body weight, height, tendon vascularity and adiposity have been identified (Kannus, 1997; Järvinen et al., 2005; Gaida et al., 2009). Also anatomic factors of the lower limb, such as malalignments, decreased flexibility, muscle weakness and leg length discrepancy can possibly predispose (Järvinen et al., 2005).

4.5 DIAGNOSTICS AND IMAGING

As described above, the clinical symptoms of midportion Achilles tendinopathy are pain and impaired function; these being accompanied by local swelling in the Achilles tendon midportion (Khan et al., 1999). Pain on palpation of the tendon and subjective reporting of pain in the midportion during Achilles tendon loading activity have shown to be good and valid tests, which facilitates further decisions on how to manage the condition (Hutchison et al., 2013). In this context, the VISA-A questionnaire is often used to determine the severity of tendinopathy (Robinson et al., 2001; Iversen et al., 2012).

Tendon tissue parameters such as thickness and structural disorganisation are visualised via traditional ultrasound (US) and the occurrence of high blood flow is detected with colour Doppler (CD) (Ohberg et al., 2001; Sengkerij et al., 2009). It should, however, be noted that there are controversies concerning the association between colour Doppler measures and clinical symptoms. Not all tendinopathic tendons show high blood flow. The different sensitivity to detect blood flow among different machines, and different timing of examination of the tendons, are of importance for the detection of high blood flow (Peers et al., 2003; de Vos et al., 2007). Magnetic resonance imaging (MRI) is also occasionally used by some clinicians (Shalabi et al., 2002).

Recently a new imaging tool called “Ultrasound Tissue Characterisation” (UTC) has been developed. It allows characterisation and semiquantification of the internal tendon architecture by comparing pixel integrity between 600 transverse images over 12 cm (van Schie et al., 2000; 2001; 2003; 2010). Based on this comparison, it renders a three dimensional image of the tendon and identifies four different types of collagen alignments (figure 5): Echotype I (green) represents intact and aligned bundles; echotype II (blue) stands for fibrils with increased waviness and a certain amount of separation of fibrils; echotype III (red) represents decreased fibrillar integrity; echotype IV (black) shows the absence of fibrillar organisation. Originally developed for racehorses, UTC has also been used on human Achilles and patellar tendons (van Schie et al., 2010; Docking et al., 2014; Rosengarten et al., 2015; van Ark et al., 2015).

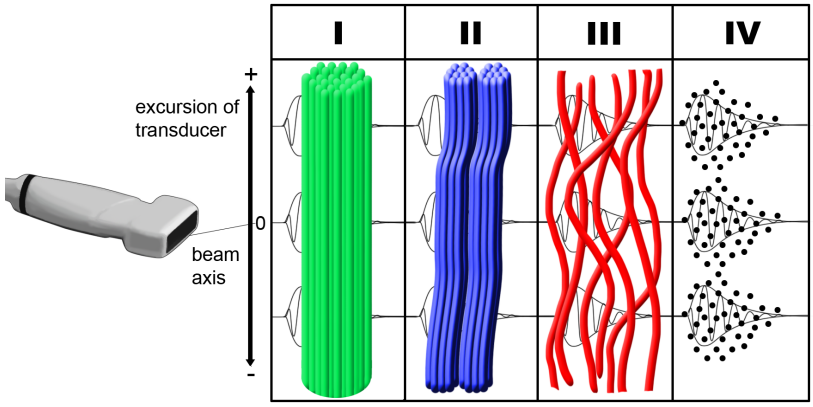


Figure 5: Types of echopixels measured with UTC. (Adapted from www.utcimaging.com)

It has been shown that symptomatic Achilles tendons have less type I and II echotypes and more type III and IV echotypes than asymptomatic Achilles tendons (van Schie et al., 2010). The architecture and integrity of the tendon matrix are based on comparison with histo-morphology of tissue specimens as reference test (van Schie et al., 2000; 2001; 2003; 2010). Thus, this tool is thought to be useful to diagnose Achilles tendinopathy and also to guide loading during pre- and post treatment, and during season in order to try to prevent from tendon disorders (Cook & Purdam, 2014; Rosengarten et al., 2015).

4.6 TREATMENTS

In the treatment of midportion Achilles tendinopathy, there are both non-surgical and surgical options. The initial treatment should consist of conservative methods, but in approximately 25 % of patients a surgical intervention is still required (Zwiers et al., 2014).

Eccentric training, mostly described as a twelve-week programme, has been shown to be an effective first-line treatment (Alfredson et al., 1998; Fahlström et al., 2003; Ohberg et al., 2004; Magnussen et al., 2009). The mechanisms behind the results are, however, not fully understood yet (Rees et al., 2009; Allison & Purdam, 2009). Recently, the focus has shifted to programmes also including concentric loading (Zwiers et al., 2014). In the midseason, it is not always recommended to use eccentrics since it can increase tendinopathic symptoms in the beginning of the treatment (Cook & Purdam, 2014). Reduction of compression and isometric exercises to maintain the tendon stimulus, but also to reduce the pain, should be introduced reasonably early as suggested by some authors (Cook & Purdam, 2014).

Beside exercise treatments there are some other non-invasive treatments, such as extracorporeal shock wave therapy (ESWT), stretching, iontophoresis, topical glyceryl trinitrate, and low-level laser. The evidence, however, is only moderate or limited for these (Zwiers et al., 2014).

There is a variety of injection treatments in use to treat midportion Achilles tendinopathy (van Sterkenburg & van Dijk, 2011). The majority of these are still to be considered at an experimental stage with very little support in scientific studies (Scott et al., 2011; Zwiers et al., 2014). Injecting corticosteroids inside or outside the tendon is very controversial, with no evidence-based studies confirming their effect, and many state that this should be completely avoided in midportion Achilles tendinopathy (Scott et al., 2011; Zwiers et al., 2014). Recently, injections of platelet-rich-plasma PRP have become very popular (Gaweda et al., 2010). However, again there is no science supporting the use. The only level 1 study performed showed no difference between injecting PRP and saline (do Vos et al., 2010; de Jonge et al., 2011b).

New research findings showing that there are very few nerves inside the tendinopathic tendon, but lots of nerves in close relation to blood vessels outside the deep (ventral) side of the tendon (Bjur et al., 2005; Andersson et al., 2007), initiated a method using sclerosing polidocanol injections. Ultrasound and Doppler-guided injections targeting regions with high blood flow (and nerves) outside the tendon was shown to reduce tendon pain in several, mostly randomised controlled, studies (Ohberg & Alfredson, 2002; Alfredson & Ohberg, 2005; Willberg et al., 2008). Also, a long-term follow-up study showed good clinical results and decreased tendon thickness and improved structure (Lind et al., 2006). These new

findings also initiated “high volume injection treatment”, in which high volumes of a mixture containing local anaestetic+ saline and cortisone are injected outside the tendon (Chan et al., 2008). The results have been promising, but more studies are needed on larger material.

In patients for whom conservative treatment fails, surgical treatment is often instituted. There are different surgical approaches in use concerning what tissue that is addressed for the treatment. The traditional method is an intra-tendinous approach, in which macroscopically abnormal tendon tissue is removed, this being combined with a relatively long rehabilitation period (Zwiers et al., 2014). The results of that method have been shown to be varying (Zwiers et al., 2014). Recently, a new method, initiated from the new research findings showing that the majority of nerves are located outside the tendon, has come into use. It is a minimally invasive surgical scraping procedure in local anaesthesia, in which US/CD findings guide the surgery targeting the regions with high blood flow and nerves outside the ventral side of the tendon (Alfredson et al., 2007; Alfredson, 2011a). This method is combined with fast rehabilitation and return to full tendon loading, and the clinical results have been shown to be good in a randomised study (Alfredson et al., 2007) and in a large material study (Alfredson, 2011a). Other procedures used a tendon stripper (Longo et al., 2008) or endoscopic debridement (Steenstra & van Dijk, 2006) targeting similar tissues. Recently it has also been found that plantaris tendon removal is beneficial in several patients with Achilles tendinopathy (Alfredson, 2011b). This will be further described below.

Chapter 5: CURRENT TOPICS - BASIS FOR THIS WORK

5.1 PLANTARIS TENDON INVOLVEMENT IN MIDPORTION ACHILLES TENDINOPATHY

In patients with midportion Achilles tendinopathy who failed to respond to the surgical scraping treatment of the ventral side of the Achilles tendon, a thickened plantaris tendon has often been found to be positioned close to the Achilles tendon, seemingly interfering with the medial side of this tendon (Alfredson, 2011b). Plantaris tendon release and removal in addition to the scraping procedure has shown very good outcome in this condition (Alfredson, 2011b; Ruergard & Alfredson, 2014). Despite these promising results there is poor knowledge on the background mechanisms of the potential plantaris tendon involvement in Achilles tendinopathy. It has been discussed that compressive forces – the plantaris tendon compressing onto the Achilles tendon - may play a role (Cook & Purdam, 2012). This is supported by the fact that the plantaris tendon has been reported to be stronger and stiffer than the Achilles tendon (Lintz et al., 2011). The peritendinous connective tissue in between the Achilles and plantaris tendons might be highly affected in this situation.

There is so far nothing known about the morphology and innervation patterns in the plantaris tendon and the peritendinous connective tissue in between the Achilles and plantaris tendons in situations with midportion Achilles tendinopathy. Information on this is however necessary in order to further explain the mechanisms behind a possible plantaris tendon involvement. Furthermore, it is of high interest to know how the Achilles tendon structure develops after plantaris tendon removal. These issues were addressed in the present thesis.

5.2 POTENTIAL ROLES OF GLUTAMATE AND ACETYLCHOLINE

When shifting the concept of tendinopathy from a classical inflammatory condition to a rather degenerative condition, the mechanisms behind pain were newly discussed (Khan et al., 2000). Khan and co-authors hypothesised that the pain might have biochemical and not only structural origin (Khan et al., 2000). Thus, in the past 15 years several research groups have extensively investigated the occurrence of numerous signalling substances that are thought to be involved in pain perception, but also the tissue changes that appear in tendinosis. The idea that those substances can cause tendinosis-related features was summarised as the "biochemical hypothesis" (Danielson, 2009, Scott & Bahr, 2009). It is believed that tenocytes can produce and release those substances and hereby interact with other tenocytes, vessels, and nerve fibres leading to tendinosis-like features such as cell proliferation/apoptosis, collagen production, and nerve/vessel ingrowth (Danielson, 2009, Scott & Bahr, 2009). Especially the possible impacts of substance P (Andersson et al., 2008; Andersson et al., 2011a; Backman et al., 2011a, 2011b) and catecholamines (Bjur et al., 2008a; Backman et al., 2013) have recently been in focus. Two other substances that have been discussed in relation to tendinopathy/tendinosis are glutamate and acetylcholine (ACh), both well-known neurotransmitters in the nervous system. Beside their key functions for nerves, both substances have also been described to have key-roles in non-neuronal tissues

(Kalariti et al., 2005; Wessler & Kirkpatrick, 2008). Recent studies give some evidence that they might even be involved in tendinopathy.

High levels of glutamate have been detected in chronic painful Achilles tendons (Alfredson et al., 1999) and injections of glutamate have been shown to provoke tendon pain (Gibson et al., 2009). Furthermore, several glutamate receptors, e.g. NMDA receptors, have been observed to be expressed by tenocytes and that the expression is increased in cells with pathologic appearancee (Schizas et al., 2010; 2012). Abnormal tenocytes also show higher expressions of vesicular transporters, such as VGluT2 (Scott et al., 2008). These transporters enable the cells to release glutamate via synaptic-like vesicles (Moriyama & Omote, 2008). There is, however, nothing known about the expression patterns in the plantaris tendon and the peritendinous tissue between the Achilles and plantaris tendons. Another drawback is the lack of knowledge concerning the effect of glutamate on tendon tissue using *in vitro* settings. Previous studies on rat supraspinatus tendons suggested apoptotic effects on tendon cells (Molloy et al., 2006). However, studies on human materiel are lacking.

