TNF-α AND NEUROTROPHINS IN ACHILLES TENDINOSIS

JOHAN BAGGE

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IN ACHILLES TENDINOSIS

JOHAN BAGGE

Department of Integrative Medical Biology, Section for Anatomy, and Department of Surgical and Perioperative Sciences, Sports Medicine Unit, Umeå University, Umeå, Sweden
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Cover: The background colour is dedicated to *Idrottsklubben Studenterna i Umeå*. The top layer on the front is based on a photo of tenocytes in an Achilles tendon. Back: The author's footprint.

Fig. 1: the structure of a tendon. Illustration by Gustav Andersson. Based on Scarr (2010).
Fig. 2: tendon stress. Based on an anatomical illustration at www.angelwellbeingclinic.co.uk [Accessed December 2012].
Fig. 3: the Achilles tendon. Illustration by Maria Bagge. Based on illustration in Gray's Anatomy, Philadelphia: Lea & Febiger, 1918
Fig. 4: TNF-α signalling pathways. Illustration by Gustav Andersson.
Fig. 8: theory. Illustration by Gustav Andersson.

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Abstract

Tenocytes are the principal cells of the human Achilles tendon. In tendinosis, changes in the metabolism and morphology of these cells occur. Neurotrophins are growth factors essential for the development of the nervous system. Tumour necrosis factor alpha (TNF-α) has been found to kill sarcomas but has destructive effects in several major diseases. The two systems have interaction effects and are associated with apoptosis, proliferation, and pain signalling in various diseases. Whether these systems are present in the Achilles tendon and in Achilles tendinosis is unknown. The hypothesis is that the tenocytes produce substances belonging to these systems. In *Studies I–III,* we show that the potent effects of these substances are also likely to occur in the Achilles tendon. We found tenocyte immunoreactions for the neurotrophins brain-derived neurotrophic factor (BDNF), the nerve growth factor (NGF), the neurotrophin receptor p75, and for TNF-α and both of its receptors, TNFR1 and TNFR2. This occurred in both subjects with painful mid-portion Achilles tendinosis, and in controls. Furthermore, we found mRNA expression for BDNF and TNF-α in tenocytes, which proves that these cells produce these substances. TNFR1 mRNA was also detected for the tenocytes, and TNFR1 immunoreactions were upregulated in tendinosis tendons. This might explain why tenocytes in tendinosis undergo apoptosis more often than in normal tendons. Total physical activity (TPA) level and blood concentration of both soluble TNFR1 and BDNF were measured in *Study IV*. The results showed that the blood concentration of both factors were similar in subjects with tendinosis and in controls. Nevertheless, the TPA level was related to the blood concentration of sTNFR1 in tendinosis, but not in controls. This relationship should be studied further. The findings of this doctoral thesis show that neurotrophin and TNF-α systems are expressed in the Achilles tendon. We believe that the functions include tissue remodelling, proliferation and apoptosis.

Populärvetenskaplig sammanfattning

Hälsenan kan angripas av olika skador och sjukdomar. Ett vanligt förekommande tillstånd är hälsenetendinopati. Patienter som ges denna diagnos har smärtor i ena eller båda hälsenorna, speciellt vid belastning såsom vid gång eller löpning. Ofta blir besvären långvariga, hos en del patienter till och med livslånga. Hälsenorna hos patienter med tendinopati kan analyseras med ultraljud eller genom att ta ett vävnadsprov som undersöks i mikroskop. Vid dessa analyser konstateras att många patienterna har förändringar i senvävnaden och man kan därmed ställa diagnosen tendinos.

Vid tendinos förändras sencellernas form. Cellerna kan också öka i antal eller drabbas av onormal celldöd. Det senare kan i värsta fall leda till att senan går av. Förutom ökning av antalet senceller ses vid tendinos även tillväxt av blodkärl och i viss mån nerver. Med hjälp av en speciell typ av undersökning (Doppler) kan man också studera blodflödet i senan. Vanligt vid tendinos är att blodflödet är förhöjt inom det skadade området.

Långvarig hälsenetendinopati ger i sig inga tecken på inflammation. Vidare är det inte helt klarlagt vare sig vad som orsakar smärtan vid denna sjudom eller vad som ger upphov till vävnadsförändringarna vid tendinos. En möjlighet att öka förståelsen för detta är att studera förekomsten av olika kemiska substanser, kopplade till smärta och vävnadsförändringar, i sencellerna.

Kemiska substanser finns också i blodet och det är möjligt att mäta nivåerna av dessa. Vidare vet man att belastning av hälsenan förvärrar smärtan och att fysisk aktivitet generellt sett ofta har stor påverkan på olika kemiska substanser i blod.

Vi analyserade vävnadsprover av hälsenor tagna från patienter med smärtande hälsenetendinos och kontrollpersoner som inte hade smärta eller förändringar i senan. Blodprover togs för att mäta nivåer av kemiska substanser och jämföra dessa mellan de två grupperna. Vidare mättes hur fysiskt aktiva patienterna med tendinos och kontrollpersonerna hade varit under ett år. Totalt innefattade studierna 34 patienter och 25 kontroller, både män och kvinnor.

Tre substanser, tillhörande två olika familjer, vilkas påverkan på hälsenan i stort sett varit okänd tidigare, studerades. Den ena substansfamiljen är de så kallade neurotrofinerna som man vet är oumbärliga för människans nervsytem. Den andra familjen är tumörnekrosfaktor (TNF)-familjen som namngetts efter sin förmåga att kunna döda tumörceller. Båda substansfamiljerna har visats ha viktiga funktioner i samband med flera allvarliga sjukdomar. De är även involverade vid inflammation och vävnadsomvandling samt kan framkalla smärta. Substanserna kan även förändra cellers ämnesomsättning, något som kan avgöra om cellen ska dö eller leva vidare. Avhandlingen omfattade två neurotrofiner (BDNF, NGF) och en TNF-substans (TNF-α), samt deras mottagarmolekyler (receptorer) som behövs för att substanserna ska göra verkan. I senvävnaden studerades alla dessa substanser och i blodet studerades BDNF och den ena receptorn till TNF-α som heter TNFR1.

Huvudresultatet i avhandlingen är att sencellerna i hälsenan, från såväl normala senor som tendinossenor, visar sig innehålla de båda neurotrofinerna och TNF-α samt deras receptorer. Observationerna gjordes med hjälp av mikroskop och markerande signalämnen, dels på proteinnivå, dels på budbärar-RNA-nivå. Den senare är bärare av en kod som styr vilka protein cellen ska kunna tillverka. Sammantaget talar fynden för att sencellerna själva kan producera de här substanserna.

För en av receptorerna till TNF-α — TNFR1, sågs en högre nivå av markörerna i tendinossenor än kontrollsenorna, vilket kan förklara varför sencellerna i de förstnämna oftare är utsatta för självdöd. För nivåerna i blod av TNFR1 och BDNF fanns ingen skillnad mellan tendinospatienterna och kontrollgruppen. Intressant nog konstaterades dock att fysisk aktivitet har samband med förhöjda nivåer av receptorn TFNR1 hos patienter med tendinos, men inte hos kontrollindivider. Detta samband kan beskrivas som att personer med tendinos som var mycket fysiskt aktiva, generellt sätt hade högre nivåer av denna receptor.

Upptäckten av neurotrofiner och TNF-α i hälsenans senceller är intressant med tanke på deras välkända funktioner i andra vävnader

och vid olika sjukdomar. Den exakta funktionen i hälsenor och vid tendinopati/tendinos är dock än så länge oklar. Förmodligen är de främst kopplade till vävnadsförändringar genom att reglera celldöd och tillväxt.

Acknowledgements

Joyous Day! I am done!

Although I am alone responsible for what is written in this thesis, the work would not have been completed without the generous help from many people. I'd like to name some of those that have helped me. Most of them work, or have worked, at the Department of Integrative Medical Biology and at the Sports Medicine Unit, Umeå University.

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It is always good to have more than one supervisor. I was lucky to have two talented people by my side. I thank Patrik Danielson for invaluable help and feedback on my work. I also thank Håkan Alfredson at the Sports Medicine Unit. He was directly responsible for getting this thesis started and finished and, in particular, for doing all the clinical work. His great knowledge of tendons has been invaluable.

This thesis is based on laboratory work, and it would not have been possible to complete it without the expertise of Ulla Hedlund, my excellent teacher in the lab. She has been the principal person in all laboratory work that was carried out. I do not know how I can thank her enough with words.

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I was privileged to collaborate with three people (co-authors) from Australia. I am thankful to Jamie Gaida at Monash University

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Anatomy is old, so were many articles, which I had trouble obtaining on my own. I thank the staff at Customer Services at the University Library/Medical Library for providing me with literature.

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My guides and friends in *IKSU friluftsf*: they consumed almost all of my time, so I should perhaps not thank them for that in this context, but they gave me everything. They are my life, and they are the reason that I stayed in Umeå for so long.

I thank past and present co-runners in Umeå and Karlstad and elsewhere. Because of them, I started and keep running, and therefore this book was written (Yes, this is true. How otherwise would I have been able to sit still for so many hours each day?). Running helps me think clearly; the greatest ideas always come to me while I am running.

At last, to the people that are closest to me: all my love goes to my family: Britt – Nils – Malin – and – Maria. For everything. And to Karin. Because you are the one you are.

To my grandmother *Ulla Bagge (1926-2007)*. She encouraged me to study medical science.

LIST OF PUBLICATIONS

This doctoral thesis is based on the following original articles. They will be referred to by their roman numerals.

I Johan Bagge, Ronny Lorentzon, Håkan Alfredson, and Sture Forsgren. (2009). Unexpected presence of the neurotrophins NGF and BDNF and the neurotrophin receptor p75 in the tendon cells of the human Achilles tendon. *Histol Histopathol* 24, 839–848.

II Johan Bagge, Patrik Danielson, and Sture Forsgren. (2012). In situ hybridization studies favouring the occurrence of a local production of BDNF in the human Achilles tendon. *Histol Histopathol* 27, 1239–1246.

III James Edmund Gaida, Johan Bagge, Craig Purdam, Jill Cook, Håkan Alfredson, and Sture Forsgren. (2012). Evidence of the TNF-α system in the human Achilles tendon: expression of TNF-α and TNF receptor at both protein and mRNA levels in the tenocytes. *Cells Tissues Organs* 196, 339–352.

IV Johan Bagge, James Edmund Gaida, Patrik Danielson, Håkan Alfredson, and Sture Forsgren. (2011). Physical activity level in Achilles tendinosis is associated with blood levels of pain-related factors: a pilot study. *Scand J Med Sci Sports* 21, e430–438.

CONTENTS

Preface

Many of my co-runners were prevented from running, or had to quit, because of prolonged Achilles tendon problems. What can be worse than not being able to be run? Many things of course, but it is an undesirable condition that no one should be affected by.

My interest in tendons was raised during my undergraduate physiotherapy studies, while reading about tendon injuries. I was fascinated that there were no good explanations for where the pain came from and what was going on inside the tendon at a cellular level. Later, I had the luck to get involved in tendon research dealing with these questions. It is a very interesting area, with a well-known history: everyone surely knows the tale of Achilles and his heel from Greek mythology, but yet not everyone knows about the theory that suggests that the Achilles tendon was an important factor in human evolution. This theory is based on the fact that the Achilles tendon is important in enabling human beings to run with such outstanding endurance.

I have thought about the Achilles tendon more or less every day during the last five years. I have learned that tendons are far more complicated than I ever could have imagined. My contribution so far to tendon research is described in this thesis. In a wider perspective, this contribution seems rather small. But, I have now left my first footprint, and I think that it might not be the last.

Achilles tendinopathy affects many people who are seriously interested in different types of physical activity, from the recreational level to the elite one. The injury causes disability and pain, which prevent activity. Still, the underlying mechanisms of tendon tissue changes are not understood, and the picture of where the pain emanates from is not clear. Several new treatment methods have developed during the past decade and they have significantly improved curing time and rate, but many patients remain patients for several years. Studies on molecular pathophysiology will increase the understanding of the underlying mechanisms behind tissue changes

and pain, and will facilitate the development of new treatments so that people can return to active lives faster. Ultimately, this type of research will also increase our knowledge of how we can prevent the disease.

In this thesis, I first give a background, starting from general aspects of normal tendons to the Achilles tendon and the major disease: Achilles tendinopathy/tendinosis, before describing briefly the methods used. An overview of each study (*I-IV*) is given in the Results, and the results are discussed in greater detail in the Discussion section.

PART I

BACKGROUND

I will start by giving some brief background information on general aspects of tendons and their diseases, and then describe in more detail the Achilles tendon, Achilles tendinosis and what this thesis really is about. I intend to give you the rationale for studying TNF-α and neurotrophins in tendinosis. Let us start from the beginning, by looking at normal tendons.

Human Tendons

General characteristics

Tendons are part of the muscle-tendon unit, which is essential for movement. The main function of tendons is to transmit force from muscle to bone, resulting in joint movement.

Tendons are made up of long collagen fibres organised in parallel bundles and fascicles. A fibre is built up of three helical procollagen polymers forming a triple helix of tropocollagen (for an overview of the anatomical structure and nomenclature, see Fig. 1).

A tendon is covered by a connective tissue sheath called the epitenon. Similarly, the collagen fascicles within the tendon tissue are covered by a connective tissue sheath, the endotenon. The whole tendon is surrounded by a sheath that is comprised of loose connective tissue (sometimes called the paratenon). This peritendinous (peri = latin for near/around) tissue lies outside the epitenon. Tendons that change direction need better lubrication and, therefore, are generally covered by a synovial sheath (Elliott, 1965; Jozsa & Kannus, 1997).

The principal cells of tendons are the tenocytes, which are a specialised type of fibroblast. They produce the extracellular matrix (ECM), which is a ground substance comprising both collagen and proteoglycans. Tenocytes are long spindle-shaped cells that are transformed from tenoblasts. The nucleus of a tenocyte occupies almost the entire cell. The tenocytes are arranged in rows in line with the collagen fibres and are connected to the ECM via integrins, which permit the cells to sense and adapt to mechanical loading (Chiquet et al., 2009).

The collagen of tendons is mainly collagen type I, which gives mechanical strength. Collagen is strong. To completely rupture a tendon requires a tensile stress of 50-100 MPa (Elliott, 1965). During running, a tendon may transmit up to 9 kN and during walking up to 2.6 kN (Komi et al., 1992). The collagen fibres are mainly arranged longitudinally. Transverse fibres also exist; these help tendons to withstand forces from several directions.

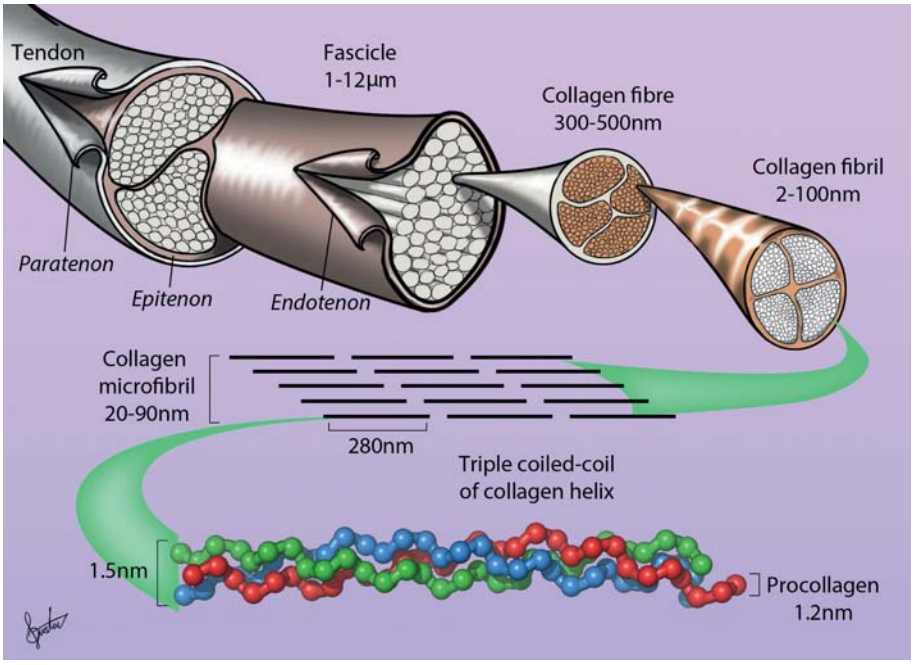

Figure 1. The structure of a tendon.

To withstand force better, collagen is also helped by proteoglycans and glycoproteins, which bind water and are therefore important for the tendon's viscoelastic properties. Elasticity is also provided by elastin fibres (Jozsa & Kannus, 1997; Jozsa et al., 1991; O'Brien, 1997). The amount of proteoglycan varies within a tendon depending on the type of force that it is exposed to. In rabbit flexor tendons, the proteoglycan content increases and the type of proteoglycan changes in regions with high compression (Gillard et al., 1977).

In its resting state, the collagen fibres of a tendon show a wavy formation, a characteristic phenomenon known as crimp. When

stretched, this formation disappears and the fibres become longitudinal. In the human Achilles tendon, the crimp angle seems to be the same in different regions of the tendon (Magnusson et al., 2002).

Tendons are less vascularised than muscles. The blood supply may vary in different regions within a tendon. Blood vessels run longitudinally within the endotenon and the epitenon, and are especially present in the peritendinous tissue. Numerous vessels also enter the tendon at the myotendinous junction. These vessels are responsible for part of the blood supply of a tendon. Tissues outside, but adjacent to a tendon, e.g., the adipose tissue and tendon sheaths, have a richer blood supply (Theobald et al., 2005). It has still not been concluded which areas are least, and which are most, vascularised. The vascular pattern is often changed in tendon pathology (Öhberg, 2003). Blood supply is also increased by acute physical activity (PA) (Cook et al., 2005).

Tendons are innervated. There are great variations between different tendons, as well as within a tendon, in the numbers of neurons and type of innervation (Jozsa & Kannus, 1997). Generally, it is believed that tendons are sparsely innervated and that nerves follow blood vessels in the peritendinous connective tissue. No innervation is seen in the tendon tissue proper, but nerves occasionally occur in the endotenon septa. Thus in healthy tendons nerves are generally absent (Schizas et al., 2010).

Innervation includes sensory nerves (free nerve endings/type IV endings) on the tendon surface, Golgi tendon organs, Vater-Pacini corpuscles and Ruffini corpuscles (Jozsa & Kannus, 1997; Stilwell, 1957). The myotendinous junction is a region rich in mechanoreceptors (Jozsa et al., 1993). Sympathetic innervation is also found in the peritendinous tissue of the patellar (Danielson et al., 2007) and the Achilles (Andersson et al., 2007; Bjur et al., 2008a) tendons.

From the proximal to the distal end, tendon anatomy varies significantly and the different regions are prone to different diseases and injuries. This thesis deals with the mid-portion of the Achilles tendon, which is described more thoroughly on page 26. The place

where tendons insert onto bone is sometimes called enthesis, a region that is prone to injury, and which has been well described (Benjamin et al., 2006). The enthesis is complex, comprising several anatomical structures. Therefore the concept of an enthesis organ has been described. One function of the enthesis organ is to share and reduce tensile forces (Benjamin et al., 2006).

Mechanical loading and metabolism

Unfortunately little is known about how loading affects diseased tendons. Heavy mechanical loading usually aggravates pain in diseased tendons, particularly in the Achilles tendon. Therefore, knowing how tendons respond to loading is essential to understanding tendon pathology. Studies of treatment with heavy load eccentric training, have shown improved tendon structure and decreased tendon thickness in patients with mid-portion Achilles tendinopathy (Öhberg et al., 2004).

One main theory about the cause of chronic tendon disease is that it is due to overloading. An extensive eccentric loading programme (see page 34) is one of the most used treatments for this condition.

How human tendons respond to loading has been reviewed by Heinemeier and Kjaer (2011). Generally, it is believed that tendons adapt to increased loads by remodelling and by trophic events, including hypertrophy, increased collagen synthesis, and hyperplasia. It appears that tendons respond to loading according to the biological law that "hyperfunction leads to hypertrophy" and, in order to meet altered demands, tissues need to respond to loading.

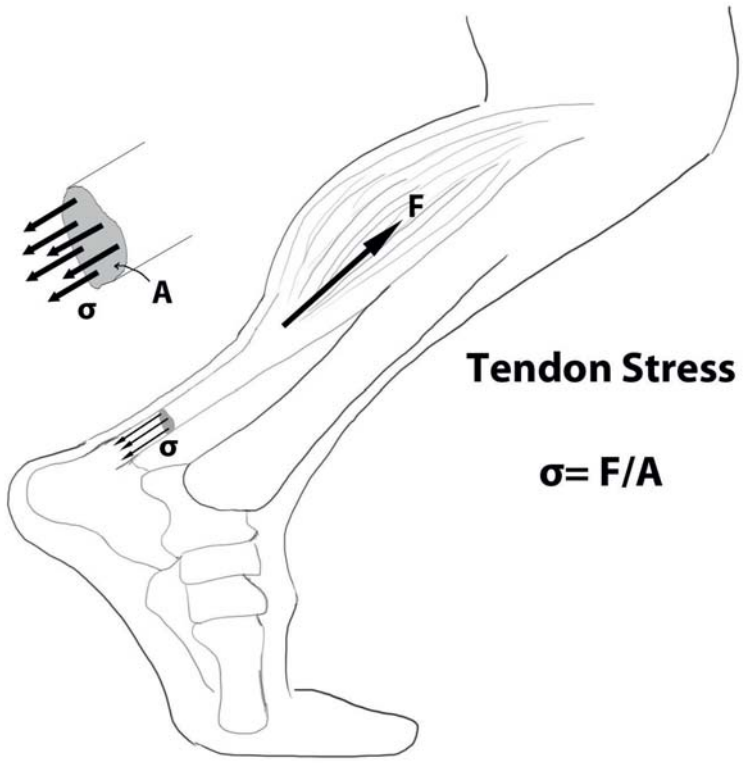

Figure 2. When a tendon is loaded by an external longitudinal force (F) from a muscle, tensile stresses (σ) will occur over the cross sectional area (A) of the tendon, creating a balance to the force. Tensile stress is defined as actual force divided by the cross sectional area in loaded state: $\sigma = F/A$ and is measured in MegaPascale (MPa) where 1 MPa = 1 N/mm^2. The stress at rupture, σ_{rup}, is defined as the maximum tensile stress a given material can resist before rupture occurs. Its value is specific for different materials, and it varies greatly with material properties. A few examples are given below.

Typical values of stress at rupture in axial direction of some materials:	
Achilles tendon tissue (Wren et al., 2001)	70 MPa
Pine wood, European (small, clear samples)	100 MPa
Structural steel (ordinary quality)	500 MPa

The Force at rupture (F_{rup}) can be computed as the tensile stress at rupture multiplied by the cross sectional area in loaded state, and is measured in Newton (N).