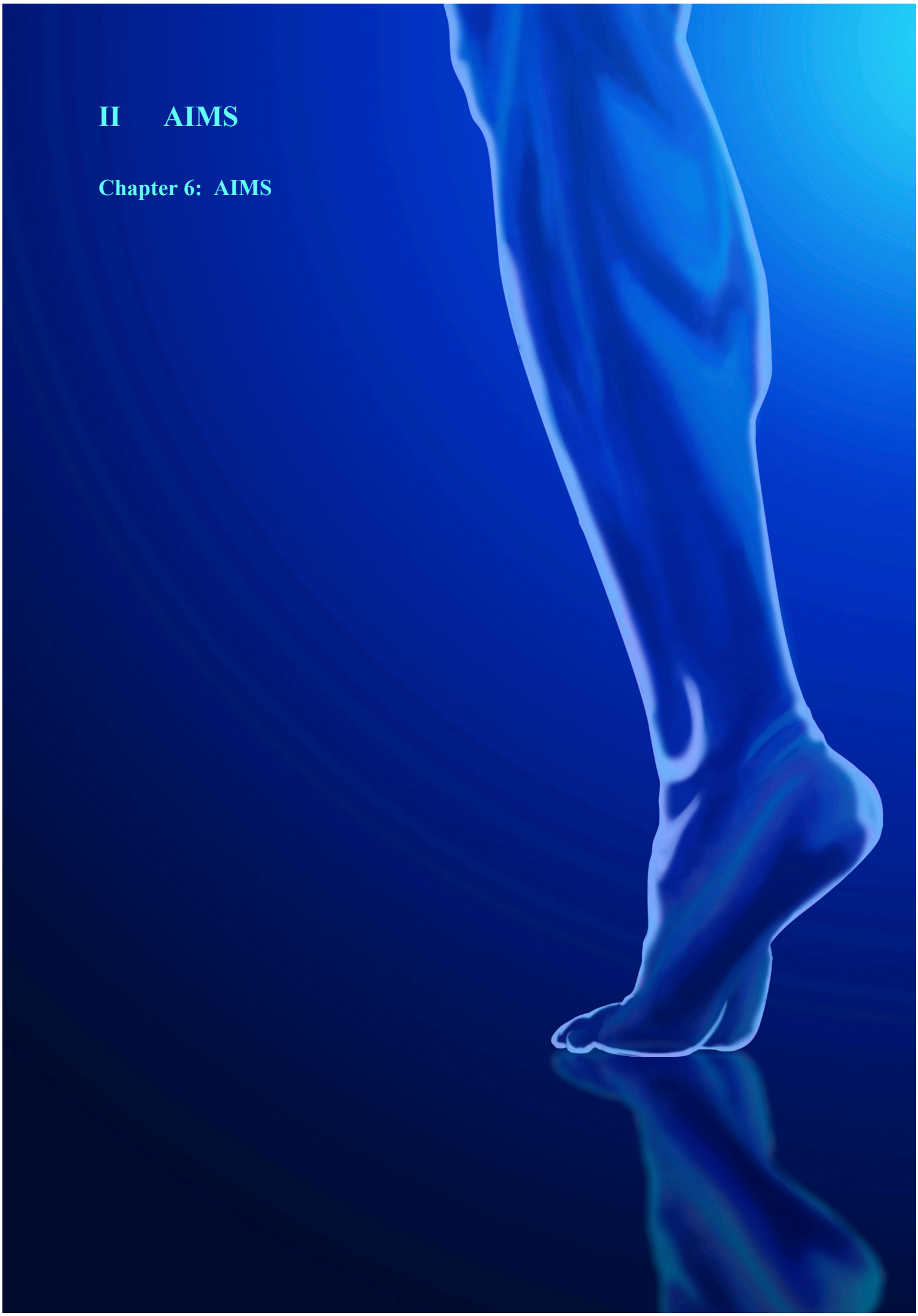
In abnormal Achilles and patellar tenocytes, it has also been found that the ACh producing enzyme choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VAChT) and muscarinic ACh receptors are upregulated (Danielson et al., 2007; Bjur et al., 2008b). *In vitro* studies have shown that acetylcholine stimulation can lead to Achilles tenocyte proliferation (Fong et al., 2013). However, there is nothing known as to whether a non-neuronal cholinergic system also exists in the plantaris tendon and the peritendinous tissue.

5.3 BILATERAL EFFECTS

Despite extensive research the mechanisms behind tendon pain are not fully understood (Rio et al., 2014). Recent studies have highlighted the importance of the central nervous system (Rio et al., 2014). This idea is supported by the observation of bilateral phenomena in relation to muscle/tendon overuse. Bilateral muscle inflammation (myositis) has been observed to occur in the rabbit triceps surae muscle after unilateral overuse (Song et al., 2012). The corresponding Achilles tendon showed tendinosis-like features bilaterally after unilateral stimulation using the same model (Andersson et al., 2011b). Sensory and motor deficits occur also in the non-injured side in patients with tendinopathies (Heales et al., 2014). The question remains if this knowledge can be used in the treatment of patients. Does unilateral surgical treatment targeting peripheral nerve structures, such as the ventral scraping procedure, result in bilateral recover?

II AIMS

Chapter 6: AIMS



Chapter 6: AIMS

6.1 AIMS

The overall aim of these studies was to characterise the plantaris tendon and the peritendinous connective tissue in between the Achilles and plantaris tendons in situations with midportion Achilles tendinopathy.

The specifies aims were to:

- Study the morphology of the plantaris tendon and the peritendinous connective tissue (I, III).
- Examine Achilles tendon structure after plantaris tendon removal and ventral-medial scraping (II).
- Examine the overall innervation patterns in the plantaris tendon and the peritendinous connective tissue, and specifically map the sensory and sympathetic innervation and the neuronal glutamate system (III).
- Evaluate bilateral effects of unilateral treatment targeting the peritendinous connective tissue (IV).
- Study the presence of a possible non-neuronal cholinergic system in the plantaris tendon and peritendinous connective tissue (I).
- Study the presence of a possible non-neuronal glutamate system in the plantaris tendon and the peritendinous connective tissue (V).
- Examine the effect of glutamate on plantaris tendon cells (V).

III MATERIAL AND METHODS

Chapter 7: PATIENTS AND CLINICAL PROCEDURES

7.1 Patients
7.2 Pre-surgical examinations
7.3 Surgical procedures
7.4 Ethical considerations

Chapter 8: ANALYSIS OF TENDON TISSUE

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8.3 Immunohistochemistry (IHC)
8.4 In situ hybridisation

Chapter 9: PLANTARIS TENDON CELL CULTURE

9.1 Isolation and culturing
9.2 Immunocytochemistry (ICC)
9.3 Exposure to NMDA and glutamate
9.4 2D strain
9.5 Lactate dehydrogenase (LDH) assay
9.6 Detection of proteins (Western blot)
9.7 Detection of RNA (RT-qPCR)

Chapter 10: STATISTICAL ANALYSIS

Chapter 7: PATIENTS AND CLINICAL PROCEDURES

7.1 PATIENTS

All patients included in the present work had suffered from ultrasound + colour Doppler (US+CD) verified midportion Achilles tendinopathy with a duration of symptoms for at least 3 months. Most patients had tried exercise treatment (eccentrics) without success. The total material includes 69 patients (23 women, 46 men; mean age: 47 years). All patients underwant a surgical procedure performed by orthopaedic surgeon Håkan Alfredson. Normally the operations were performed 24 hours after a clinical assessment. The majority of patients (n=56) underwent a surgical procedure including plantaris tendon removal (figure 6) and scraping of the ventro-medial part of the Achilles tendon (see below, table 1). A subpopulation of the patients (n=13) was treated with scraping only. One plantaris tendon was taken from a healthy individual (women, 27 years).

Patients were excluded if they had other diseases or injuries related to the lower limb, such as acute or chronic inflammatory diseases, tendon tears, and previous major trauma to the ankle joints.

7.2 PRE-SURGICAL EXAMINATIONS

All Achilles tendons were examined using a high-resolution greyscale ultrasound (US) and colour+Doppler (CD) (Antares-Siemens, Germany), with a linear multifrequency (8-13 MHz) probe. Tendon thickness, structure and blood flow were evaluated. Tendons with high blood flow showed at least one region with localised high blood flow in the sagittal and axial planes. Furthermore, careful examination of the medial side, starting high up in the mid calf and going downwards, was performed to look for the plantaris tendon. Scans were performed by co-author Håkan Alfredson. All patients included in this study had a thickened Achilles tendon together with irregular tendon structure, hypoechogenity and localised high blood flow.

The Achilles tendon from patients included in study II (n=8) were further evaluated using "Ultrasound Tissue Characterisation (UTC)". Scans were collected in a distal to proximal direction, and were performed by co-author Lorenzo Masci. The details of the procedure are described in study II. Via UTC algorithms (UTC 2010, UTC Imaging) the dynamics of grey levels of corresponding pixels in contiguous images over 25 images were quantified. The scans were furthermore analysed for regions with disintegration (echopixels type III and IV) within the midportion of the Achilles tendon (superficial, ventral, medial or lateral). In the present work (study II) on patients who underwent plantaris tendon removal and scraping of the ventral Achilles tendon (procedure see below) scans were performed before and 6 months after surgery. All patients included in study II showed disintegration primarily in the medial aspect of the Achilles tendon at baseline.

7.3 SURGICAL PROCEDURES

The patients in the studies I, II, III and V underwent a surgical procedure, in local anaesthesia, consisting of plantaris tendon removal and scraping of the ventro-medial aspect of the Achilles tendon (Alfredson, 2011a; Alfredson, 2011b). The medial aspect of the tendon was hereby visualised via a minor longitudinal incision on the medial side of the Achilles tendon midportion. This region was then carefully inspected to evaluate the presence and precise location of the plantaris tendon. In all patients included in this work the plantaris tendon was positioned very close to the medial aspect of the Achilles tendon, seemingly compressing it (figure 6 A). In some cases, the plantaris tendon was "invaginated" into the Achilles tendon. The peritendinous connective tissue in between the Achilles and plantaris tendons often contained fat tissue (figure 6 B), not seldom infiltrating into the medial Achilles tendon. The plantaris tendon with adjacent peritendinous connective tissue was carefully released, followed distally and proximally, and cut at both ends. The regions of the ventro-medial side of Achilles tendon showing Ultrasound+Doppler-verified high blood flow additional scraping was performed.

Patients in study IV (6 women, 7 men; mean age 53 years; range 34-70) underwent a scraping procedure without plantaris removal. The incision was done on the lateral side and the ventral Achilles tendon was then released from the peritendinous connective tissue (Alfredson, 2011a).

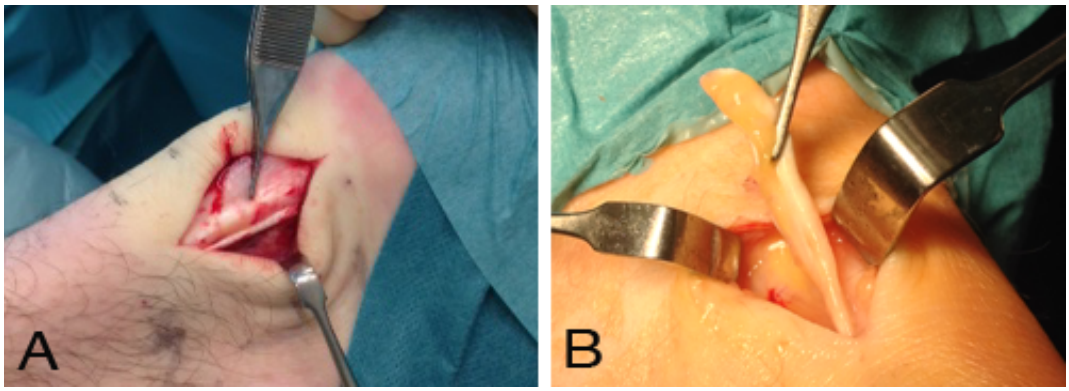

Figure 6: Plantaris tendon positioned very close to the medial Achilles tendon (A). Plantaris removal performed (B). Fatty richly vascularised peritendinous connective tissue is present in between the Achilles and plantaris tendons (B).

7.4 ETHICAL CONSIDERATIONS

All experiments were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was received from all patients. Ethical approvals were obtained from the Ethical Committee at the Medical Faculty of Umeå University, the Regional Ethical Review Board in Umeå (project nr. 04-157M, 2011-83-32M) and the Ethical Board of Queen Mary's University in London (UK).