The axial ultimate tensile stress of the tendon is described in Fig. 2. One law of physics states that the force at rupture of a sample of material is proportional to the cross-sectional area (CSA). Thus, in tendons too, tensile strength is dependent on the CSA. However, the internal structure in tendons may influence tensile strength as well. This is supported by the observation that the plantaris tendon has higher stress at failure than the Achilles tendon (Lintz et al., 2011). The internal structure is likely to be improved by loading (Michna & Hartmann, 1989). So this can explain why CSA is not always increased in response to exercise. Despite these facts, trained athletes have larger tendon CSA compared with those of non-athletes (Kongsgaard et al., 2005; Rosager et al., 2002).

It can be assumed that increased CSA essentially depends on increased collagen content. Collagen synthesis can be measured and is often used to indicate the rate of tendon metabolism. It has been shown that markers for both synthesis and degradation of collagen type I in tendons are upregulated after 4 weeks of military training (Langberg et al., 2001). Moreover, collagen type I turnover in the peritendinous connective tissue of the Achilles tendon is increased after 7 weeks of immobilisation (Christensen et al., 2008). One study that directly measured collagen synthesis in human patellar tendons also suggests that collagen is upregulated by exercise (one-legged kicking) (Miller et al., 2005).

The metabolism of tendons is a controversial topic. Tendons have enzymes for all major energy pathways (Jozsa & Kannus, 1997). Blood flow can be used to indicate metabolism. In the 19th century, it was believed that tendons had no blood supply and therefore had no metabolism. Subsequently, it has been acknowledged by many authors that tendons do have a significant metabolism (for references see Jozsa & Kannus, 1997). For example, dynamic plantar flexion increases Achilles peritendinous blood flow (Boushel et al., 2000).

Glucose uptake can also be used to indicate metabolism. It has been shown, e.g. by PET scanning, that voluntary plantar flexion

increases glucose uptake by the Achilles tendon (Bojsen-Møller et al., 2006).

Mechanotransduction

Mechanotransduction is a principle of how tissues respond to mechanical loading, leading to biochemical and electrical signalling (Chiquet et al., 2009). A cell's response to mechanical stimuli depends on the type and intensity of the stimuli. This theroy is based on an asumption that forces exerted by the ECM surrounding a cell are responded to by a force of the same magnitude by the cell (Chiquet et al., 2009).

One description made by Khan and Scott (2009) is that "[m]echanotransduction is generally broken down into three steps: (1) mechanocoupling, (2) cell–cell communication and (3) the effector response." Mechanotransduction is an important theory in the concept of mechanotherapy — in which movement is used to achieve tissue healing. The authors stress that the term mechanotherapy should be reintroduced (Khan & Scott, 2009).

Mechanotransduction is mainly studied *in vitro* by comparing cell lines subjected, or not subjected, to different loading regimens. In brief, forces are transmitted from the ECM to the cytoskeleton and to the nucleus. This leads to an alteration of the composition of integrins and G-proteins, which then initiate a complex biochemical cascade, including effects on gene expression, protein synthesis and differentiation (for a review, see Wang, 2006). Such initiation includes both cytokines and growth factors. Mechanotransduction is complex, and the exact mechanism of the way in which tension leads to biochemical signalling in tenocytes is not understood.

Common diseases of human tendons

Tendons can be affected by various diseases or injuries of local or systemic origins. These diseases may be divided into acute or non-acute. The term chronic is often used to describe tendon problems that persist for longer than 3 months.

Perhaps the most well-known tendon injury is the total rupture of the Achilles tendon. In most cases this injury is a spontaneous rupture, and degenerative changes are often found in these tendons (e.g. as seen in histological examinations of 397 ruptured human Achilles tendons) (Kannus & Jozsa, 1991). In sports, a total rupture is not often preceded by tendon pain (Fahlström et al., 1998). Ruptures can be caused by external force directly applied to the tendon. Partial tendon ruptures also occur. Such ruptures can be difficult to detect clinically. Microruptures are another type of rupture, involving only a single fibre or a few fibres. Although such ruptures are minimal, it has been suggested that repeated microruptures cause tendinopathy. Microruptures constitute an important factor in the overuse theory, which attempts to explain how overuse may lead to chronic tendinopathy (Leadbetter, 1992). However, this is a theory and it has not been proved that microruptures precede tendinopathy.

Tendinopathy is a family name for many different tendon diseases. It simply means a painful and swollen tendon with impaired functions (van Dijk et al., 2011). It does not say anything about the structure of the tendon (Maffulli et al., 1998). Recently it was proposed that tendinopathy should be divided into different stages, and then classified according to the ongoing cellular processes (Cook, 2011).

Diseases relating to the tissues surrounding tendons are commonly referred as tenosynovitis, paratenonitis and peritendinitis. It has been suggested that injuries in the tissue surrounding the Achilles tendon should be named paratendinopathy (van Dijk et al., 2011).

The names used to describe chronic tendon diseases are sometimes confusing because they do not relate to the underlying pathological changes within the tendon. This is particularly so for

"tendinitis" which is often used despite the fact that inflammatory cells have not infiltrated into the tendon (Maffulli et al., 1998). A sub-diagnosis of tendinopathy is tendinosis, which implies structural changes, but no inflammation. Tendinosis is described more in detail on page 28.

Tendon diseases relating to the distal tendon, are referred to as enthesopathies or insertional tendinopathies. In these regions, other diseases can be retrocalcaneal and superficial calcaneal bursitis.

The Achilles tendon

The Achilles tendon is the largest tendon in the human body, and it also has the largest fibres. The average fibre diameter is 26,0 μm, but this can vary two-fold (Jozsa & Kannus, 1997). The main function of the Achilles tendon is to transfer force from the calf muscles to the heel bone, which leads to plantar flexion.

The Achilles tendon is formed by the two tendons of the soleus and gastrocnemius muscles and inserts broadly at the posterior aspect of the calcaneal bone (see Fig. 3). Soleus is a flat, broad muscle, lying ventral to the gastrocnemius muscle and originates in the upper third of the fibula and mid tibia. The gastrocnemius muscle has a medial and a lateral head, originating from the medial and lateral femur condyles (Cummins & Anson, 1946).

Together the two muscles are named triceps surae, and are of importance for support and propulsion in normal gait (Anderson & Pandy, 2003; Gottschall & Kram, 2003). The principal function of the triceps surae muscle is plantar flexion of the foot. Triceps surae also supinates the foot, i.e., the combination of plantarflexion, adduction and internal rotation. Another function of the gastrocnemius muscle is knee flexion because it originates above, and runs behind, the knee joint.

The fibres of the Achilles tendon are twisted up to 90 degrees laterally (White, 1943). The magnitude of the twist depends on the anatomical relationship between the soleus and gastrocnemius muscles

(Cummins & Anson, 1946). This anatomical relationship varies between individuals.

The Achilles tendon is innervated both by the nerve from its attaching muscles, the tibial nerve, and by branches from the sural nerve (Stilwell, 1957).

Structures lying close to the distal part of the Achilles tendon are Kager's fat pad and the superficial and deep bursae. Other tendons run ventral, medial and lateral to the Achilles tendon. One long tendon that runs medial to the Achilles tendon is the tendon of the plantaris muscle (Fig. 3). This muscle and its tendon are described below. The plantaris tendon has been highlighted lately as important in Achilles tendinopathy and is sometimes removed surgically with good results (Alfredson, 2011a).

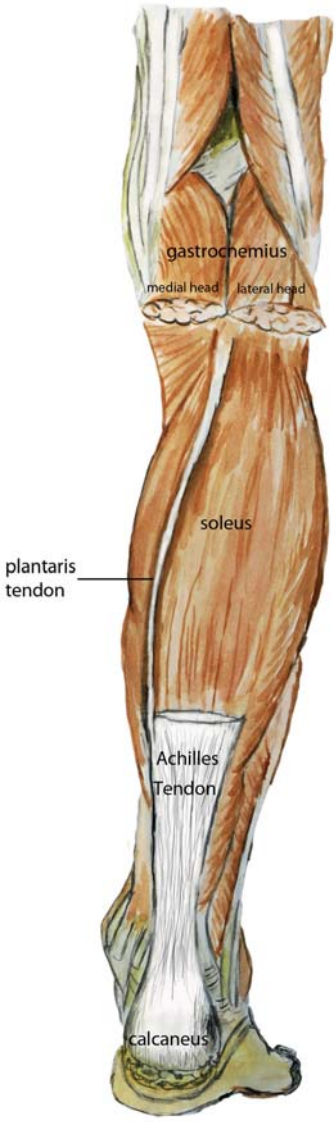

Figure 3.

The course and insertion of the plantaris tendon vary considerably. Most often the plantaris tendon is separated from the Achilles tendon but sometimes they anastomose. It has been suggested that the plantaris tendon is missing in 7% of the population. This was found in a study of 750 cadavers (Daseler & Anson, 1943) but, in a recent study, the tendon was found to be present in all 107 lower extremities examined (van Sterkenburg et al., 2011).

The plantaris tendon is the tendon of a small muscle. It is unlikely that this small muscle contributes significantly to the mechanical force in plantar flexion. The plantaris muscle lengthens by 40% compared with the gastrocnemius muscle that only lengthens by 15% when the foot is moved

from full plantar flexion to full dorsiflexion (Peck et al., 1984).

Like other small muscles, the plantaris muscle is considered to be important for proprioceptive feedback to the central nervous system (Peck et al., 1984). This is because it is believed that it has higher muscle spindle density compared with that of its larger synergistic muscles (von Hoyer, 1963; Voss, 1971). However, the importance and function of muscle spindles is not understood. And neither is the role of the plantaris muscle.

Achilles Tendinopathy/Tendinosis

Diagnosis and classification

Morphological changes in tendons are described as tendinosis (Perugia et al., 1976; Puddu et al., 1976). Tendinosis will be used to describe the Achilles conditions of the patients included in this thesis. All the patients had structural changes in the mid-portion of their Achilles tendon, together with chronic pain in the Achilles tendon.

The term tendinosis is used for this thesis because it describes the condition concerned more specifically than tedinopathy, i.e. because tissue changes were verified objectively. It is more exact than tendinopathy, although it would also be correct to use tendinopathy for these patients' conditions. Hence, tendinosis is one type of tendinopathy, but tendinopathy is not always tendinosis (van Dijk et al., 2011). Here, the terms tendinosis and tendinopathy are, nevertheless, used interchangeably when appropriate.

The diagnosis of tendinosis is established using ultrasonography, MRI or biopsy.

This thesis is restricted to tendinosis of the mid-portion of the Achilles tendon, i.e. the part that is 2 to 7 cm proximal to the Achilles

tendon insertion onto the calcaneal bone. Hence, insertional Achilles tendinosis was not studied.

Chronic mid-portion Achilles tendinopathy is a diagnosis that is easy to verify clinically. The patients have a long history of persistent tendon pain and dysfunction. There is tender swelling of the mid-portion. Especially in the later stages, excessive physical activity (PA) aggravates the pain (Cook et al., 2002).

The severity of pain in Achilles tendinopathy can be evaluated using a visual analogue scale (VAS), in which the patients score the severity of pain during tendon loading activity on a 100 mm scale (on which 0 represents no pain and 100 is the worst pain). The severity of Achilles tendinopathy and the function of the Achilles tendon can be tested by the Victorian Institution of Sports Assessments – Achilles questionnaire (VISA-A questionnaire) (Robinson et al., 2001). This questionnaire has been validated in a Swedish version (Silbernagel et al., 2005).