Table 1: Patients undergoing plantaris removal and ventro-medial scraping (papers I, II, III, V)

Code	Sex	Age	Papers				Notes	
Umea: 15 women, 33 men; mean age: 47 years (range 18-69)								
UPF 1	female	58	I		III		Umeå	
UPF 2	female	50	I		III	V	Umeå	Bilateral procedures
UPF 3	female	56	I		III		Umeå	
UPF 4	female	52	I		III		Umeå	
UPF 5	female	57	I		III		Umeå	Bilateral procedures
UPF 6	female	60	I		III		Umeå	
UPF 7	female	57	I		III		Umeå	
UPF 8	female	69			III	V	Umeå	
UPF 9	female	55			III	V	Umeå	
UPF 10	female	53			III		Umeå	
UPF 11	female	46			III		Umeå	
UPF 12	female	40			III		Umeå	
UPF 13	female	44			III		Umeå	
UPF 14	female	64				V	Umeå	
UPF 15	female	38			III		Umeå	
UPM 1	male	53	I		III		Umeå	
UPM 2	male	44	I		III	V	Umeå	
UPM 3	male	38	I		III		Umeå	Bilateral procedures
UPM 4	male	50	I		III		Umeå	
UPM 5	male	35	I		III	V	Umeå	Bilateral procedures
UPM 6	male	40	I		III		Umeå	
UPM 7	male	43	I		III		Umeå	
UPM 8	male	52	I		III	V	Umeå	
UPM 9	male	18	I		III		Umeå	
UPM 10	male	41	I		III	V	Umeå	
UPM 11	male	49			III	V	Umeå	
UPM 12	male	61			III	V	Umeå	Bilateral procedures
UPM 13	male	50			III	V	Umeå	Bilateral procedures
UPM 14	male	64			III		Umeå	
UPM 15	male	51			III		Umeå	
UPM 16	male	57			III	V	Umeå	
UPM 17	male	56			III		Umeå	
UPM 18	male	51			III	V	Umeå	
UPM 19	male	46			III		Umeå	
UPM 20	male	40			III		Umeå	
UPM 21	male	45			III		Umeå	
UPM 22	male	41			III	V	Umeå	
UPM 23	male	34			III		Umeå	
UPM 24	male	50			III	V	Umeå	
UPM 25	male	42			III		Umeå	
UPM 26	male	25			III		Umeå	Bilateral procedures
UPM 27	male	40			III		Umeå	Bilateral procedures
UPM 28	male	40			III		Umeå	
UPM 29	male	52			III		Umeå	
UPM 30	male	44			III	V	Umeå	Bilateral procedures
UPM 31	male	60			III		Umeå	Bilateral procedures
UPM 32	male	35				V	Umeå	
UPM 33	male	39			III		Umeå	
London: 1 women, 7 men; mean age 39 years (range 26-56)								
LPF1	female	47		II			London	
LPM1	male	39		II			London	
LPM2	male	31		II			London	Bilateral procedure
LPM3	male	30		II			London	
LPM4	male	39		II			London	
LPM5	male	50		II			London	
LPM6	male	56		II			London	
LPM7	male	26		II			London	

Chapter 8: ANALYSIS OF TENDON TISSUE

8.1 TISSUE PROCESSING

Plantaris tendon (n=56) samples from 46 patients (10 patients underwent bilateral procedure) were processed for tissue analysis. All samples contained the plantaris tendon with adjacent peritendinous connective tissue including infiltrated fat tissue. The amount of peritendinous tissues however varied between the samples. For four patients a "tissue block" was taken containing the plantaris tendon, the peritendinous tissues and a small part of the medial Achilles tendon.

Immediately after the samples were taken, they were fixed overnight at 4˚C in a solution of 4 % formaldehyde in 0.1 M phosphate buffer (pH 7.0). Then the specimens were three times washed in Tyrode's solution containing 10 % sucrose, at 4˚C (one washing overnight), subdivided into specimens (at least three per tendon), mounted on a thin cardboard in OCT embedding medium (code: 45830; Miles Laboratories, Naperville, IL, USA) and frozen at -80˚C until sectioning.

Tissue sections of 7 μm thickness were cut using a cryostat (Leica Microsystem CM 3000, Heidelberg, Germany). The sections were mounted onto super frost slides (code: 041200; Menzel Gläser, Braunschweig, Germany) or slides pre-coated with crome-alume gelatine, and frozen at -20°C until use. In total, 137 tendon specimens from 46 patients and from 56 plantaris tendons were further processed and analysed.

8.2 GENERAL MORPHOLOGY (H&E STAINING)

To characterise the morphological features, sections were stained with haematoxylin and eosin (H&E). Initially, the slides were kept in Harris haematoxylin solution (code: 01800; Histolab, Göteborg, Sweden) for 2 minutes. Thereafter they were two to three times rinsed in distilled water, then dipped in 0.1 % acetic acid for a few seconds and washed in running warm water for around 5 minutes. After that, counterstaining with 1% Eosin in 70 % ethanol for 1 minute was performed. Finally the sections were dehydrated in absolute ethanol (3 times) and mounted in Pertex (code: 00840; Histolab).

8.3 IMMUNOHISTOCHEMISTRY (IHC)

Sections were immunohistochemically evaluated for innervation patterns (III), occurrence of immune cells (I,III), and components of cholinergic (I) and glutamate systems (III, V). The expression of scleraxis, a transcription factor for tenocyte-like marker tenomodulin (V), and tyrosine hydroxylase (TH) (IV) was also determined.

The innervation patterns were defined by using antibodies against general nerve marker PGP9.5, marker for sensory (CGRP) and sympathetic (TH) nerve fibres, glutamate receptor NMDA (NMDAR1) and Schwann cell marker S-100β. Furthermore, antibodies against macrophages (CD68), mast cells tryptase and T-cell and neutrophil marker (MCA805) were used. Regarding the cholinergic system, expression of choline acetyltransferase (ChAT) was

evaluated. Concerning glutamate signalling, antibodies against NMDAR1, but also glutamate itself and vesicular transporter VGluT2, were used. All antibodies are listed in table 2.

The staining procedures are described in detail in studies III and IV. The staining prcedure included the use of acid potassium permanganate for 2 minutes to enhance the visualisation of specific immunofluorescence reaction sites (Hansson & Forsgren, 1995). This step was not performed when using the antibody against S-100β. Primary antibodies are, as described above, shown in table 2; normal sera and secondary antibodies are stated in table 3 and 4 respectively.

When using antibodies being raised in goat, donkey normal serum and fluorescein isothiocyanate (FITC) conjugated donkey anti goat was applied. For rabbit antibodies swine normal serum and tetramethylrhodamine isocyanate (TRITC) conjugated swine anti rabbit was used. For mouse antibodies the serum was from rabbits and the secondary antibody was TRITC rabbit anti mouse.

For control purposes, antisera were preabsorbed overnight (4°C) with the corresponding blocking peptide (table 5). Replacements of primary antibodies with PBS were regularly performed.

Evaluation of the stainings was performed using a light microscope (Zeiss Axioscope) equipped with epifluorescence optics and an attached Olympus DP 70 digital camera.

8.4 PROCEDURE FOR *IN SITU* HYBRIDISATION

A digoxigenin (DIG)-hyperlabeled oligonucleotide probe (ssDNA) for detection of VGluT2 mRNA was used on a subset of samples (n=4). The sequence of the antisense probe was CCTTG TACAA ATTCC TCTTT CTTTT CCCAA CCACT AGGCC AACCT CCA (GeneDetect, Auckland, New Zealand), and is complementary to nucleotides 2066 to 2113 of the coding sequence of human VGluT2. All the following procedures were performed according to an established protocol (Panoskaltsis-Mortari & Bucy, 1995) using an alkaline phosphatase-labelled anti-DIG antibody (code: 11093274910; Roche, Germany) for mRNA detection. An antisense probe recognising all species β-actin (code: GD5000-OP; GeneDetect, New Zealand) was used as a positive control probe. The corresponding sense DIG-hyperlabeled ssDNA probe was used in previous studies (Scott et al., 2008; Spang et al., 2012).

Plantaris tendon samples were freshly cut into 10 μm crysections using a cryostate (the knife was washed in 70 % Ethanol in DEPC treated H_2O before use). Afterwards they were air-dried at room temperature for 30 minutes and then post-fixed for 60 minutes in 4 % paraformaldehyde diluted in 0.1 M sterile filtered PBS. Then they were rinsed in 2x SSC two times for 10 minutes and incubated in 0.2 M HCI at room temperature for 8 minutes in order to inhibit the endogenous alkaline phosphatase (AP)-activity. After that, the sections were acetylated at room temperature for 15 minutes in a mixture of 195 ml diethylpyrocarbonate (DEPC)-water, 2.7 ml triethanolamine, 0.355 ml concentrated HCI and 0.5 ml acetic anhydride. Then another wash in 2x SSC followed. Eventually the denaturated ssDNA probes were added (see above). Before that the probes were denaturated at 80ºC for 5 minutes in 15 μl hybridisation solution and cooled on ice. Finally the slides were covered with cover slips, sealed with nail polish, and then incubated at 56ºC overnight.

On the second day, sections were rinsed in 2x SSC (2 times, for 10 minutes each) and incubated in STE-buffer for 5 minutes at room temperature. Sections were then incubated with 100 µl of RNase A solution at 37ºC for 30 minutes followed by a series of washes: the first in 2x SSC supplemented with 50 % formamide at 56ºC for 20 minutes; 2 washes in 1x SSC for 5 minutes at room temperature; 2 washes in 0.5x SSC for 5 minutes each. After that, sections were transferred into buffer 1 for 5 minutes and then again into the same buffer supplemented with 4 % horse normal serum for 60 minutes in a humid chamber at room temperature. Then 100 µl of the the alkaline phosphatase (AP)-labelled anti-digoxigenin (DIG) antibody, diluted 1:500 in buffer 1 with 5 % horse serum, was added and incubated for 60 minutes in a humid chamber at room temperature. Another set of washing steps followed: first in buffer 1 (2 washes, 10 minutes each) and then in buffer 2 (2 washes, 5 minutes each). Finally, freshly sterile-filtered (22 µm filtered) substrate solution was added. The sections were incubated upside-down to avoid deposits in the tissue at 4ºC overnight. The substrate solution reacted here with the enzyme alkaline phosphatase, which then resulted in a detectable reaction.

On the third day, in order to stop the colour reaction, the slides were placed in buffer 3. Before sections were mounted using Pertex, a counterstaining with methyl green was accomplished. The sections were washed for 30 seconds in 75 % ethanol, followed by 30 seconds in 95 % ethanol, 4-5 seconds in 0.5 % methyl green and finally three times dipped in pure ethanol.

More details concerning the solutions are stated in table 6.

Table 2: Primary antibodies used

Antigen	Code	Source	Type*	Dye**	Papers			
CD68	M0814	DAKO, Glostrup, Denmark	Mouse	TRITC	I			
ChAT	AB144P	Chemicon, Temecula, CA, USA	Rabbit	Alexa488	I			
CGRP	sc-8856	Santa Cruz, CA, USA	Goat	FITC		III		
CGRP	ab47027	abcam, Cambridge, UK	Rabbit	TRITC		III		
Fibroblast	CBL271	Chemicon, Temecula, CA, USA	Rabbit	TRITC	I			
Glutamate	G-143	RBI, Natrick, MA, USA	Rabbit	TRITC				V
Mast cell tryptase	ab2378	abcam, Cambridge, UK	Mouse	TRITC		III		
NMDAR1	ab134308	abcam, Cambridge, UK	Rabbit	TRITC				V
NMDAR1	sc-1467	Santa Cruz, CA, USA	Goat	FITC		III		V
pNMDAR1	sc-12890	Santa Cruz, CA, USA	Goat	FITC				V
PGP 9.5	7863-0504	Serotec, Oxford, UK	Rabbit	TRITC		III		
S-100β	S2657	Sigma-Aldrich, St. Louis, USA	Mouse	TRITC		III		
Scleraxis	ab58655	abcam, Cambridge, UK	Rabbit	TRITC				V
T-Cell/Neutrophils	MCA805G	Serotec, Oxford, UK	Mouse	TRITC	I			
TH	P40101	Pel-Freez, Rogers, AR, USA	Rabbit	TRITC		III	IV	V
VGluT2	sc-26026	Santa Cruz, CA, USA	Goat	FITC		III		V
VGluT2	ab101760	abcam, Cambridge, UK	Goat	FITC				V

* Goat antibodies were diluted in PBS whereas all others were diluted in 0.1 % BSA in PBS
** FITC = fluorescein isothiocyanate; TRITC = tetramethylrhodamine isocyanate

Table 3: Normal sera used

Serum	Code	Source	Papers			
Donkey	017-000-121	Jackson I.R., West Grove, PA, USA	I	III		V
Rabbit	X0902	DAKO, Copenhagen, Denmark	I	III		V
Swine	014-000-121	Jackson I.R., West Grove, PA, USA	I	III	IV	V

Table 4: Secondary antibodies used

Dye	Host	Reactivity	Code	Source	Papers			
Alexa 488	donkey	goat	A-11055	Invitrogen, CA, USA	I			
FITC	donkey	goat	705-095-147	Jackson IR, West Groove, USA	I	III		V
TRITC	swine	rabbit	R0156	DAKO, Glostrup, Denmark		III	IV	V
TRITC	rabbit	Mouse	R0270	DAKO, Glostrup, Denmark	I	III		V