Bilateral Achilles tendinopathy is frequently seen. In an 8-year follow-up study, Achilles tendinopathy was found to develop in the previously uninjured tendon in 41% of the patients who initially had unilateral Achilles tendinopathy (Paavola et al., 2000).

Examples of differential diagnoses to mid-portion Achilles tendinopathy include: partial or total Achilles ruptures, acute paratenonitis, chronic inflammatory conditions and presence of accessory soleus muscle. Further differential diagnoses are: diseases or injuries affecting the lower mid-portion of the Achilles tendon, partly including the enthesis, such as involvement of the deep and superficial bursae, and the upper calcaneus (Haglund deformity). Entrapment of the sural nerve and referred pain from the vertebral column are other differential diagnoses. It should be noted that plantaris tendinosis can cause local interference with the medial side of the Achilles tendon, and often co-exists with mid-portion Achilles tendinopathy (Alfredson, 2011a).

Epidemiology

Achilles tendinopathy is often regarded as a sports injury, and it is commonly reported in epidemiological sport injury studies. However, inactive people are also affected (Rolf & Movin, 1997) and constitute a high proportion of the visits to clinics. There are few studies on the prevalence and incidence of Achilles tendinopathy in the general population.

I have found about 60 epidemiological (prevalence and incidence) studies on Achilles tendinopathy — almost all were on sport populations. Most of the studies did not have a clear definition of tendinopathy, i.e., the studies state what the diagnosis is, but give no further definitions. The studies that had a clear definition of Achilles tendinopathy, and that studied the incidence or prevalence of Achilles tendinopathy are summarised in Table 1. Studies that investigated tendinopathy at competitions and tournaments were excluded because such studies are associated with selection bias; prevalence in such studies may be underestimated. Also, in contrast to chronic tendinopathy, which develops over months, these tendinopathies are likely to be acute inflammatory tendinopathies.

One study that is not listed in Table 1 is the study by Kujala and coworkers (2005). They reported that, in Finland, the historical cumulative incidence of Achilles tendinopathy in former elite runners is 52%.

The incidence and prevalence of mid-portion Achilles tendinopathy/tendinosis has not been determined although, from the clinical perspective seen in northern Sweden, it is evident that subjects younger that 25 years of age are rarely affected. The condition is seen mainly among people in the age group 35-60 years, and appears to be equally common in men and women. The latter fact is supported by the study by de Jonge and coworkers (2011). People who are not physically active, and who often have a high body mass index (BMI), are increasingly affected (personal communication with Håkan Alfredson, November 2012).

Table 1. Prevalence and incidence studies with clear definition of mid-portion Achilles tendinopathy

Study	**Population/level**	**No. of participants** (m/f)	**Mean Age, y** (SD)	**Design**	**Study length** (months)	**Diagnosis**	**Cases** No. (m/f) and %	**Comments**
de Jonge et al., 2011	general population	45012 (21390/23622)	>21	r	12	medical records	94 (44/50) 2.1%	historical incidence
Hirschmüller et al., 2012	runners/ recreational	427 (285/142)	43 (11)	p	12	email, phone (interviews)	29 (20/9) 7%	33 % drop out
Van Ginckel et al., 2009	runners /novice	129 (19/110)	39 (10)	p	2.3	clinical	10 (2/8) 8%	subjects in training
Waldecker et al., 2012	general population, non-sport	697 (280/417)	50 15-88	p	NA	clinical	52 (21/31) 3.7%	patients at one clinic, time period not clear

m= men, f = female, p = prospective, r = retrospective, NA = not available

Aetiology and prevention

It is an important task to find the cause of Achilles tendinopathy, particularly because it would probably help us find a way to prevent the disease.

It is proposed that both intrinsic and extrinsic factors are important in Achilles tendinopathy. One old theory states that biomechanical abnormality is a risk factor for sports-related injuries. Results from a retrospective study show that training errors may account for 75% of all Achilles tendon injuries in runners (an injury was referred to as gradual onset of pain and swelling 2-3 cm proximal to the insertion) (Clement et al., 1984). Results from the same study suggest that functional overpronation is a risk factor. However, this has not been verified to be a cause of tendinopathy, and Åström (1997) concluded in his thesis that "[a]bnormal biomechanics, notably hyperpronation, is not important in chronic Achilles tendinopathy. . ."

The overuse theory is the main aetiological theory: it states essentially that the reparative capability of the tendons is exceeded by repetitive microtrauma (Leadbetter, 1992). Another theory describes a relationship to biochemical substances. This theory is described in the molecular pathophysiology section on page 38.

Genetic studies have gained attention. It has been found that a polymorphism within one gene (COL5A1) is associated with chronic Achilles tendinopathy (Mokone et al., 2006). Hence, genetic risk factors may exist. Other suggested risk factors for Achilles tendinopathy are systemic diseases, such as rheumatism, diabetes, and obesity (Ames et al., 2008; Gaida et al., 2009b). Serum lipid profile has also been suggested to be related to Achilles tendinopathy (Gaida et al., 2009a).

The aetiology of Achilles tendinopathy is complex and the condition may have many different causes, which are yet to be determined. Several suggestions have been made, but no prospective

study has been done; studies are of cross sectional or retrospective design, which generate hypotheses and possible risk factors. Therefore, the conclusions drawn from such studies are controversial. Moreover, the existing aetiological studies do not define the type of Achilles tendinopathy studied.

It is desirable to find an answer to the question whether Achilles tendinopathy can be prevented or not. Kraemer and Knobloch (2009) conducted an interesting cross-over study which shows that, in women soccer players, it is possible to decrease Achilles tendon ("time-loss") injuries by 90% with a proprioceptive training program. Another study found that stretching and eccentric exercise (25 repetitions with both straight and bent knee, 2-3 times/week) of the triceps surae muscle may not reduce the risk of Achilles tendon injury in elite soccer players (Fredberg et al., 2008). In that study, injury was defined as "any physical complaint sustained by a player resulting from a soccer match or soccer training" (concerning tendons).

Patients experiencing early-stage tendinopathy ("reactive tendinopathy") may derive greater benefit, with less possibility of exacerbation, by performing isometric exercises rather than eccentric training (personal communication with Craig Purdam, Jill Cook and Per Jonsson, November 2012). It is possible that chronic tendinopathy could be prevented if the patients are treated in this way at this early stage.

If the aetiology of Achilles tendinopathy is uncertain, the prevention is even more so.

Treatment

In 2011, Jill Cook highlighted that tendinopathy is a complex diagnosis and that treatment must be adapted to meet the possible different aetiological aspects and subgroups of tendinopathy, and that this must be addressed in future research. Thus, choosing the appropriate treatment is a challenge for clinicians (Cook, 2011).

Several approaches exist, including both conservative and surgical alternatives, the former being the first choice of treatment.

Here I will give some brief information on the most important aspects of treatment for tendinopathy, and specifically for mid-portion Achilles tendinopathy. Treatments may, or may not, be similar to those for tendinopathies at other sites.

In the treatment of mid-portion Achilles tendinopathy, rest diminishes the tendon pain, but rest is not a cure. When patients return to tendon-loading activity, the tendon pain returns. A wait-and-see policy is inferior to both eccentric exercise and shockwave therapy in patients with chronic mid-portion Achilles tendinopathy (Rompe et al., 2007).

Alfredson and coworkers (1998) presented a modified variant of the programme described by Stanish and coworkers (1986) to be used on patents with mid-portion Achilles tendinopathy. In brief, the programme includes 3 sets of 15 repetitions of eccentric exercise, both with bent and straight knee, 2 times per day for 3 months. The exercise should be performed with loads high enough to cause pain in the tendon. Eccentric exercise is a widely used and accepted treatment for mid-portion Achilles tendinopathy, and can also be used for other tendinopathies (Jonsson, 2009).

The mechanisms underlying this treatment are not clear, although some theories have been suggested (Rees et al., 2008; Allison & Purdam, 2009), including a possible traumatic mechanism on nerves and blood vessels outside the ventral part of the tendon where the tendon and fat tissue interface (personal communication with Håkan Alfredson, November 2012).

Usually the programme needs to be performed daily for 3 months or more, which may lead to low compliance. However, a 27 % reduction in pain can be seen clinically as early as after 6 weeks (Roos et al., 2004), and compliance is increased by follow-up visits to the clinic and clear explanation of the structure and milestones of the rehabilitation programme that will motivate patients to continue the treatment (personal communication with Craig Purdam, Per Jonsson, and Jill Cook, November 2012). The effectiveness of eccentric exercise

is reported to vary considerably from study to study (Kingma et al., 2007; Magnussen et al., 2009; Rowe et al., 2012; Sussmilch-Leitch et al., 2012; Woodley et al., 2007). Eccentric exercise combined with other therapies, such as laser or prolotherapy, may be more effective than eccentric training alone, as described in short term follow-up studies, though these benefits may be due to attention bias (Sussmilch-Leitch et al., 2012; Yelland et al., 2011). In a recent systemic review of conservative treatment for mid-portion Achilles tendinopathy, 42 studies were identified (Rowe et al., 2012). These studies were described as of varying quality, covering 11 different treatment methods. The conclusion in this review was that there is strong evidence for the effectiveness of eccentric exercise and shockwave therapy, moderate evidence for the effectiveness of low-level laser therapy, and limited evidence of it for orthoses.

Other treatments include injection treatments. Intratendinous corticosteroid injections are controversial due to the possible adverse effect, i.e. tendon rupture. Case reports that claim this relationship exist. No prospective study has been done, most likely for ethical reasons. Nevertheless, animal studies have shown several negative effects. Intratendinous corticosteroid injections to rabbit Achilles tendons led to local necrosis of the collagen and the tenocytes. No necrosis was found in the contralateral control tendons injected with saline solution (Balasubramaniam & Prathap, 1972). Moreover, the negative effect of such injections is further reinforced by the fact that corticosteroid injections decrease the tensile strength of rabbit tendons (Hugate et al., 2004), and results from one cell culture study showed that corticosteroids have negative effects on avian tendon collagen synthesis (Saarni, 1977).

Based on the results from animal studies, intratendinous corticosteroid injections have been more or less abandoned as treatment of human Achilles tendons. Although intratendinous injections have been abandoned, little is known about the effects of injecting corticosteroids into the peritendinous tissue (Mahler & Fritschy, 1992). Only one randomised controlled trial of Achilles

tendinopathy exists. This reported that this injection treatment had no benefit over placebo (as reviewed by Coombes et al., 2010).

For other tendinopathies, such as lateral epicondylalgia, it has been shown that, in the long term, peritendinous corticosteroid injections are no more effective than a wait-and-see policy (Coombes et al., 2010).

Alfredson and Öhberg (2005) developed an ultrasound and Doppler-guided method for injecting the sclerosing agent polidocanol, targeting the regions with high blood flow and peritendinous sympathetic and sensory nerves outside the ventral side of the Achilles mid-portion. A randomised controlled trial showed good clinical results with this treatment compared with lidocaine + adrenaline injections (Alfredson & Öhberg, 2005). This good clinical result suggests that the pain in this condition comes from regions with nerves and vessels located outside the ventral side of the tendon. Nevertheless, the injection technique is difficult and operator-dependent, and often 2 to 3 injection treatments at intervals of 6 to 8 weeks are needed, which makes the total rehabilitation period relatively long.

Another injection treatment that has recently been introduced is the intratendinous injection of platelet-rich plasma (PRP) (Coombes et al., 2010). The method has become popular, but there is little molecular evidence to support the use of this method, and a recent randomised controlled trial showed no better results with PRP injections than with saline injections (de Jonge et al., 2011).

If conservative treatment fails, surgery is indicated. Historically, various surgical procedures (most including tenotomy) have been used, but prospective studies are few and the success rate is negatively associated with the quality of the study (Tallon et al., 2001). These surgical methods are associated with long rehabilitation periods, considerable costs, high failure rates, and difficulties predicting an individual's outcome (Andres & Murrell, 2008). Therefore, new and better surgical methods have been developed during the past decade. These new methods include mini-invasive surgical scraping (Alfredson, 2011b) alone or in combination with extirpation of the plantaris tendon (Alfredson, 2011a). The surgical scraping method is based on

the same principle as for sclerosing polidocanol injections, i.e., that the nerves and blood vessels located in the regions with high blood flow outside the ventral part of the Achilles tendon are affected. Both surgeries are performed with ultrasound and Doppler-guidance. The patients can start to walk after one day postoperatively, then gradually increase the tendon load and return to full activity after 2-6 weeks. Short-term follow-up results indicate that these new treatments are promising (Alfredson, 2011a, 2011b).

There is a wide panorama of different treatment methods that can be used to treat chronic mid-portion Achilles tendinopathy. Forthcoming research will help to determine which the most effective and most appropriate treatment is.

To understand what causes tendinopathy, and how different treatments work, it is important to know what is going on in the tendons at a molecular level. I will give a brief introduction to the general morphology of diseased tendons, and then present molecular aspects of the pathophysiology in tendinopathy.

General morphology

Traditionally, tendinosis is defined as intratendinous degeneration, which can be hypoxic, mucoid or myxoid, hyaline, fatty, fibrinoid, calcific, or any combination of these (Jozsa & Kannus, 1997). Characteristics of morphological changes in tendinosis are: tenocyte hyperplasia, changes in tenocyte appearance (cells become rounded, widened, wavy, or larger), and increased number of blood vessels (Bjur et al., 2005; Movin et al., 1997; Åström & Rausing, 1995). In the late stages, the tenocytes undergo apoptosis and necrosis, and the collagen fibres become homogenized or disrupted. Consequently, acellular areas may be seen. Calcification as well as fibrocartilaginous metaplasia with formation of chondrocyte-like cells are also seen in late stages. Tendon ossification exists but is rare (Jozsa & Kannus, 1997).

Molecular pathophysiology and pain

In diseased tendons, the tendon matrix metabolism is likely to be altered (Riley, 2008). For example, there is upregulation in collagen type I and collagen type III mRNA expression in chronic Achilles tendinosis (Ireland et al., 2001). It has been suggested that several factors control matrix turnover in tendinopathy. These include growth factors, cytokines and neuropeptides (Riley, 2004).

Several authors conclude that there is no inflammation in chronic tendinopathy. For example, Alfredson and coworkers found that prostaglandin E2 (PGE2) is not upregulated in chronic mid-portion Achilles tendinosis (Alfredson et al., 1999). However, it cannot be ruled out that there are inflammatory components at some stages. Schubert and coworkers, e.g., found that the numbers of B lymphocytes, T lymphocytes, and macrophages in Achilles tendon tissue is higher in patients with Achilles tendinosis than in patients with Achilles tendon ruptures without previous Achilles tendon pain (Schubert et al., 2005).

Pain is subjective. Peripheral nociceptors can be triggered by mechanical or chemical stimuli. Nociceptors have contact with the brain via neurons to the dorsal horn. From the dorsal horn, the signals are transmitted via the thalamus to the cortex, where the stimuli become conscious. The sensitisation of pain signals can occur at any place along the way, from the peripheral receptors to the final perception in the brain (Kuner, 2010). Pain can actually be produced in the absence of an appropriate stimulus (Torebjörk et al., 1992). While these are general concepts of pain, it is unknown how pain signalling is altered in tendinosis. For a long time it has been suggested that tendon pain can be evoked by stimulating free nerve endings (Weddell & Harpman, 1940). Thus, in tendinopathy, increased tendon swelling may lead to mechanical stimuli of nociceptors.

One biochemical theory has been proposed to explain the cause of pain in tendinopathy (Khan et al., 2000). This theory suggests that biochemical substances produced in the tendon tissue trigger

nociceptors. Whether the biochemical effects are causative or secondary to other events is unclear (Danielson, 2009).

In nociceptors there is often a presence of one or both of the neuropeptides substance P (SP) and calcitonin-gene related peptide (CGRP) (Snider & McMahon, 1998). SP, among other substances, is described as important in the biochemical theory of tendinosis (Danielson, 2009). Because both SP and CGRP nociceptors are present in the Achilles tendon (mainly in the peritendinous tissue), these nociceptors can be stimulated there. Interestingly, the number of SP-positive nerves fibres is increased in Achilles tendinosis (Schubert et al., 2005).

Local substance production

Researchers in our group have studied the expression of signal substances in the tenocytes in the Achilles tendon, e.g., studied enzymes/transporters related the production/transport of the neutrotransmitters acetylcholine, catecholamine and glutamate (Bjur et al., 2008a, 2008b; Scott et al., 2008). The studies indicate that these substances are produced in tenocytes, especially in tendinosis. Also, receptor expression for these substances is seen in the tenocytes. Altogether, these findings strongly indicate that there is local production of nerve-related signal substances within the Achilles tendon tissue, and that the presence of receptors in the tenocytes provides a basis for local effects. Similar findings apply to the patellar tendon (Danielson, 2007).

The presence of SP mRNA, as well as the preferred SP receptor (the neurokinin-1 receptor), at both protein and mRNA levels, has also been observed in the tendons cells of the human Achilles tendon (Andersson et al., 2008). The function of SP has been studied in an animal model (Andersson et al., 2011) and in tendon cell cultures (Backman et al., 2011b). These studies suggest that SP may have a role in tendinosis development (Andersson et al., 2011) and that SP can be an autocrine regulator of tenocyte proliferation

(Backman et al., 2011b). It has also been shown that endogenous SP production in the Achilles tendon increases with loading in the animal model (Backman et al., 2011a).

Other groups of substances, for which little is known about the relationship with tendons, are cytokines and growth factors. These are known to be involved in pain-modulating mechanisms, and have pro-inflammatory, apoptotic as well as proliferative effects. Two of these substances families are described below.

TNF-α

General characteristics

Tumour necrosis factor alpha (TNF-α) belongs to the TNF superfamily (TNFSF), which comprises 19 ligands and 29 receptors (Aggarwal et al., 2012). The members of this family are mainly expressed by various types of immunoactive cells and the members have diverse roles in our body; most of them have both beneficial and harmful effects. All members have pro-inflammatory effects, which are partly related to the activation of nuclear factor kappa beta (NF-κB).

Here I will give a brief introduction to TNF-α and its two receptors, all of which were studied in this thesis.

In 1975, a substance with the ability to induce necrosis of mouse sarcomas was initially named tumour necrosis factor (Carswell et al., 1975), and subsequently renamed TNF-α (Pennica et al., 1984). There is also a beta version (TNF-β); the two versions share 50% amino acid homology (Pennica et al., 1984). Another name for TNF-β is lymphotoxin, which was described 1968 (Kolb & Granger, 1968; Ruddle & Waksman, 1968). TNF-α was discovered to be a molecule that corresponds to cachectin in mice (Beutler et al., 1985).

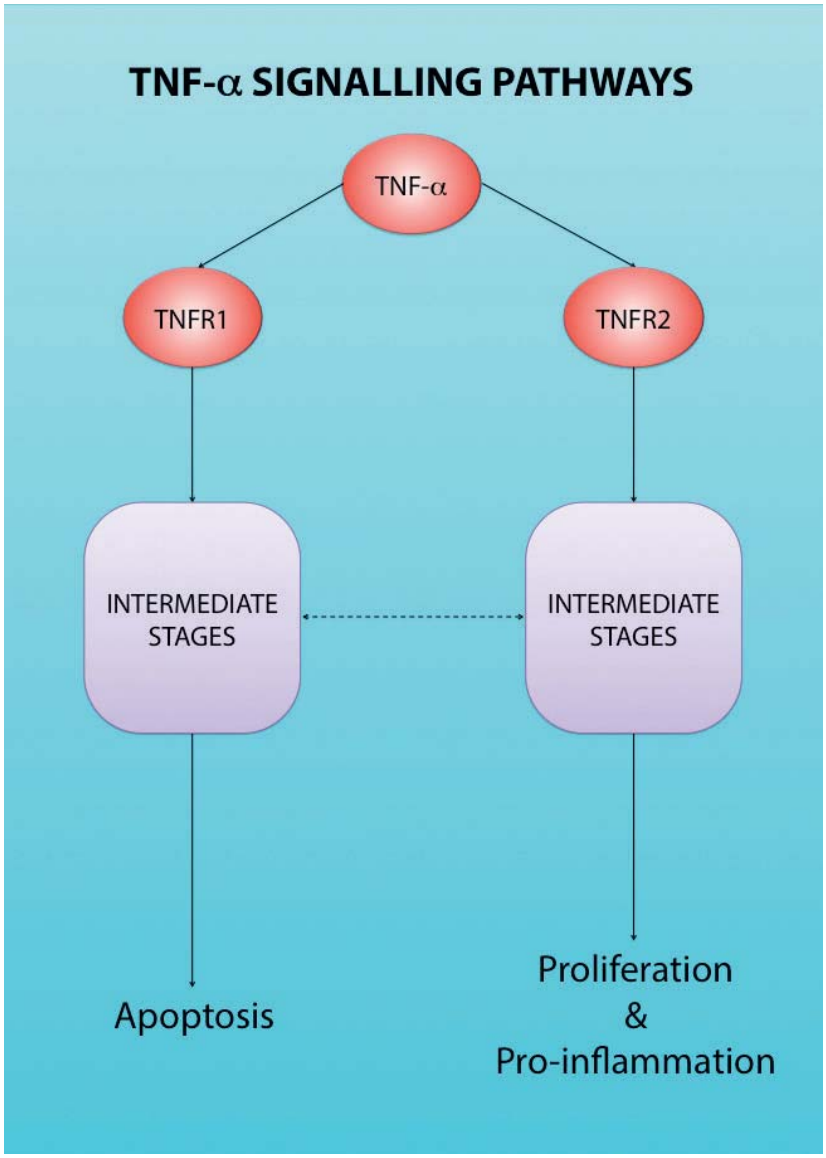

Figure 4. Schematic illustration of the two main pathways for TNF-α signalling. Generally, activation of TNFR1 leads to apopotosis whereas that of TNFR2 leads to proliferation and inflammation. However, the two receptors can have both effects. The pathways are complex and involve several steps, here called "intermediate stages".

TNF-α is synthesized as a prohormone, subsequently cleaved to yield the mature 17-kD cytokine (Pennica et al., 1984). Early studies on mice and rabbits reported that the principal cells that produce it are macrophages (Matthews, 1978; Männel et al., 1980). However, other cells that produce TNF-α are natural killer (NK) cells, and T and B lymphocytes (Aggarwal et al., 2012). TNF-α appears both as a transmembrane and as a soluble ligand and, therefore, it can signal over a wide area. There are two possible different effects for the two forms. There are several signal pathways for TNF-α, which include activation of the NF-κB and apopotosis pathways. NF-κB pathways are related to pro-inflammation, proliferation and cell survival (see Fig 4).