Table 5: Antigens used for preabsorption control

Antigen	Code	Source	Used with ab	Concentration	Papers			
ChAT	AG220	Chemicon, CA, USA	AB144P	20µg/ml	I			
CGRP	sc-8856-P	Santa Cruz, CA, USA	sc-8856	20µg/ml		III		
NMDAR1	sc-1467-P	Santa Cruz, CA, USA	sc-1467	20µg/ml		III		V
VGluT2	sc-26026-P	Santa Cruz, CA, USA	sc-26026	20µg/ml		III		V

Table 6: Used solutions for in situ hybridisation

Solution	Ingredients
0.25% Acetic anhydride / 0.1 M triethanolamine-HCl buffer	0.93 g triethanolamine-HCl powder dissolved in 45 ml DEPC-H_2O with 6 drops of 10 N NaOH (pH 8.0); diluted to 50 ml with DEPC-H_2O
20x SSC	3 M NaCl and 0.3 M sodium citrate (pH 7.0), autoclaved
2x SSC, 50% formamide	25 ml formamide and 5ml 20x SSC diluted to 50 ml with double-distilled H_2O
4% paraformaldehyde (PFA)	2 g of PFA dissolved in RNase free H_2O (25 ml) with PBS (25 ml)
Buffer 1	100mM Tris-HCl (pH 7.5), 150mM NaCl; diluted to 1 l with DEPC-H_2O; filtered
Buffer 2	100mM Tris-HCl (pH 9.5), 100mM NaCl, 50mM $MgCl_2$ x 6 H_2O, diluted to 1 l with RNase free H_2O and filtered
Buffer 3 (stop buffer)	10mM Tris-HCl (pH 8.0) and 1mM EDTA diluted up to 1 l with RNase free H_2O; filtered
Hybridisation solution	500 µl formamide, 200 µl 20x SSC, 50 µl 20x Denhardt's solution, 50 µl of herring sperm DNA (10 mg/ml) (heat denatured the same day), 20 µl bakers yeast RNA (10 mg/ml) and 175 µl of dextran sulfate 50%
RNase A solution	40 µg RNAse A /ml STE
STE buffer	500 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5)
Substrate solution	1 ml buffer 2, 20 µl NBT/BCIP (Roche, Mannheim, Germany), 10 µl levamisole solution (1 mM) (Vector Labs, Burlingame, CA, USA)

Chapter 9: PLANTARIS TENDON CELL CULTURE

9.1 ISOLATION AND CULTURING

Plantaris tendon samples from six patients were further processed for cell-culture. Tendon samples were kept in Dulbecco's Modified Eagle Medium (D-MEM) (code: 11960; Invitrogen, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (code: 25030; Invitrogen), 1 % pen-strep (code: 15410; Invitrogen) and 0.2 % L-Glutamine (code: 25030; Invitrogen) when transported to the laboratory and then prepared for cell culture. First the samples were carefully washed under sterile conditions in Hank's Balanced Salt Solution (HBSS) (code: 14170; Invitrogen). Any attached tissues such as fat tissue or peritendinous loose connective tissue was removed. Then the samples were divided into equally small pieces using a sterile knife, and after that enzymatically digested overnight at 37°C with collagenase (Clostridiopeptidase A; code: C-0130; Sigma, St. Louis, MO, USA) in D-MEM in a final concentration of 2 mg/ml. The digested products were centrifuged at 500 relative centrifugal force (rcf) for 5 minutes. The supernatant was discarded and the pellet was dissolved in D-MEM with supplementation (10 % FBS, 1 % pen-strep, and 0.2 % L-Glutamine).

The cell culture was initially performed in a petri dish which was placed in a humidified atmosphere of 5 % CO_2 and 37°C. After 48 hours the medium was replaced. When the cells were confluent, they were scraped and transferred into a culture flask, in which they were further cultured. After being confluent they were enzymatically detached using 0.05 % trypsin (code: 15400; Invitrogen) in HBSS. After that they were split into 1:3 ratio. In this stage (passage 2) they were kept until confluence and then frozen in liquid nitrogen until use. Cells in passage 2-4 were used for experiments. For all experiments, cells were serum-starved with medium containing 1 % FBS one day before stimulation. This condition was kept during the entire experiment. Serum starvation leads to a reduced metabolism of the tendon cells i.e. reduced proliferation (Backman, 2013).

9.2 IMMUNOCYTOCHEMISTRY (ICC)

Immunocytochemistry was performed to identify tenocyte-like markers in the culture by immunostaining for scleraxis. Furthermore the expression of glutamate signalling components such as glutamate, NMDAR1, pNMDAR1 and VGluT2 were studied (table 2).

Cells were trypsinised and seeded in an 8-well culture slide (code: 354108; BD Bioscience, Heidelberg, Germany) overnight. On the next day the cells were fixed with 2 % paraformaldehyde (PFA) diluted in 0.1 M PBS (pH 7.4) for 5 minutes. Then they were washed four times, one minute each, in 0.01 M PBS. After that the cells were blocked in 5 % serum (table 3) for 15 minutes and then incubated with the primary antibody at 37 °C for 60 minutes. Then a washing step followed. After that the cells were again blocked in 5 % serum and eventually incubated with the secondary antibody (table 4) at 37°C for 30 minutes. In the end, the cells were washed once more and then mounted using mounting medium (code: P36962; Life Technologies, Carlsbad, CA, USA) supplemented with 4',6-diamidino-2-phenylindole (DAPI).

9.3 EXPOSURE TO NMDA AND GLUTAMATE

For stimulation, cells were seeded in 6-well plates, 250,000 in each well, and kept in a humidified atmosphere of 5 % CO_2 and 37°C overnight. From the next day cells were daily exposed to either glutamate (code: G8415; Sigma) or N-Methyl-D-aspartate (NMDA) (code: M3262; Sigma) in concentrations ranging from 5-500 µM for 1, 2 and 3 days. During the whole experiment the cells were kept in D-MEM with or without supplementations (FBS, pen strep, L-glutamine). The medium together with the diluted stimulation reagents was changed every 24 hours.

Supernatant was daily collected until day 3 to evaluate lactate dehydrogenase (LDH) concentration (see below). After 24 hours cells were further processed for the analysis of protein expression of scleraxis, c-PARP and c-caspase 3 (Western Blot, see below). After 8 hourse cells were taken and processed for the evaluation of scleraxis RNA (qPCR, see below).

9.4 2D STRAIN

Stretching of tendon cells was performed via a FlexCell system using BioFlex culture plates (code: BF-3001C, Bioflex), consisting of a collagen I pre-coated elastic membrane. Strain was applied via vacuum induced deformation of the membrane downwards at the outside edges of a 25 mm diameter cylindrical loading post. The cylindrical shape of the loading post leads to an equibiaxial strain. The actual strain on each individual cell depends on its orientation along the axis. The average strain, however, is 37 +/- 8 % (Wall et al., 2007). For more details see Backman (2013).

In this study, the loading procedure was performed with a frequency of 1 Hz and 10 % strain for 2 hours (Scott et al., 2011; Backman et al., 2011b). Cells were daily strained for 3 days. Two hours after the last loading session on day 3, the cells were processed for detection of RNA for the glutamate synthesising enzymes glutaminase (GLS) and glutamic-oxaloacetic transaminase 1 (GOT1) (qPCR, see below).

9.5 LACTATE DEHYDROGENASE (LDH) ASSAY

For measuring cell death as a response to the exposure to glutamate and NMDA, a lactate dehydrogenase (LDH) assay (code: G1780; Promega, Fitchburg, WI, USA) was used. Supernatant was collected after 24 hours of stimulation and directly analysed. According to the instructions, 50 µl of the sample was pipetted into a 96-well plate and mixed with 50 µl reconstituted substrate mix. Then incubation for 30 minutes in a light protected condition followed before 50 µl of stop solution was added. The absorbance was read at 490 nm.

9.6 DETECTION OF PROTEINS (WESTERN BLOT)

In this work Western blot was performed in order to measure the expression of proteins for NMDAR1, scleraxis, c-caspase 3 and c-PARP after 24 hours of stimulation with NMDA. Untreated cells were compared with cells subjected to NMDA at concentrations of 10, 100 and 500 μM.

After 24 hours cells were washed in sterile PBS and then frozen at -80°C. On the next day cells were scraped in RIPA lysis buffer (150 mM Sodium chloride, 1 % triton, 0.5 % Sodium deoxycholate, 0.1 % Sodium Dodecyl Sulphate (SDS), 50 mM Tris, pH 8.0) supplemented with protease inhibitor cocktail at a concentration of 1:200 (all from Sigma) and transferred into an eppendorf tube. After vortexing, incubation was performed on ice for 30 minutes. In order to get rid of the cell debris the lysate was centrifuged at 11,300 rcf for 5 minutes. The supernatant was then collected and a small volume was used to measure the total protein concentration via a protein assay dye reagent concentrate (code: 500-0006; Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) (code: A9647; Sigma) as a standard.

Supernatant was diluted in Laemmli sample buffer (code: 161-0737; Bio-Rad), supplemented with beta-mercaptoethanol and then heat-denatured at 95°C for 5 minutes. Beta-mercaptoethanol was mainly added to reduce the disulphide bonds and the heat-denaturation to disrupt both the secondary and tertiary structures. After that the samples were mixed well and immediately used for Western blot.

Samples of similar total protein concentrations were loaded onto sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins after their molecular weight (in kilo Dalton; kDa). The pre-made TGX gels used were run at 160 V for approximately 45 minutes in running buffer. Details concerning the solutions can be seen in table 7.

After that the proteins were transferred from the gel to a polyvinylidene fluoride transfer membrane (PVDF) (code: 10600023; GE Healthcare Life Science) for 60 minutes at 100 V. In order to see if the transfer was successful the membrane was stained with ponceau red (0.1 % ponceau red, 1 % acetic acid and MilliQ water) which detects proteins in the membrane. Ponceau staining was removed via washing in MilliQ water.

The membrane was then blocked in TBS-T supplemented with 5 % non-fat milk or 5 % BSA at room temperature for 60 minutes. After that the primary antibody (table 8), which was diluted in TBS-T with 5 % milk/BSA (same condition as blocking), was added and incubated overnight at 4°C on constant agitation.

On the second day the membrane was washed in TBS-T for 3 x 5 minutes. Then the secondary antibody (table 9) with conjugated horseradish peroxidase (HRP), diluted in TBS-T + 5 % milk/BSA, was added. Incubation lasted for 60 minutes at room temperature. Eventually the membrane was again washed and then exposed to a chemiluminescence substrate (code: 28-9068-38; GE Healthcare Life Science) for 5 minutes and immediately analysed.

In order to confirm an equal loading the membrane was stripped using stripping buffer (code: 21059; Thermo Scientific, Waltham, MA, USA). This was done at room temperature for 15 minutes under constant agitation. After that the membrane was thoroughly washed in TBS-T for 3 x 5 minutes and then blocked before being re-stained with the antibody against loading control β-actin.

9.7 DETECTION OF RNA (RT-qPCR)

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to measure levels of scleraxis mRNA after 8 hours of stimulation with NMDA. Furthermore levels of GLS and GOT1 were evaluated after 3 days of straining (table 10).

For extraction of RNA a RNeasy Mini Kit (code: 74106; Qiagen, Hilden, Germany) was used. The cells were first washed in PBS and then lysed using RLT buffer supplemented with 1 % mercaptoethanol. After that one volume of 70 % ethanol was added and then thoroughly mixed before transferred to the RNeasy spin columns. The tubes were then centrifuged for 15 seconds at 8,000 rcf. After that RW1 washing buffer was added and the tubes were again centrifuged for 15 seconds at 8,000 rcf. Then two additional washing steps in the RPE washing buffer, were performed. After the first wash centrifugation was undertaken for 15 seconds at 8,000 rcf and after the second 8,000 rcf for 2 minutes and then 13,400 rcf for 1 minute was applied. Eventually RNase free water was added to collected the RNA in a new tube by centrifugation at 13,400 rcf for 1 minute.

The collected RNA was then reversely transcripted into complementary DNA (cDNA) using a High-capacity cDNA Reverse Transcription kit (code: 4368813; Applied Biosystems, Warrington, Cheshire, UK). RNA (1000 ng) was here diluted with 2 µl of RT buffer, 2 µl of the RT random primers, 1 µl of the multiscribe reverse transcriptase, 0.8 µl of the dNTP mix and nuclease free water to get a final volume of 20 µl for each reaction. The cycler conditions were as follows: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C and eventually refrigerated to 4°C. The cDNA was either immediately used or stored at -20°C.

Finally, samples of equal cDNA concentration were diluted with 2x TaqMan Fast Advanced master mix, the 20x TaqMan MGB probe and nuclease free water. The settings for amplification were 95°C for 20 seconds in the first cycle, followed by 2 step cycles with the denaturation at 95°C for 1 second (first step), and annealing, extension at 60°C for 20 seconds (second step).

Table 7: Solutions used for Western blot

Solution	Ingredients
TBS-T (1.5 L)	1.82 g Tris-Base. 8.76 g NaCl. 1,5ml Tween-20 pH 7.4
Running buffer (1.0 L)	3.03 g Tris-base. 14.4 g glycine. 1 g SDS pH 8.3
Transfer buffer (1.0 L)	3.03 g Tris-base. 14.4 g glycine. 200 mL Methanol

Table 8: Primary antibodies used for Western blot

Antigen	Code	Source	Type	Dilution	Paper
β-actin	4967	Cell signal, Danvers, MA, USA	Rabbit	1:2000	V
c-PARP	9541	Cell signal, Danvers, MA, USA	Rabbit	1:1000	V
c-Caspase-3	9664	Cell signal, Danvers, MA, USA	Rabbit	1:1000	V
NMDAR1	ab134308	abcam, Cambridge, UK	Mouse	1:1000	V
Scleraxis	ab58655	abcam, Cambridge, UK	Rabbit	1:1000	V

Table 9: Secondary antibodies used for Western blot

Secondary ab	Code	Source	Dilution	Paper
HRP-conjugated goat anti-rabbit	7074	Cell signal, Danvers, MA, USA	1:2000	V
HRP-conjugated horse anti-mouse	7076	Cell signal, Danvers, MA, USA	1:2000	V

Table 10: Probes used for RT-qPCR

Probe name*	Code	Source	Paper
SCXA/B	Hs00243225	Applied Biosystems, Warrington, Cheshire, UK	V
GLS	Hs00248163	Applied Biosystems, Warrington, Cheshire, UK	V
GOT 1	HS00157798	Applied Biosystems, Warrington, Cheshire, UK	V
β-actin	4352935	Life Technologies, Carlsbad, CA, USA	V

***** SCX = Scleraxis, GLS = Glutaminase, GOT = glutamic-oxaloacetic transaminase

Chapter 10: STATISTICAL ANALYSIS

Statistics were used in study II and V. In study II, when comparing UTC echopixels before and 6 months after surgery, differences in the mean of (1) type I+II (green+blue) and (2) type III+IV (red+black) were determined. For this puropose an independent sample t-test was used.

In study V, when comparing the amount of RNAs between different groups, a one-way ANOVA test with Bonferri post-hoc test was performed.

Significance was predetermined to $p<0.05$.

IV RESULTS

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Chapter 11: GENERAL MORPHOLOGY (Study I, III)

11.1 PLANTARIS TENDON TISSUE PROPER

Stainings for morphology revealed tendinosis-like tissue patterns in the vast majority of examined plantaris tendons (40 out of 46 patients evaluated; study I+III). These features included tenocyte hypercellularity, collagen disorganisation, and occurrence of frequent blood vessels in the tendon proper (figure 7 A). Furthermore, the tenocytes varied in their appearance. They often exhibited widened/rounded and wavy appearances (figure 7 B,C). Those being rounded were often seen lining up in rows (figure 7 C).

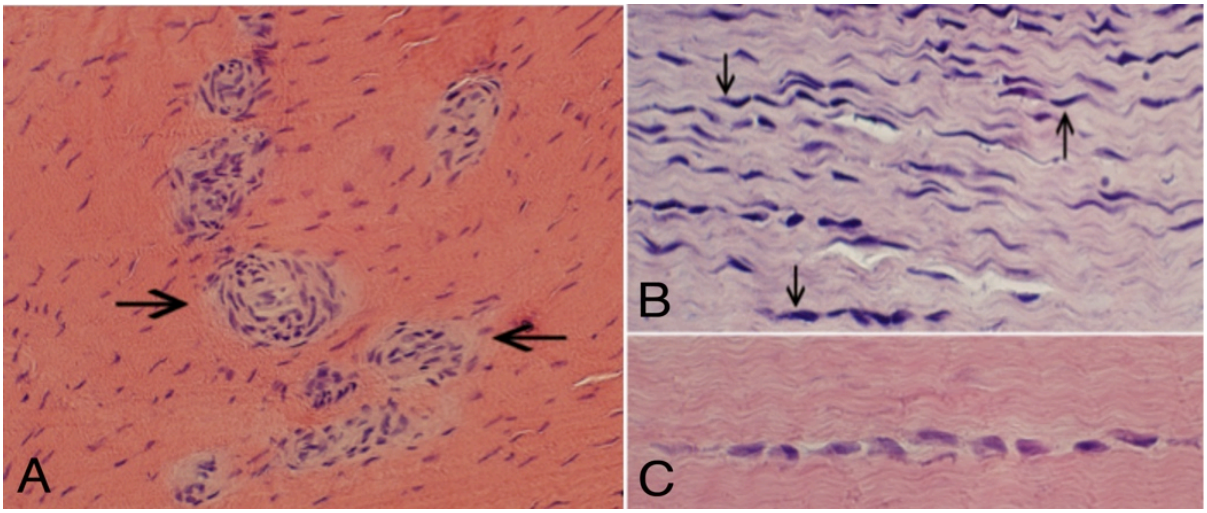


Figure 7: Plantaris tendon tissue proper of patients with midportion Achilles tendinopathy. Blood vessels (arrows) and a large numbers of tenocytes can be seen (A). The tenocytes often show wavy (B, arrows) or rounded (C) shapes. They are often organised in rows (C). (Adapted from study I, figures 1 and 2)

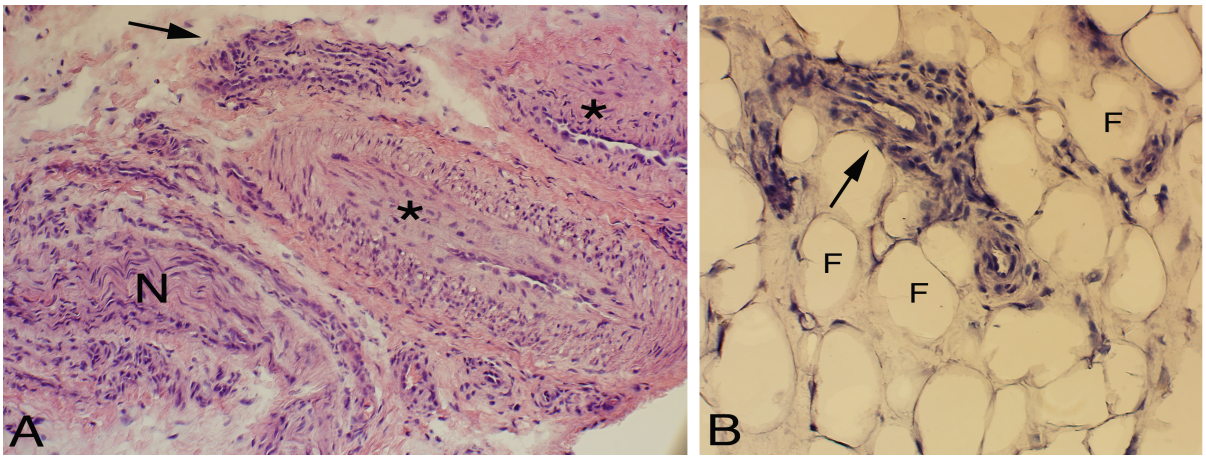


Figure 8: Peritendinous connective tissue in between the plantaris and Achilles tendons from patients with midportion Achilles tendinopathy. Large numbers of large (asterisks) and small (arrows) blood vessels (A,B) and occurrence of fat tissue (F) can be observed (B). N=nerve (A). (Adapted from study III, figure 1)

None of these features were seen in the plantaris tendon of the healthy individual (study I). In this tendon the collagen fibres were seen to be aligned, and the tenocytes, few in number, had a normal slender shape. Furthermore, in six plantaris tendons from patients with Achilles tendinopathy we observed a rather normal tissue pattern as well (study III).

11.2 PERITENDINOUS CONNECTIVE TISSUE

The peritendinous connective tissue located in between the plantaris and Achilles tendons was found to contain large numbers of blood vessels, including large and small arterioles/venoles and fine blood vessels (figure 8 A,B), and infiltrated fat tissue (figure 8 B). Furthermore, the cells occurring in this tissue were defined to mainly be fibroblasts but to some extent be mast cells and macrophages. The amount of peritendinous connective tissue that could be evaluated varied between the different specimens. In 70 out of 137 patients there was enough of this tissue present to further evaluate for innervation patterns (see below).

The peritendinous connective tissue of the healthy control showed infrequent blood vessels. In the specimens from the six patients exhibiting rather normal tendon tissue patterns, blood vessels occurred.

Chapter 12: ACHILLES TENDON STRUCTURE AFTER SURGERY (Study II)

12.1 STRUCTURAL CHANGES

Six months after surgery, Ultrasound Tissue Characterisation (UTC) showed a statistically significant ($p<0.001$) increase in the mean number (in percentage) of organised echopixels (echo-type I+II) from 83.33 (range 74-95) to 91.33 (76-97) in the Achilles tendon (figure 9). The mean number (in percentage) of disorganised echopixels (echo-type III+IV) decreased from 16.66 (range 5.1-29.9) to 8.67 (range 3.6-14.2) (figure 9).

12.2 PATIENT SATISFACTION AND VISA-A SCORES

Seven out of eight patients evaluated (8/9 tendons) expressed satisfaction with the result of the treatment. Concerning the VISA-A scores, there was a significant increase ($p< 0.001$) from 56.8 (range 34-73) pre-operatively to 93.3 (range 87-100) postoperatively.

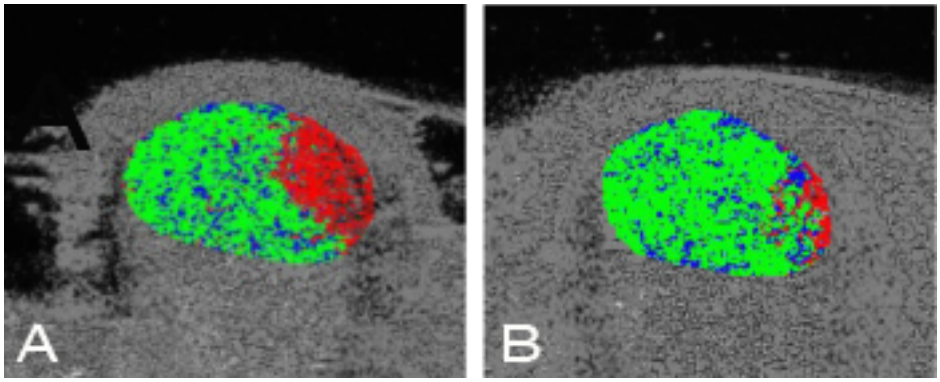


Figure 9: UTC findings in the Achilles tendon before and six months after surgical plantaris removal and ventro-medial scraping in one patient with midportion Achilles tendinopathy. (Adapted from study II, figure 4)

Chapter 13: INNERVATION PATTERNS (Study III)

13.1 PLANTARIS TENDON TISSUE PROPER

Single nerve fibres and thin nerve fascicles were detected in the zones of connective tissue inside the tendon tissue proper (figure 10) as seen by stainings for the general nerve marker PGP9.5. These observations were made in 48 out of 137 examined specimens (35.0 %) conforming to 37/56 (66.1 %) examined plantaris tendons and 32/46 patients (69.6 %) (table 11). Thus, there were nerve structures in the internal connective tissue sheets in more than two third of patients. However, the magnitude of presence of the nerves varied along the tendon. In only one third of examined tendon specimens, nerve fibres were found. This shows the importance of evaluating more than one level along the tendon in order to draw conclusions.

Nerve structures in internal connective tissue sheets were infrequent in samples of the six patients showing an almost normal morphology. In only 1/12 tendon specimens (from 6 tendon samples) these nerve structures could be detected (table 11).