Work done earlier reported that only abnormal cells were affected by TNF-α. Subsequently it was shown that normal cell lines can also be affected (Collins et al., 1986). Nevertheless, it has been suggested that TNF-α can differentiate between normal cells and abnormal cells and that it induces growth in the former and death in the latter (Vilcek et al., 1986).

TNF-α binds to two different receptors: TNF receptor 1 and TNF receptor 2 (TNFR1 and TNFR2). They are also referred to as CD120a, p55 and CD120b, p75 receptors, respectively. This p75 receptor is not the same as the p75 neurotrophin receptor ($p75^{NTR}$), although this also belongs to the TNFSF.

TNFR1 contains a death domain and is expressed in many cell types. TNFR2 is more restricted, and appears in immunoactive, endothelial and nerve cells (for references, see Aggarwal et al., 2012). The two types of receptors are both transmembrane and soluble receptors (sTNFR1, sTNFR2). It is believed that, in different ways, the receptors are related to two main pathways for TNF-α, where TNFR1 induces apoptosis and TNFR2 is responsible for the pro-inflammatory effects (Fig. 4). However, data are inconclusive, and both receptors might have both effects (Aggarwal et al., 2012). Experiments on TNF-α-deficient mice have shown that their immune system is damaged

and that these mice are vulnerable to infections (Pasparakis et al., 1996; Marino et al., 1997).

Diseases and treatments

TNF-α was originally discovered as an antitumour agent but is now known to be involved in many different diseases, including cancer. Examples of other groups of diseases where it is involved are neurological, cardiovascular, pulmonary, autoimmune and metabolic diseases. TNF-α is expressed in tumours and microglia cells, and it is produced in cells in failing hearts. It is also produced in various cells in diabetes, obesity, and in autoimmune diseases such as multiple sclerosis, arthritis, and Crohn's disease. Mutations of the TNF-α gene have been linked to, for example, cerebral infarction. The role of TNF-α in all these diseases is not fully understood (for references, see Aggarwal et al., 2012).

Anti TNF-α treatment is commonly used in the clinic for several diseases, mainly inflammatory, and is related to the use of either anti-TNF-α monoclonal antibodies or a soluble receptor that binds to TNF-α. Numerous adverse effects, principally related to higher risk of different infections and tumours, are related to these treatments (Aggarwal et al., 2012).

Pain

TNF-α is involved in chronic pain. For example, TNF-α is a known mediator of hyperalgesia (Watkins et al., 1994). TNF-α is important in both the pathogenesis of neuropathic pain as well as pain perception, acting via central mechanisms, such as shown in a study on the rat brain (Ignatowski et al., 1999). Wild type mice show elevated levels of pain compared with TNF-α knock-out ones (Yamashita et al., 2008). Interestingly, early pain reduction was ascribed to changes within the

brain rather than in the affected peripheral joints in response to anti-TNF treatment (Hess et al., 2011). It is unclear whether the nociceptive outcome is more dependent on the amount of the cytokine or on the activation/inhibition of their specific receptors (Andrade et al., 2011).

Tendons

There is some information concerning the expression and effects of TNF-α in relation to tendons. This is a brief review of the literature.

Expression and concentration

The concentration of TNF-α in encapsulating and invasive tenosynovium wrist extensor tendons in patients with rheumatoid arthritis undergoing tenosynovectomy is similar to the concentration of TNF-α in wrist synovium (Jain et al., 2001). Moreover, Hosaka and collaborates (2002) showed that 64% of the tenocytes of the inflamed equine superficial digital flexor tendon (SDFT) express TNF-α, but that normal SDTF tenocytes are mainly negative for TNF-α. They showed later that the level of TNF-α mRNA is 2.7 times higher in inflamed tendon tissue than that in normal tendon tissue (Hosaka et al., 2005). In this study, the authors also showed that TNFR1 is weakly immunoexpressed in inflamed SDFT, and that only few tenocytes of normal tendons cells are immunoreactive for TNFR1.

Low concentrations of TNF-α are found in human peritendinous tissue of both ruptured tendons and in contra-lateral, unaffected tendons (Ackermann et al., 2012).

Eliasson and collaborators (2009) found in a rat tendon rupture model that, during healing, loading of the Achilles tendons initially suppresses TNF-α gene expression.

TNF-α is expressed in human cultured tendon tenocytes derived from different tendons (hamstrings, patellar, finger, and Achilles tendons) in men and women 20 to 84 years old (John et al., 2010)

Effect

In the cell culture study mentioned earlier, it was shown that TNF-α decreases collagen type I expression and that it induces the expression of MMP-1 as well as IL-10 mRNA, and enhances IL-10 receptor 1 protein mRNA expression in tenocytes (John et al., 2010). In this study, it was also shown that TNF-α upregulates IL-1β, TNF-α and IL-6. Thus, it has effects on itself.

TNF-α depresses apoptosis of cultured tenocytes from quadriceps femoris muscle tendon taken from patients with osteoarthritis, but not in controls (biceps femoralis taken during ACL reconstructions) (Machner et al., 2003). In both groups, TNF-α reduces the expression of TNFR1 in tenocytes.

Hosaka and coworkers (2004) studied TNFR1 and TNFR2 expression in TNF-α-treated cultured tenocytes derived from histologically normal SDFTs and reported that tenocytes treated with TNF-α immunostained more intensely for TNFR1 than untreated tenocytes do. TNFR2 stained equally in the tenocytes treated with TNF-α and in the controls.

In a stress deprivation model on rat patellar tendon, it was seen that the absence of stress to the tendon increases the expression of TNF-α in the fibroblasts. The authors concluded that not only elevated stress (as seen in other studies), but also the absence of stress, can elevate the levels of TNF-α (Uchida et al., 2005).

In cultures of tenocytes derived from patients with chronic Achilles tendinopahty, TNF-α upregulates the gene expression for Toll-like receptor 2, MMP-1 and MMP-9 (de Mos et al., 2009).

Effect of treatment

TNF-α blockade decreases the concentration of MMP-1 in the tenosynovium in a patient with rheumatoid arthritis (Jain et al., 2002). In a non-randomised, non-controlled pilot study, athletes with chronic Achilles tendinopathy were treated with TNF-α blocker injections (Adalimumab) (Fredberg & Ostgaard, 2009). Tendon thickness, walking pain and pain at rest was evaluated at 1, 4 and 12 weeks. There was no significant effect on tendon thickness and walking pain.

One week after injection, pain at rest was, however, reduced from 4.3 to 3.0 and after 12 weeks to 3.6 (the latter was not statically significant) (Fredberg & Ostgaard, 2009).

Blocking of TNFR1 in rat supraspinatus tendon reduces TNF-α staining at 4 and 8 weeks but not at 2 weeks (Gulotta et al., 2011). This blockade results in greater ultimate loads at failure. No difference in the ultimate stress at failure was seen at any time point.

Information missing

Most of the results discussed above on the expression of TNF-α and its receptors are from studies on cell cultures, inflamed conditions, animals, or peritendinous tissue. It should be emphasized there is also no information on whether the tenocytes in the Achilles tendon exhibit TNF receptors or not. Although there is some information on TNF-α and tendons, there is no information on whether TNF-α is produced in human tendons *in vivo* or not.

Neurotrophins

General characteristics

The neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 and neurotrophin 4/5 (NT-3 and NT-4/5). In addition, NT-6 (Götz et al., 1994) and NT-7 (Nilsson et al., 1998; Lai et al., 1998) are found in fish. The neurotrophin receptors include the tyrosine kinase receptors TrkA, TrkB, and TrkC, and the neurotrophic receptor p75 ($p75^{NTR}$,

hereafter referred to as p75). For this thesis, I chose to study NGF, BDNF and the receptors TrkB and TrkA and p75. BDNF turned out to be the most interesting neurotrophin to study. The next section is some background information on the neurotrophins studied in this thesis.

The first neurotrophin discovered was NGF. The discovery was made by Rita Levi-Montalcini and Hamburger (1951) in studies on mouse sarcomas implanted in chick embryos. The group made extensive research on this growth factor and Levi-Montalcini was awarded the Nobel Prize in 1986 for the discovery. They showed that isolated nerve cells cannot survive without the presence of NGF, and that NGF was especially important in early growth and differentiation. Even back then, they described that NGF acted to stimulate excessive growth rather than functional activity and that this was detrimental rather than beneficial to the organism. They suggested that NGF could possibly be produced by any cells of mesenchymal type throughout the organism (Levi-Montalcini, 1965). The second neurotrophin indentified was BDNF, which was described by Barde and coworkers (1982).

Neurotrophins are synthesized as precursors (proneurotrophins) that are cleaved to mature, biologically active neurotrophins (Edwards et al., 1988; Lee et al., 2001). Even though the major source of neurotrophins is neurons, they are also produced by different cell types throughout the human body, not least inflammatory cells (Vega et al., 2003). BDNF is also produced in platelets (Yamamoto & Gurney, 1990) and in cells in peripheral tissues such as the kidney and the ovaries (Ernfors et al., 1990). Normal primary chick fibroblasts are a source of NGF (Young et al., 1975).

Receptors

There are two types of receptors for NGF and BDNF. The low affinity receptor p75 binds both neurotrophins with similar (low) affinity, whereas the high affinity tyrokinase receptors TrkA and TrkB bind to

NGF and BDNF, respectively, with high affinity (Barde, 1990). The p75 receptor is a member of the tumour necrosis receptor superfamily.

The receptors give rise to different functions. The p75 receptor on its own has two different main functions. On the one hand, it promotes cell survival and nerve regeneration and, on the other, it is pro-apoptotic (Dechant & Barde, 1997). Trk receptors can promote the survival of neurons, and can also be involved in modulating differentiation and apoptosis events (for a review, see Patapoutian and Reichardt, 2001).

It has been shown that the function of the p75 receptor is upregulated during conditions of stress. In one study, the authors argued that its functions peak at levels of mild stress (Bradshaw et al., 2012).

Effect of physical exercise

The blood concentration of BDNF is influenced by physical exercise. Some studies show that there is a transient increase in plasma or serum BDNF concentration following an acute bout of aerobic exercise. Others studies found, however, that trained men have lower BDNF blood concentrations. The effect of different types of exercise on the BDNF blood concentration is controversial, but it seems as if the type, mode and intensity of physical exercise are important for how the BDNF blood concentration is altered (for a systematic review, see Knaepen et al., 2010).

Pain

Both NGF and BDNF are known pain mediators. Together they have pain-modulating effects on both peripheral and central levels (Bennett, 2001; Merighi et al., 2008; Nicol & Vasko, 2007; Woolf et al., 1994).

For example, BDNF is increased in the rat dorsal horn following peripheral inflammation (Garraway et al., 2003).

It has been proposed that the TrkA receptors promote the survival of neurons (Patapoutian & Reichardt, 2001) and are expressed on nociceptors (Thompson et al., 1999). Humans that have mutations in the TrkA gene lose pain sensation (Indo et al., 1996). Investigation of NGF in the treatment of chronic pain conditions is by Phase III studies proved to have not only pain-reducing effects, but also adverse effects (Lane et al., 2010).