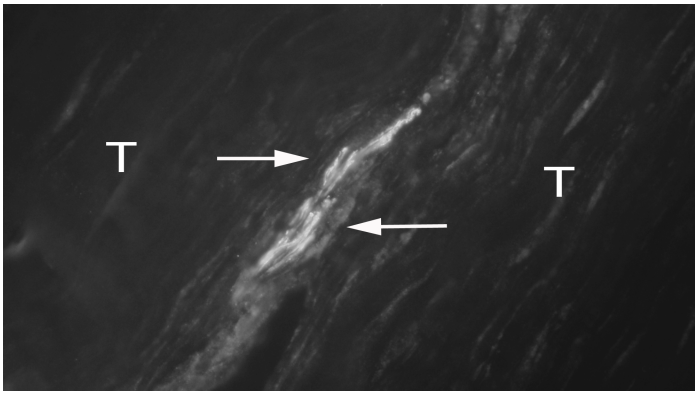


Figure 10: Tendon tissue proper of plantaris tendon. There are PGP9.5 positive immuno-reactive fine nerve fibres (arrows), which are located in a zone of loose connective tissue. Tendon tissue (T) containing tenocytes is seen to the left above and to the right below. (Adapted from study III, figure 3)

Table 11: Ratios of existence of PGP9.5 positive reactions in the zones of connective tissue of the tendon tissue proper in numbers of patients, legs and specimens.

Nerves in internal sheet	Patients	Tendons	Specimens
All patients	32/46 (69.6 %)	37/56 (66.1 %)	48/137 (35.0 %)
Tendinosis Group	31/40 (77.5 %)	36/50 (72.0 %)	47/125 (37.0 %)
Group with normal morphology	1/6 (16.7 %)	1/6 (16.7 %)	1/12 (8.3 %)

13.2 PERITENDINOUS CONNECTIVE TISSUE

Based on the PGP9.5 immunoreaction pattern, the peritendinous connective tissue in between the plantaris and Achilles tendons in tendinopathy patients was observed to be richly innervated. This included occurrence of large nerve fascicles (figure 11 A), isolated nerve fibres, and nerve structures in blood vessel walls or just outside the vessels. Some PGP9.5 immunoreactions were also seen in the peritendinous connective tissue of the tendons showing an almost normal morphology.

13.3 NERVE CHARACTERISATION

Immunoreactions for CGRP, indicating sensory nerve fibres, were observed in freely coursing nerve fibres and in fibres in nerve fascicles. The CGRP immmunoreactive nerve fibres were present both in the peritendinous connective tissue and in zones of the connective tissue inside the tendon tissue proper. Sympathetic nerve fibres, demarcated by showing TH immunoreactions, were sparse in number and could only be seen in the peritendinous connective tissue, most often in a perivascular location. Immunoreactions for NMDAR1 could be seen in a subgroup of nerve fascicles. These reactions were only observed in the peritendinous connective tissue.

13.4 PARTIAL LACK OF AXONS

Most of the nerve fascicles in the peritendinous connective tissue regularly exhibited a rather homogenous pattern of PGP9.5 immunoreactions (figure 11 A). However, there were some nerve fascicles that showed a partial lack of immunoreactions (figure 11 B). Double staining with S-100β showed on the other hand a homogenous S-100β immunoreaction, including immunoreaction in the PGP9.5 negative spaces (figure 11 B).

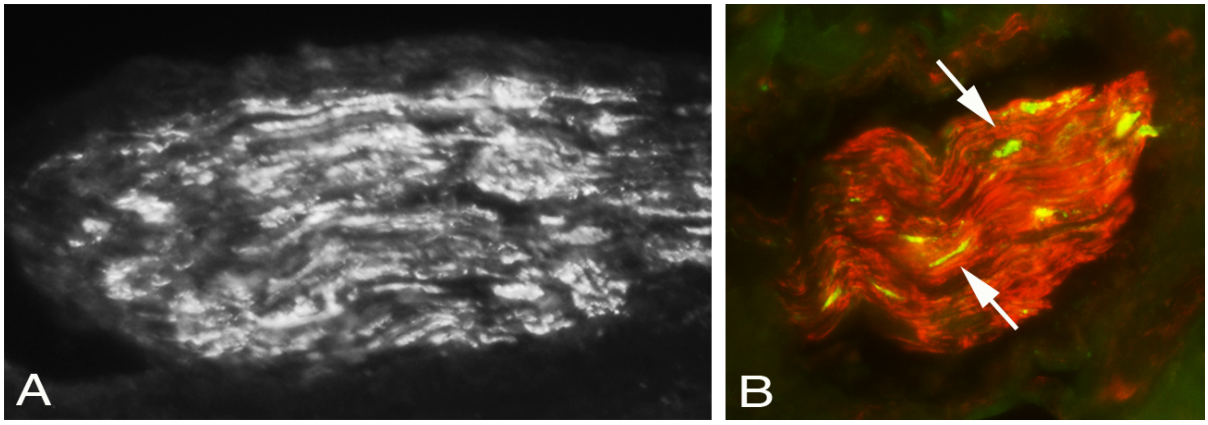


Figure 11: Nerve fascicles located in the peritendinous connective tissue in between Achilles and plantaris tendons, stained for PGP9.5 (A,B). Homogenous immunoreactions (white) are seen in (A). Inhomogenous PGP9.5 (green) pattern is seen in another nerve fascicle (B). Double staining PGP9.5 with S-100β (red) in (B) reveals that, whilst only parts of axons are PGP9.5 immunoreactive, there is a generalised reaction for S-100β. (Adapted from study III, figures 2 and 4)

Chapter 14: BILATERAL OBSERVATIONS (Study IV)

14.1 MORPHOLOGY IN BOTH LEGS

The morphology in the specimens from the Achilles and plantaris tendons from three patients, who were operated due to Achilles tendinopathy, were examined (Achilles tendon biopsies from two patients; plantaris tendon biospies from two patients). Tendon tissue of both right and left sides (Achilles and plantaris tendons) exhibited tendinosis-like tissue patterns. The peritendinous connective tissue located ventrally to the Achilles tendon contained large numbers of blood vessels on both sides. Immunoreactions for tyrosine hydroxylase (TH) were also present in tenocytes in specimens from both the Achilles and plantaris tendons from both legs.

14.2 CLINICAL OUTCOME AFTER UNILATERAL SCRAPING

11 out of 13 patients reported of 97 % satisfaction (range 82-100) as a result of the surgical treatment. Five of these eleven were satisfied after 6-8 weeks, four patients after 10-18 weeks and two after 26 weeks. At the final follow up (mean: 37 months) 10 out of the 13 patients were pain-free in both Achilles tendons during Achilles tendon loading. In one patient, minor pain was still present. This, however, did not prevent from running activities.

The VAS scores, measuring pain during Achilles tendon loading activity, decreased from mean 76 (range: 55-98) pre-operatively to mean 7 (range 0-23) after the surgery at the final follow up.

The eleven patients that were satisfied with the treatment also reported pain relief in the non-treated leg. Thus unilateral treatment resulted in bilateral recovery in 11 out 13 patients. An operation on the contralateral side was thus not warranted for these patients.

Two patients needed surgery in the leg that was originally not operated.

Chapter 15: SIGNALLING SUBSTANCES (Studies I,V)

15.1 PRESENCE OF A NON-NEURONAL CHOLINERGIC SYSTEM

Marked expression of choline acetyltransferase (ChAT) was found in tenocytes (figure 12 A) and in cells in the peritendinous connective tissue (figure 12 B). The specifity of these reactions was confirmed by preabsorption stainings and control staining using PBS instead of the primary antibody.

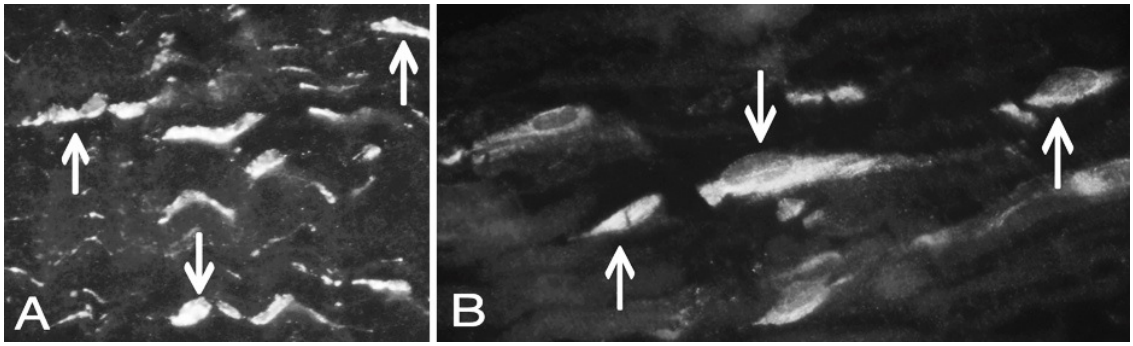


Figure 12: Plantaris tendon tissue (A) and peritendinous connective tissue (B) stained for ChAT. Positive immunoreactions are seen in tenocytes (arrows, A) and cells in the peritendinous tissue (arrows, B). (Adapted from study I, figures 4 and 6)

15.2 PRESENCE OF A NON-NEURONAL GLUTAMATE SYSTEM

Tenocytes in the plantaris tendon tissue proper and cells in the peritendinous connective tissue were found to express NMDAR1, VGluT2, pNMDAR1, and glutamate as seen via immunohistochemistry. Reactions in tenocytes could especially be observed in wavy shaped cells but also in cells showing a rounded appearance. VGluT2 mRNA detected by *in situ* hybridisation was found in tenocytes and in peritendinous cells. Tendon cells in culture derived from the plantaris tendon tissue exhibited immunoreactions for NMDAR1, VGluT2, pNMDAR1 and glutamate as well. The existence of NMDAR1 protein was further confirmed by Western blot. The findings are shown in study V (figures 1-3).

In preabsorption stainings for VGluT2 and NMDAR1, and control stainings, using PBS as replacement for the primary antibody, immunoreactions were abolished.

15.3 EFFECT OF NMDA RECEPTOR AGONIST (NMDA)

No significant differences in LDH levels were detected between cells exposed to glutamate and NMDA, an agonist of the NMDA receptor, and cells without any stimulation. This result was observed after 1,2 and 3 days. Furthermore Western blot showed no presence of c-caspase 3 and c-PARP after 24 hours of stimulation.

However, a significant decrease of scleraxis mRNA could be detected after 8 h of stimulation with NMDA. Scleraxis protein was furthermore found to be decreased after 24 h of exposure to NMDA, as seen with Western blot.

15.4 EFFECT OF LOADING ON THE GLUTAMATE SYSTEM

The mRNA for glutamate synthesising enzymes glutamic-oxaloacetic transaminase 1 (GOT1) and glutaminase (GLS) were both significantly increased after 3 days of loading as seen with qPCR.