Diseases

It has been suggested that neurotrophins are important in several diseases, e.g., Alzheimer's disease (Allen et al., 2011), inflammatory diseases of the skin (Raap & Kapp, 2010) and depression (Neto et al., 2011). BDNF plasma levels are associated with multiple risk factors for the metabolic syndrome and cardiovascular function (Golden et al., 2010). NGF might be involved in chronic arthritis (Aloe et al., 1992). BDNF is also related to inflammation-modifying effects. Inflammatory cells in the intestine do, e.g., show NGF and BDNF immunoexpression in ulcerative colitis patients (Johansson et al 2008). Nevertheless, in the synovial tissue of the knee joint of rheumatoid arthritis patients, there is no correlation between the concentration of BDNF in tissue homogenate and the degree of inflammation in that tissue (Grimsholm et al., 2008). Interestingly, the blood concentration of BDNF in those patients decreases in response to anti-TNF treatment. This shows that there is a relationship between the two systems that are studied in this thesis, the TNF-α system and the neurotrophin system.

Neurotrophins and tendons — information missing

Hitherto, almost nothing had been known about neurotrophins in tendons. In one study it was shown that BDNF expression in rat Achilles tendon rupture is downregulated if the limb is immobilised compared with if the limb is mobilised (Bring et al., 2010). No information is available on whether the neurotrophins BDNF and NGF, and their receptors, are expressed in human tendons or not. This is a drawback, because the neurotrophins have both proliferative/tropic and apoptosis-modulating effects. Therefore, the neurotrophin system was analysed in this thesis.

Interaction effects

TNF-α and neurotrophins have common effects in different tissues. They also have interaction effects on each other, as well as with other substances. An interaction effect means that two substances have additive, synergistic or antagonistic effects.

It is known that NGF and TNF-α have interaction effects (Takei & Laskey, 2008). For example, NGF is involved in TNF-α upregulation (Manni & Aloe, 1998). In contrast, cytokines stimulate NGF production (Safieh-Garabedian et al., 1995) and TNF-α can stimulate BDNF secretion in human peripheral blood monocytes (Schulte-Herbruggen et al., 2005).

With regard to the interaction effects with other substances, it is known that NGF can release SP from the spinal cord (Malcangio et al., 1997). TNF-α also has interaction effects with SP. For example, SP leads to TNF-α mRNA expression in human dental fibroblasts (Yamaguchi et al., 2008).

Physical Activity

As physical activity (PA) was measured in *Study IV*, a brief introduction to it is given here.

PA is defined as any bodily movement produced by skeletal muscles that results in energy expenditure (EE) (Caspersen et al., 1985). However, PA is much more complicated than EE because the different patterns (combinations of frequency, duration and intensity) of PA can result in the same EE.

In order to determine the importance of PA in health and disease, it is essential to measure PA. Measurement methods include: behavioural observations (e.g. video analysis), self-reports (questionnaires and activity diaries), physiological markers (e.g. heart rate monitors), motion sensors (pedometers, accelerometers) and indirect calorimetry. In addition, the global positioning system (GPS) is used. All the methods can be used alone or in combination (Plasqui & Westerterp, 2007).

One technique that is commonly used is double-labelled water (DLW) which is a form of indirect calorimetry and is the gold standard for measurement of EE in everyday life ("free-living" = not controlled/ not in laboratory). The method is expensive and is therefore not used in large scale studies.

Accelerometers are devices used to monitor PA patterns (intensity, duration and frequency) objectively, and can also give an estimation of EE (Westerterp, 2009).

Questionnaires, or diaries, are widely used in large-scale studies and can be used in different set-ups, recordings spanning from hours to several years. Questionnaires are cheap and can give contextual information as well as an estimation of EE in free living (Westerterp, 2009). The Past Year Total Physical Activity Questionnaire (PYTPAQ) was used in this thesis. This questionnaire has been validated against accelerometers and physical activity logs of 154 healthy Canadian men and women aged 35–65 years (Friedenreich et al., 2006).

In this thesis (*Study IV*) total physical activity (TPA) was studied. This parameter is a combination of occupational, transport, household and recreation physical activity. As such, TPA corresponds to nearly all PA in free-living.

In Summary

The background to tissue changes in chronic mid-portion Achilles tendinosis is poorly understood, so is the origin of the pain. It is known that both TNF-α and neurotrophins may be associated with apoptosis, growth, and remodelling, and that both factors have a relationship to pain. They are expressed in various tissues in the human body, but little is known about their relationship with tendons for these substances. It is not known whether these systems are expressed in the human Achilles tendon or not. There is also, at least for neurotrophins, a relationship to PA.

The rationale for this thesis was based on research that proposes: (1) tendons are active structures and respond to both load and immobilisation. (2) Marked tissue changes occur, and (3) the tendon metabolism is changed in Achilles tendinosis. (4) TNF-α and neurotrophins are substances that have homeostasis-controlling and pain-signalling functions in other tissues and in other diseases. (5) Pain is sensitised at a local level in Achilles tendinopathy. (6) Studying the expression of TNF-α and neurotrophins and their receptors is essential to determining whether they are also important in the Achilles tendon and in Achilles tendinosis.

AIMS

The general aim of this thesis was to investigate the neurotrophin and TNF-α systems in Achilles tendinosis and in the normal Achilles tendon. I have chosen to study the neurotrophins NGF and BDNF, in particular BDNF, and their receptors TrkA, TrkB and p75, as well as TNF-α and its two receptors TNFR1 and TNFR2. More specifically, the main issues to be answered were whether:

- TNF-α and the neurotrophins are produced in the tenocytes in the Achilles tendon in subjects with tendinosis and in those with normal tendons (controls).

- The receptors for these substances are expressed in the tenocytes of the Achilles tendon in subjects with tendinosis tendons and in controls.

- The blood concentrations of BDNF and sTNFR1 are increased in subjects with tendinosis compared with in controls.

- Physical activity in subjects with tendinosis and in controls is associated with the blood concentrations of BDNF and sTNFR1.

PART II

MATERIALS AND METHODS

This part describes briefly the methods used to answer questions related to the aims of this thesis

Subjects

This thesis includes both human subjects with chronic painful mid-portion Achilles tendinosis and human control subjects with pain-free, normal Achilles tendons. Achilles tendon tissue and blood samples were taken from both groups, and analysed.

Table 2. Subjects' mean age

	All Studies		**Study I**		**Study II**		**Study III**		**Study IV**	
	p	c	p	c	p	c	p	c	p	c
m/f	18/16	12/13	9/6	2/3	1/1	0/2	13/10	2/4	14/8	10/9
Age, y (SD)	49 (12)	44 (12)	43 (11)	45 (3)	41 (16)	47 (0)	50 (10)	41 (10)	50 (13)	45 (13)

p = patient, c = control, m = male, f = female

Tendinosis

In total, 18 men and 16 women with chronic mid-portion Achilles tendinosis were included in this thesis. We wanted to include both men and women of a wide age range in order to be able to generalize the results to the whole population who develop tendinosis. Nevertheless, tendinosis is seldom seen in subjects younger than 25 years of age. All the subjects in this group were active recreationally or did sports. Hereafter, these subjects, or patients, are referred to as subjects with tendinosis, or simply patients, and when referring to tendons from this

group, the term "tendinosis tendons" is used. For more details on individual characteristics, see Table 2.

An experienced orthopaedic surgeon performed the clinical examination and diagnosed tendinopathy, which was then confirmed by examination with ultrasonography (localized widening of the tendon, irregular structure and focal hypoechoic areas) (Acuson Sequoia 512 with 8–13 MHz linear array transducer). Additionally, colour Doppler ultrasonography was used to detect whether blood flow was high inside or outside the Achilles mid-portion.

Controls

A total of 12 men and 13 women that were active recreationally or in sports were included as controls (hereafter referred to as control subjects or controls, see Tables 2 and 3 for more details). These subjects had pain-free and structurally normal Achilles tendons, as verified by clinical examination and ultrasonography. The subjects in the control group were selected to match the tendinosis group in age and gender. When referring to tendons from this group, the term "control tendons" will be used.

Table 3. Study distribution

Patient	Study				Contol	Study			
1	I			IV	1				IV
2				IV	2				IV
3			III		3				IV
4			III	IV	4				IV
5	I		III	IV	5				IV
6				IV	6				IV
7			III	IV	7				IV
8	I				8				IV
9	I		III		9				IV
10			III	IV	10				IV
11			III	IV	11				IV
12	I			IV	12				IV
13			III		13				IV
14				IV	14				IV
15			III	IV	15				IV
16	I			IV	16				IV
17			III	IV	17				IV
18			III	IV	18				IV
19			III	IV	19				IV
20	I		III		20			III	
21	I		III	IV	21	I	II	III	
22	I	II	III		22	I		III	
23	I	II	III		23	I	II	III	
24	I		III	IV	24	I		III	
25	I		III	IV	25	I		III	
26			III	IV					
27	I			IV					
28	I								
29				IV					
30			III	IV					
31			III						
32			III						
33			III						
34	I								

Recruitment

All subjects with tendinosis were patients at the Sport Medicine Unit in Umeå, Sweden. They were recruited consecutively during 2007. Control subjects were recruited from the general population during 2002 and 2003 (biopsies) and 2007 (blood samples).

The inclusion and exclusion criteria for patients and controls are listed in Table 4.

Table 4. Inclusion and exlusion criteria

	Inclusion criteria	Exclusion criteria
p	• Age 18 or older. • More than 3 months of Achilles tendon pain. • Clinical symptoms: tender and thickened mid-portion Achilles tendon. • Structural tendon abnormalities, verified by ultrasonography. • High tendon blood flow, verified by colour Doppler.	• Acute and chronic inflammatory disease. • Disease or injury affecting the lower extremities other than Achilles tendinopathy. • Smoking habits. • Achilles paratenonitis. • Partial or complete Achilles tendon rupture. • Bursitis. • Haglund deformity.
c	• Age 18 or older. • Normal Achilles tendon structure, verified by ultrasonography. • Normal tendon blood flow, verified by colour Doppler.	• The same exclusion criteria as the subjects with tendinosis, plus: • Past or present Achilles tendon pain.

p = patient, c = control

Physical Activity Level

In *Study IV*, the level of PA was measured in subjects with tendinosis and controls using The Past Year Total Physical Activity Questionnaire (PYTPAQ). For our study, we used the parameter total physical activity (TPA). This parameter is the combination of occupational, transport, household and recreation PA. PA is estimated in metabolic equivalent hours per week (MET-h/week) (Ainsworth et al., 2000).

Sampling, Fixation and Sectioning

Biopsies from tendinosis tendons were taken during surgery performed by an experienced orthopaedic surgeon specialised in Achilles tendinopathy. The biopsies were taken from the lateral side of the ventral part of the mid-portion of the Achilles tendon (2-6 cm cranial to the insertion onto the calcaneal bone). For ethical reasons biopsies from the controls were taken via mini-incision in the skin from the dorsal part of the tendon. In both groups, the samples were approximately 2 mm wide and 1–5 mm long. However, the control biopsies were generally smaller than the tendinosis biopsies.

The majority of the tissue samples were chemically fixed before freezing. Only a few were directly frozen at −80°C after the sampling process, i.e. processed chemically unfixed. Chemical fixation was done by immersion overnight at 4°C in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. Then, the samples were thoroughly washed overnight in Tyrode's solution containing 10% sucrose. They were then mounted and frozen in propane, chilled with liquid nitrogen, and stored at −80°C until sectioning. Theses samples were used for *Study I–III*. As described earlier, some samples were processed chemically unfixed. They were not mounted but were frozen

immediately in liquid nitrogen for later analysis with enzyme-linked immunosorbent assay (ELISA).