V DISCUSSION

Chapter 16: MAJOR FINDINGS

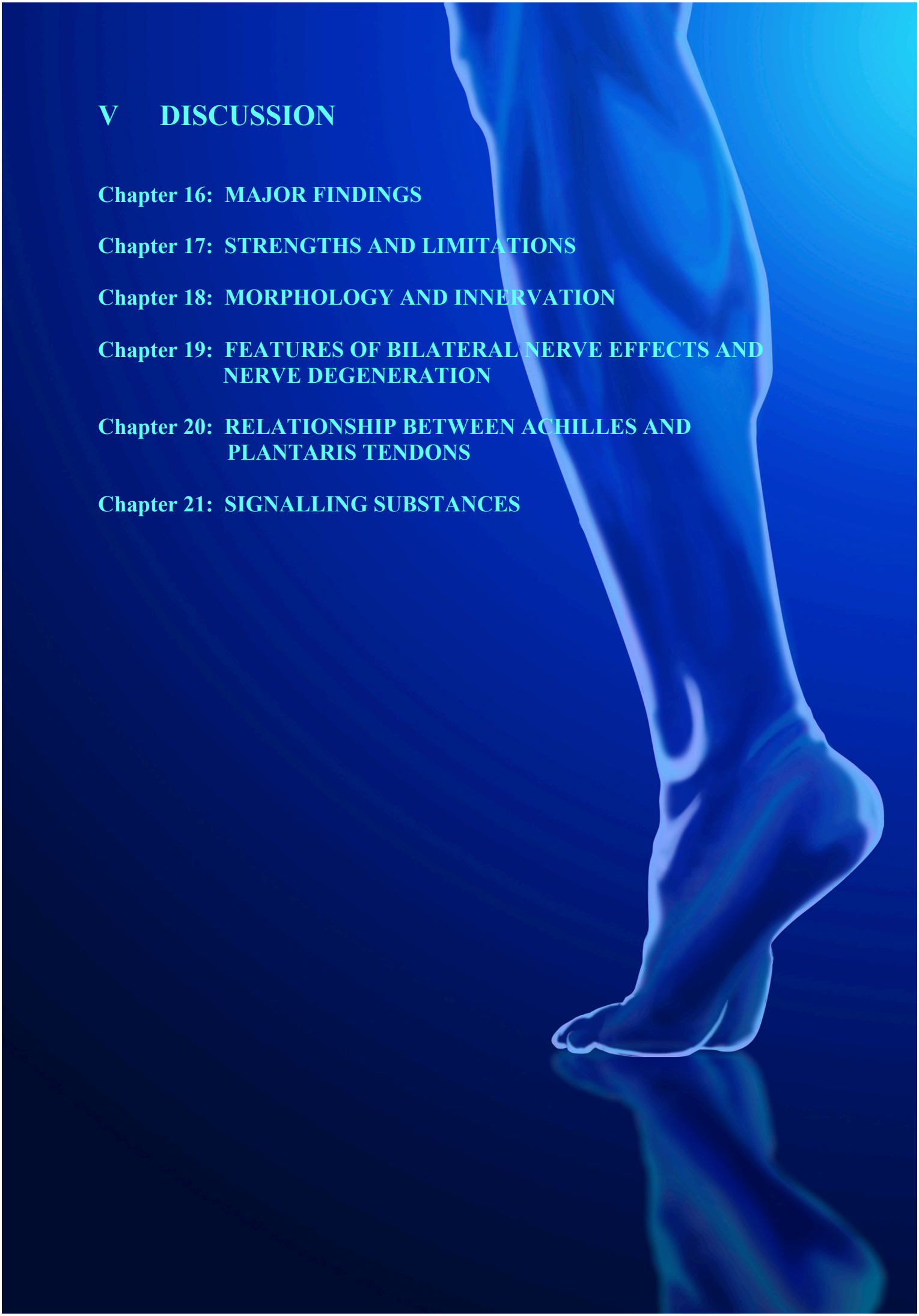
Chapter 17: STRENGTHS AND LIMITATIONS

Chapter 18: MORPHOLOGY AND INNERVATION

Chapter 19: FEATURES OF BILATERAL NERVE EFFECTS AND NERVE DEGENERATION

Chapter 20: RELATIONSHIP BETWEEN ACHILLES AND PLANTARIS TENDONS

Chapter 21: SIGNALLING SUBSTANCES



Chapter 16: MAJOR FINDINGS

This work was primarily designed to characterise the plantaris tendon and the peritendinous connective tissues located in between the plantaris and Achilles tendons in patients with chronic painful midportion Achilles tendinopathy. The involvement of the plantaris tendon in this condition has recently been highlighted and plantaris tendon removal has shown to lead to good clinical outcomes (Alfredson, 2011b; van Sterkenburg et al., 2011b; Pearce et al., 2012). The present thesis has now provided information that help to further understand the relationship between the two tendons and the effect of surgical interventions.

The results of the study show that the plantaris tendons exhibit tissue patterns that resemble those occurring in the Achilles and patellar tendons when affected by tendinopathy/tendinosis (Khan et al., 1999; Maffulli et al., 2004). That includes hypercellularity, multiple blood vessels, and disorganisation of the collagen structure. The fatty peritendinous connective tissue in between the Achilles and plantaris tendons contained large numbers of blood vessels and cells being identified as fibroblasts, but also macrophages and mast cells occurred. Furthermore, we could show that surgical release of the plantaris tendon together with ventro-medial scraping of the Achilles tendon can improve the collagen structure of the Achilles tendon.

This study has further addressed the innervation patterns and found that the plantaris tendon tissue proper exhibits nerve fibres in the zones of connective tissue, indicated by immunoreactions for PGP9.5. Nerve fibres being positive for sensory marker CGRP were also seen. Nerve fibres were seen to a greater degree in tendinosis tendons than in tendons with a normal appearance. The vast majority of innervation was, however, seen in the peritendinous connective tissue. That included presence of large nerve fascicles, isolated nerve fibres, and nerves in perivascular location. Further characterisation revealed that the nerve structures are mainly sensory nerve fibres, but also to some extent sympathetic nerve fibres as well as nerve fibres being positive for glutamate receptor NMDAR1. In some nerve fascicles there was an inhomogeneous pattern of PGP9.5 immunoreactions, which differed from the patterns seen in other fascicles. Another unexpected finding was that unilateral treatment, targeting the peritendinous connective tissue, can result in bilateral recovery based on patient's satisfaction and pain relief.

Studies on the cholinergic and glutamate systems showed evidence of the presence of components such as ChAT, VGluT2 and the receptor NMDAR1. Reactions for these were observed in tenocytes and peritendinous cells in tissue sections and in plantaris tendon cells *in vitro*. *In vitro* studies on the effect of glutamate on plantaris tendon cells showed that glutamate/NMDA does not induce cell death but instead lead to a down-regulation of scleraxis, a marker for tendon cells. Loading of the cells led to higher expression of glutamate producing enzymes in the cells.

Chapter 17: STRENGTHS AND LIMITATIONS

This is the first study examining the morphological and nerve-related characteristics of the plantaris tendon and the adjacent peritendinous connective tissue in patients with chronic painful midportion Achilles tendinopathy. As this area has become of high interest in relation to midportion Achilles tendinopathy (Alfredson, 2011b), this study is innovative and provides new information on a possible interference between the Achilles and plantaris tendons in this condition. Another strength is that plantaris tendon samples were studied at several locations along the tendon length. This provides a more representative presentation of the morphology and innervation for the tendon, compared to when only one sample is examined. To the best of our knowledge no other human tendinopathy tendon has been evaluated along its course. Furthermore this thesis has shown how the Achilles tendon structure was affected by surgical removal of the plantaris tendon. This is very important information since it helps to clarify a possible interference between the two tendons. It also verifies the outcome of the surgical treatment. The use of the new imaging tool Ultrasound Tissue Characterisation (UTC) also strengthens this thesis.

One major weakness in the present thesis is the relative lack of control specimens from healthy individuals. This was solely due to ethical reasons. Although it has been suggested that the plantaris is a vestigial muscle (Wiedersheim, 1893), the full impact of plantaris tendon removal on knee and ankle function is not fully understood. Therefore, we believe that plantaris tendon removal should this far only be performed in situations when the plantaris tendon is located very close to the medial aspect of the Achilles tendon indicating a possible plantaris tendon involvement in midportion Achilles tendinopathy. Another point that might lead to criticism, is that the evaluation of the peritendinous connective tissue was very much descriptive. This was due to the huge variations in size of this tissue between individual patients, which made extensive quantifications impossible. The results of our study show that the surgical treatment used improves Achilles tendon structure. However, we cannot definitely say if these results were achieved from the plantaris tendon removal, the scraping procedure or by a combination of both procedures.

Chapter 18: MORPHOLOGY AND INNERVATION

This work shows that plantaris tendons positioned close to the medial aspect of the Achilles tendon in patients with midportion Achilles tendinopathy exhibit morphological tissue changes that resemble those seen in Achilles and patellar tendinosis (Khan et al., 1999; Maffulli et al., 2004). In material from 50 out of 56 plantaris tendon specimens, we found large numbers of tenocytes, multiple blood vessels, and collagen disorganisation in the tendon tissue proper. This strongly indicates a co-existing plantaris tendinosis occurring in a subgroup of patients with midportion Achilles tendinopathy (figure 13). Thus, it is possible that the plantaris tendon may contribute to the features of midportion Achilles tendinopathy.

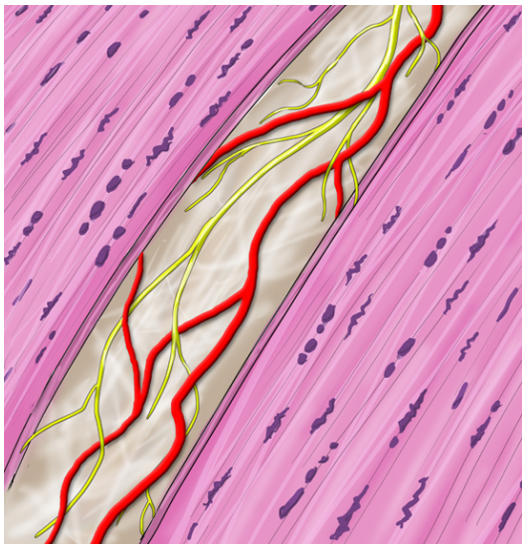


Figure 13: Plantaris tendon lying close to the medial aspect of the Achilles tendon. Teninosis-like tissue patterns are seen in both tendons; Achilles tendon to the left, plantaris tendon to the right. The peritendinous connective tissue located in between the tendons (middle) contains large numbers of blood vessels (red) and nerve structures (yellow). (Adapted ftom study III, figure 7)

Interestingly, we found nerve fibres in zones of the connective tissue within the plantaris tendon tissue proper. These features were rather pronounced in tendons that had a clear tendinosis tissue pattern. It has previously been reported that an ingrowth of sensory nerve fibres can occur in Achilles and patellar tendinopathy (Schubert et al., 2005; Lian et al., 2006). However, in another study by Bjur and co-workers (2005) on the Achilles tendon, there was no difference in the occurrence of PGP9.5 reactions between healthy and tendinosis tendons. Thus, it remains uncertain if nerve ingrowth is a main characteristic of tendinopathy. In any case, a presence of nerve fibres was noted internally in the plantaris tendon in this study. These nerve fibres detected in the zones of connective tissue in the plantaris tendon tissue proper and that were of sensory type (CGRP-immunoreactive) may have an impact on pain symptoms. This might explain why plantaris removal has been shown to lead to good clinical outcome (Alfredson, 2011b; Ruergard & Alfredson, 2014)

Previous studies on the innervation patterns in Achilles and patellar tendons have identified the peritendinous connective tissues as the regions with the majority of blood vessels and nerve structures (Bjur et al., 2005; Danielson et al., 2006; Andersson et al., 2007). In the present study on the peritendinous connective tissues in between the Achilles and plantaris tendons, we found large numbers of blood vessels, fat tissue, and nerve structures

(figure 13), as evidenced by H&E stainings and immunostainings for general nerve marker PGP9.5. This marked innervation in the form of large nerve fascicles, isolated nerve fibres, and innervation located in perivascular areas, was found to be frequently positive for sensory nerve marker CGRP and to some extent for sympathetic marker tyrosine hydroxylase (TH) and glutamate receptor NMDAR1. It cannot be excluded that this tissue is actively involved in the pain symptoms. Consequently, there are two possible pain generators, the innervation in the plantaris tendon and that in the peritendinous connective tissue, which alone or together can be responsible for the pain involved in midportion Achilles tendinopathy.

Beside the occurrence of vessels and nerves, we also found numerous cells in the peritendinous connective tissue. These were identified to be mainly fibroblasts. However, we also found several macrophages and mast cells. The occurrence of white blood cells in peritendinous connective tissues ("paratendinopathy/paratendinitis") is not unusual and has been described to often co-occur with tendinosis in tendons exposed to chronic overuse (van Dijk et al., 2011). Several studies have highlighted the importance of the peritendinous connective tissues in response to exercise and loading (Langberg et al.,1998, 1999; Kjaer et al., 2000). Repetitive heavy tendon loading puts high demands on these tissues. Invasive procedures for patients with midportion Achilles tendinopathy often target the peritendinous tissues, especially the ventral peritendinous connective tissue which has been shown to contain large numbers of vessels and nerves (Andersson et al., 2007; Alfredson et al., 2007). Ultrasound guided injections of sclerosing substance Polidocanol targeting the regions with high blood flow and nerves outside the tendon have resulted in good clinical outcomes (Alfredson & Ohberg, 2005). Surgical scraping in the same region has shown similar good results (Alfredson et al., 2007). Altogether, it is reasonable to suggest that the peritendinous connective tissue in between the Achilles and plantaris tendons may also be actively involved in the pathogenesis and pain symptoms related to this condition. The possible interference between the Achilles and plantaris tendons, which might influence the inter-positioned peritendinous tissue, is thus of high interest and will be discussed in the next chapters.

Chapter 19: FEATURES OF BILATERAL NERVE EFFECTS AND NERVE DEGENERATION

An interesting observation was that the unilateral surgical scraping targeting the ventral peritendinous connective tissue resulted in bilateral recovery in patients with chronic painful midportion Achilles tendinopathy. This was a very unexpected finding and highlights the involvement of the central nervous system. The effect of cross over effects after unilateral exercise is well known, (Lee & Carroll, 2007; Zult et al, 2014) and it has been shown that there are bilateral cortical adaptions after unilateral strength training (Goodwill et al., 2012). However, little is known about the situation in chronic tendon pain and the impact of unilateral treatment on bilateral recovery. Some authors have strongly suggested that the central nervous system should be taken into account when discussing pain mechanisms in tendinopathy (Rio et al., 2014). Animal studies using a unilateral exercise protocol have reported degenerative tissue changes in triceps surae muscle and Achilles tendon not only in the exercised leg but also on the contralateral side (Andersson et al., 2011b; Song et al., 2012). There is thus evidence that the central nervous system can be important also for tendon pain. However, the results of this study, in parallel with previous studies (Andersson et al., 2007), also show that the peritendinous tissues, containing large numbers of nerve structures, might be an important tissue in perceiving and possibly transmitting the pain signals. More studies are needed to further examine the relationship between nerves in the peripheral tissues and the central nervous system in relation to tendinopathy.

We also found signs of nerve degeneration in tendinopathic plantaris tendons. Thus, some nerve fascicles exhibited a relative lack of PGP9.5 immunoreactions. These empty areas, however, were homogenously stained for Schwann cell marker S-100β. These features raise a possibility for the occurrence of nerve degeneration together with Schwann cell proliferation in the examined peritendinous tissue. This is supported by reports that Schwann cells activation and proliferation often occurs as a response to nerve damage and that S100β protein can play essential roles in axonal repair and regeneration of the central nervous system (Hu et al., 2003; Duobles et al., 2008; Yardan et al., 2011). The possibility that these features may be involved in the process of tendinosis is a new aspect that has not been reported before. It is thus possible that not only nerve ingrowth, as previously suggested (Schubert et al., 2005; Lian et al., 2006), but also nerve degeneration are features of chronic tendon pain. So far it can only be speculated about mechanisms and impact on the pain and the nerve influence on the whole. Future studies are needed to clarify the importance of the findings for the nerves. One possibility is that the nerve changes can be related to a tissue stress in the peritendinous connective tissue. In comparison, features of both nerve degeneration and proliferation occur in muscle tissue that is under stress, namely in response to marked muscle overuse as seen experimentally (Song et al., 2012,2013). We in our laboratory have also seen features favouring occurrence of nerve degeneration in another tissue that is under stress and that gives pain sensations; the tissue at the lateral epicondyle for patients with tennis elbow (unpublished observations).

Chapter 20: RELATIONSHIP BETWEEN ACHILLES AND PLANTARIS TENDONS

The results in this thesis support the hypothesis of a plantaris tendon involvement in situations with midportion Achilles tendinopathy (Alfredson, 2011b). The question that still remains is how this involvement actually manifests itself. One possibility is that the plantaris tendon, known to be stronger and stiffer than the Achilles tendon (Lintz et al., 2011), compresses onto the medial Achilles tendon (Alfredson, 2011b). Compression is known to play a role in tendinopathy in the insertional part of the Achilles tendon and might also be involved in the midportion (Cook & Purdam, 2012). Another, rather similar scenario that has been discussed, is a friction-induced inflammatory reaction occurring when the Achilles and plantaris tendons interfere with each other (Van Sterkenburg & van Dijk, 2011; Pollock et al., 2014). In both cases, a continuous inflammatory response in the peritendinous connective tissue leading to chronic pain may be the consequence. We have indeed seen macrophages and mast cells in the peritendinous connective tissue. This finding can indicate an inflammatory condition ("paratendinopathy/paratendinitis") occurring in the peritendinous connective tissue in between the Achilles and plantaris tendons and which might be related to compressive/shearing forces resulting from the interference between the Achilles and plantaris tendons. However, whether these cells also occur in normal plantaris tendons needs to be further evaluated. In this thesis, we have also shown that plantaris tendon removal together with ventro-medial scraping does not only improve pain symptoms but also improve the collagen structure in the medial Achilles tendon. This might support the idea of the existence of a force originating from the plantaris tendon and transmitting onto the medial side of the Achilles tendon.

The scraping of the ventral peritendinous tissue was originally performed from the lateral side and has shown good clinical outcome with a high patient satisfaction rate (Alfredson, 2011a). In re-operations, undertaken from the medial side, on patients that were not satisfied with the results and complained of medial pain, a thickened plantaris tendon positioned close to the Achilles tendon was often found (Alfredson, 2011b). Plantaris tendon removal showed good results with pain free patients. In a one-year follow-up after plantaris removal together with scraping, all 47 out of 47 patients examined went back to full Achilles tendon loading (Ruergard & Alfredson, 2014). It is obvious that scraping alone can cure a lot of patients, but in some patients this might not be enough. The reason can be that there is a plantaris tendon markedly interfering with the Achilles tendon in these cases. Other studies in which plantaris removal/release has been made, have also reported good results on clinical outcome (van Sterkenburg et al., 2011b; Pearce et al., 2012; Calder et al., 2014). A drawback concerning interpretations of the treatments is that it is difficult to say if the good outcome results come from the plantaris removal, the scraping, or from both procedures.

Studies on cadavers and studies using imaging tools have extensively described the shape and location of the plantaris muscle/tendon and have found high individual variations. For the tendon part, up to nine different insertion sites have been defined (Daseler & Anson, 1943; Harvey et al., 1983; Schlicht & Morrison, 1992; Nayak et al., 2010; van Sterkenburg et al., 2011a). Although the majority of plantaris tendons insert into the calcaneus bone without touching the Achilles tendon, there are several individuals who have structural connections between these two tendons. It might thus be that the plantaris tendon in patients suffering

from plantaris tendon associated midportion Achilles tendinopathy is either structurally connected to the Achilles tendon or at least closely positioned. In patients having a very pronounced structural connection, plantaris removal might be the only way of long-term curing and that additional scraping of the peritendinous connective tissue is favourable. From the clinical experience we also know that patients with plantaris tendon issues respond poorly to exercise treatment. However, not all patients with midportion Achilles tendinopathy have a very pronounced structural connection between the plantaris and the Achilles tendons. In many patients the plantaris tendon is thickened and positioned close to the medial aspect of the Achilles tendon without merging with it. In this context it should be recalled that we found tendinosis-like tissue features in these plantaris tendons indicating a co-existing plantaris tendinosis/tendinopathy. It might be that the plantaris tendon develops tendinosis independently of the Achilles tendon due to chronic overuse. The thickening of the plantaris tendon, a feature that develops in tendinopathic tendons (Khan et al., 1999), might then reduce the distance between the two tendons and eventually fascilitate an interference.

The full mechanism of the interference between the Achilles and plantaris tendons in midportion Achilles tendinopathy needs further evaluations. Future surgical studies comparing plantaris removal without scraping with pure scraping procedure should be performed in order to fully determine the impact of plantaris tendons in midportion Achilles tendinopathy.

Chapter 21: SIGNALLING SUBSTANCES

In the present study, we have shown the presence of a non-neuronal cholinergic system in the plantaris tendinosis tendon and the peritendinous connective tissue in between the plantaris and Achilles tendons. Immunoreactions for choline acetyltransferase (ChAT), the ACh producing enzyme, were detected in tenocytes in the tendon tissue proper and in cells in the peritendinous connective tissue. This might indicate an autocrine/ paracrine ACh signalling within the tendon tissue proper, which might be involved in tissue changes occurring in tendinosis. This is further supported by the observation that ChAT immunoreactions were most frequently seen in tenocytes exhibiting an abnormal rounded and wavy appearance. In studies on the Achilles and patellar tendons it has also been shown that the components of the cholinergic system are more pronounced in tenocytes with abnormal appearance in tendinosis tendons as compared to those of healthy control tendons (Danielson et al., 2007; Bjur et al., 2008b). *In vitro* studies on Achilles tendon cells have reported cell proliferation in response to ACh exposure (Fong et al., 2013). The results of our study suggest that the cholinergic system might also play a role in the plantaris tendon. The fact that there were ChAT immunoreactions in cells in the peritendinous connective tissue can be related to trophic and/or inflammation-modifying effects. It is nowadays considered that effects via the non-neuronal cholinergic system can be related to an anti-inflammatory effect (Kawashima & Fuji, 2004, 2008).

We have furthermore detected several components of a non-neuronal glutamate system in the plantaris tendon and the peritendinous connective tissue. Immunoreactions for glutamate, the vesicular glutamate transporter VGluT2, and the NMDA receptor (NMDAR1) were observed in tenocytes in the tendon tissue proper, the cells in the peritendinous connective tissue, and in cultured tendon cells (*in vitro*) derived from the plantaris tendon. It has previously been speculated that locally produced glutamate might be involved in tissue changes related to tendinosis (Scott et al., 2008). Immunoreactions for VGluT2 and NMDAR1 have been found in the tenocytes of Achilles and patellar tendons (Scott et al., 2008; Schizas et al., 2010, 2012). In tendinosis tendons with abnormal tenocyte appearance, these proteins were found to be up-regulated, suggesting a role in tendinosis (Scott et al., 2008; Schizas et al., 2010, 2012).

In an i*n vitro* study on the effect of glutamate on tendon cells, derived from rat supraspinatus tendons, it was reported that glutamate might be involved in apoptotic processes (Molloy et al., 2006). Apoptosis is considered to be one of the features occurring in tendinosis tendons (Lian et al., 2006; Millar et al., 2009). In our study, however, we could not observe signs of cell death after exposure to glutamate or NMDA receptor agonist N-acetyl-D-aspartate (NMDA). Furthermore, we could not detect any cleaved caspase 3 or cleaved PARP, both indicators for apoptostic processes, in the stimulated cells. An explanation for the disparity of the results might originate in the different conditions used for exposure. Extracellular magnesium is known to be able to block the NMDA receptors (Burnashev et al., 1992). In a test phase we thus cultured the plantaris tendon cells in medium (HBSS) without magnesium. The survival rate of the cells was very low in this pilot experiments. Therefore we decided to use D-MEM either with or without supplementations (1 % FBS) for culturing and stimulation. This condition should be closer to the physiological condition in tendons, where certain levels of magnesium are found (Kannus, 2000).

Instead of cell death we observed that scleraxis, a transcription factor for the regulation of tenomodulin expression (Shukunami et al., 2006), is down-regulated in cultured primary plantaris tendon cells in response to glutamate receptor stimulation. Tenomodulin is a type II transmembrane glycoprotein that is considered to be a fairly specific marker for tenocytes (Docheva et al., 2005; Jelinsky et al., 2010). Studies have shown that scleraxis is essential for the differentiation of tendon stem cells into tenocytes (Docheva et al., 2005; Alberton et al., 2012). The down-regulation in response to glutamate receptor activation, might indicate a glutamate-induced change in phenotype of these cells. It has actually been shown that under certain conditions, tendon stem cells can become non-tenocyte like cells such as adipocytes and osteocytes (Zang & Wang, 2010). Interestingly, osteogenesis and adipogenesis are tissue features that often occur in tendinosis tendons (Järvinen et al., 1997; Khan et al., 1999). The results of this work give some evidence that glutamate might be able to induce phenotype changes and to actively contribute to tendinosis tissue changes.

We have also seen that straining of tendon cells results in an increased production of glutamate synthesising enzymes GLS and GOT1, which may result in higher glutamate levels in tendon cells. This might further indicate that tendinosis features such as phenotype changes, in response to chronic tendon loading, can be mediated via glutamate. However, in this context it should be recalled that beside its role as signalling g substance, glutamate is also an amino acid and is important for protein synthesis (Nedergaard et al., 2002). The increase of glutamate in tendon cells in response to strain may also be a sign of a general increase in metabolic rate. Further studies are needed to clarify the full impact of glutamate on the possible phenotype change in tendon cells.

VI CONCLUSION

Chapter 22: CONCLUSION

Chapter 22: CONCLUSION

In conclusion, these studies on patients with midportion Achilles tendinopathy show that:

- There is a co-exisiting plantaris tendinosis including the existence of internal nerve structures in a subgroup of patients.
- Surgical plantaris tendon removal together with scraping can improve Achilles tendon structure in these patients.
- The peritendinous connective tissue in between the Achilles and plantaris tendons is highly innervated by mainly sensory nerve fibres.
- Several nerve fascicles in the peritendinous connective tissue show a partial lack of axons.
- Unilateral treatment for Achilles tendinopathy – scraping the peritendinous connective tissue – can result in bilateral outcome.
- That there are non-neuronal cholinergic and glutamate systems in the plantaris tendon and the peritendinous connective tissue.
- Exposure to glutamate may influence the phenotype of plantaris tendon cells.

It can furthermore be concluded that:

- There is evidence for an interference between the Achilles and plantaris tendon in patients with midportion Achilles tendinopathy.
- Nerve degeneration might be a feature of tendinopathies.
- The peritendinous connective tissue can be an important tissue related to pain and neuronal processing.

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