Before being processed for morphology or immunohistochemistry, samples were cut using a cryostat (Leica Microsystem CM 3000, Heidelberg, Germany). Consecutive series of 7 μm thick sections were produced. The sections were mounted on slides pre-coated with crome-alun gelatin, left to dry, and then stored at −20°C. For *in situ* hybridization, series of 10 μm thick cryosections were cut using the same cryostat (with a knife washed in 70 % EtOH in DEPC-H_2O), and mounted on slides (nr. 041300, Menzel-Gläser, Braunschweig, Germany).

Morphology Staining

Samples were stained with haematoxylin and eosin (H&E) in order to reveal general tendon morphology. For this purpose, we took parallel sections to those undergoing immunohistochemistry staining or *in situ* hybridisation. Haematoxylin stains the basophilic structures, such as cell nuclei and nucleic acid, blue. Eosin dye stains the cytoplasmic content and extracellular proteins various shades of red.

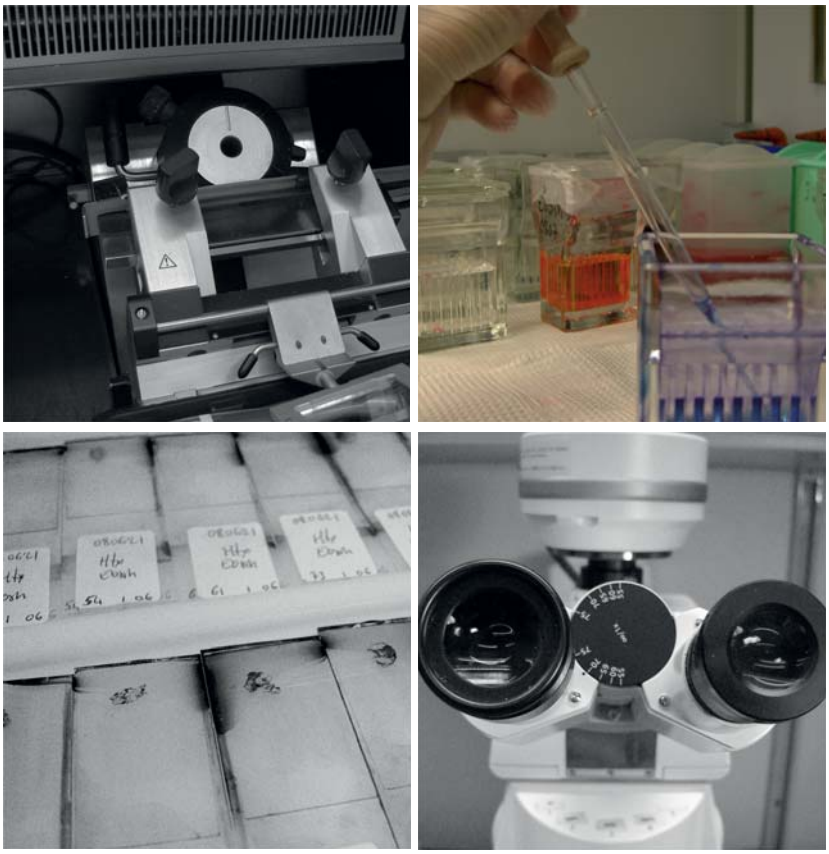

Figure 5. From sectioning to microscopy

Immunohistochemistry

Primary antibodies

Several polyclonal antibodies were used as primary antibodies for the immunohistochemical staining. For detailed information on these antibodies see Table 5.

Immunofluorescence

Immunofluorescence was done in order to detect BDNF, NGF, TrkA, TrkB, p75, TNF-α, TNFR1, TNFR2, Ki67 and Caspase-3 (*Studies I-III*). A standardised protocol was used, including pretreatment with potassium permanganate — a procedure that enhances specific immunofluorescence reactions (Hansson & Forsgren, 1995). This pretreatment was not done for sections to be processed for p75, TrkA and TrkB. Then the samples were rinsed in PBS, incubated in Triton detergent solution, then incubated in normal serum diluted in PBS supplemented with 0.1 % bovine serum albumin (BSA), incubated in primary antibody, rinsed in PBS (PBS/BSA), then incubated again with normal serum and incubated with secondary antibodies (TRITC/SAR or FITC/DAG). BSA was omitted from all dilutions (normal serum, primary and secondary antibodies) if the primary antibody was cultivated in goats (i.e. antibodies against TNF-α, TNFR1, TNFR2, and Caspase-3). Finally, sections were mounted on slides with a microscopy mounting medium (H-1000, Vector Laborations, Inc, Burlingame, Ca, USA).

Table 5. Details of primary antibodies and immunohistochemical staining procedures

Antigen	Code	Manufacturer	Raised in	Diluton	PBS/BSA	Nomal serum	Secondary antibody	Potassium permanganate	Study
BDNF	(N-20): sc-546	Santa Cruz Biotechnology, Inc., CA, USA	rabbit	1:100	PBS, BSA	swine	TRITC/SAR	yes	I, II
NGF	(H-20): sc-548	Santa Cruz Biotechnology, Inc., CA, USA	rabbit	1:200	PBS, BSA	swine	TRITC/SAR	yes	I
TrkA	(763): sc-118	Santa Cruz Biotechnology, Inc., CA, USA	rabbit	1:50	PBS, BSA	swine	TRITC/SAR	no	I
TrkB	(794): sc-12	Santa Cruz Biotechnology, Inc., CA, USA	rabbit	1:50	PBS, BSA	swine	TRITC/SAR	no	I
p75	N 3908	Sigma, Saint Louis, USA	rabbit	1:50	PBS, BSA	swine	TRITC/SAR	no	I
TNF-α	ab6671	Abcam, Cambridge, UK	rabbit	1:25	PBS, BSA	swine	TRITC/SAR	yes	III
TNF-α	(N-19): sc-1350	Santa Cruz Biotechnology, Inc., CA, USA	goat	1:50	PBS	donkey	FITC/DAG	yes	III
TNFR1	sc-1070	Santa Cruz Biotechnology, Inc., CA, USA	goat	1:100	PBS	donkey	FITC/DAG	yes	III
TNFR2	sc-1074	Santa Cruz Biotechnology, Inc., CA, USA	goat	1:100	PBS	donkey	FITC/DAG	yes	III
Ki-67	Ab9260	Millipore, CA, USA	rabbit	1:25	PBS, BSA	swine	TRITC/SAR	yes	III
Caspase-3	sc-1225	Santa Cruz Biotechnology, Inc., CA, USA	goat	1:50	PBS	donkey	FITC/DAG	yes	III

Tetramethyl Rhodamine-Thiocyanate-conjugated (TRITC) swine anti-rabbit (SAR) IgG (code: R0156, Dako, Denmark)
Fluorescein Iso-Thiocyanate-conjugated (FITC) donkey anti-goat (DAG) IgG (code: 705-095-147, Jackson ImmunoResearch Laboratories, Inc., PA, USA)

PBS = Phosphate buffered saline, BSA = Bovine serum albumin

Control staining

We did three different types of control stainings in order verify the specificity of the reactions:

- Preabsorption using the antigen for which the antibody had been designed. This was done by letting the primary antibody be preabsorbed with the antigen before putting it on the section. The preabsorbed antibodies were used in parallel with non-preabsorbed antibodies for ordinary immunofluorescense staining.
- Omission of the primary antibody (substituted with PBS or PBS/BSA), in order to rule out any cross-reactions with the secondary antibody.
- Staining of specimens of reference tissue, such as human colonic tissue and rheumatoid arthritis tissue, for which results are well established.

TUNEL Staining

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining (Promega Corporation, Madison, Wisc., USA; code: G3250) was used on selected samples in *Study III*. The procedures were performed according to the manufacturer's instructions. This system is designed specifically to detect apoptotic cells in a cell population and measures nuclear DNA fragmentation. Positive and negative controls were used. A mounting medium with DAPI (H-1500, Vector Laborations, Inc, Burlingame, Ca, USA) was used to visualize all the nuclei.

In Situ Hybridization

In situ hybridization was used in *studies II–III*. Digoxigenin (DIG)-hyperlabelled oligonucleotide probes (ssDNA) were used to detect human BDNF, TNFR1 and TNF-α mRNA. For specifications, see Table 6. *In situ* hybridization was done according to an established protocol (Panoskaltsis-Mortari & Bucy, 1995), using an alkaline phosphatase (AP)-labelled anti-DIG antibody for detection, with a few modifications (Danielson et al., 2007). The corresponding sense DIG-hyperlabelled ssDNA probes were used as negative controls and a β-actin probe (see Table 6 for details) was used as a positive control. All sections were evaluated by two observers. For further details of the procedures, see the original papers of *Studies II–III*.

Table 6. Details of anti-sense probes used for *in situ* hybridization

Probe	Code	Sequence
BDNF	GD 1259-OP	AGTTCCAGTGCCTTTTGTCTATGCCCCTGCAGCC TTCCTTGGTGTAACCC
TNF-α	GD 1178-OP	GCCCTCTGATGGCACCACCAGCTGGTTATCTCTC AGCTCCACGCCATT
TNFR1	GD 1001-DS	TCCTCGATGTCCTCCAGGCAGCCCAGCAGGTCC ATGTCGCGGAGCACG
β-actin	GD 500-OP	GCCGATCCACACGGAGTACTTGCGCTCAGGAGG AGCAATGATCTTGAT

Manufacturer for the probes was Gene Detect, New Zealand. Dilution: 50 ng in 15 µl hyrbidization solution

Microscopy and Photography

The sections were evaluated using a Zeiss Axioscope 2 plus microscope, equipped with epifluorescence optics. This microscope was used to evaluate sections of the tissue samples after staining with H&E, immunofluoresence and *in situ* hybridisation. In addition, a confocal microscope (Leica TSP-2; Leica, Heidelberg, Germany) and LEICA LCS software (Leica, San José, CA, USA) were used when analysing TUNEL staining in *Study III*. Photos were taken from representative sections with a digital camera mounted on the microscope (Olympus DP70), or with the confocal microscope system (Leica) and were cropped and adjusted for contrast and brightness in Adobe Photoshop, versions 6.0 to 11.0 (Adobe Systems Inc., San Jose, CA, USA). Some images were converted into grayscale images.

ELISA

Blood collection

For *Study IV*, blood was collected from 22 patients and 19 controls. The blood was collected in a 4 mL serum separating tube (Vacuette 454067, Greiner Bio-One GmbH, Kremsmünster, Austria) via antecubital venopuncture between 07:00 and 09:00 a.m. following a 10–12 h overnight fast. The blood was allowed to clot at room temperature for 30 minutes, centrifuged at 1300 *g* for 10 minutes and then stored in aliquots at −80°C. Samples were only frozen and thawed once.

Table 7. ELISA assays

Antigen	Code	Manufacturer	Detection limit (pg/mL)	Study
BDNF	CYT306	Chemicon, Temecula, California, USA	7.8	IV
sTNFR1	DRT100	R&D Systems, Minneapolis, USA	0.8	IV
TNF-α	EH3TNFA	Pierce Biotechnology, Rockford, USA	2.0	III

Tissue homogenisation

For *Study III*, Achilles tendon samples from 6 Achilles tendinosis patients were collected and analysed by ELISA. The samples were initially homogenized in a prepared buffer solution (for details, see *Study III*). Tendon tissue and buffer were mixed in a 1:20 ratio and this was done on ice. Immediately after homogenization, the samples were centrifuged at +4°C, 13,000 *g*, for 15 minutes. The remaining supernatant was then transferred to a new eppendorf tube and stored at −80°C.

Methods

Commercially available ELISA kits were used to measure levels of BDNF and sTNFR1 in blood from tendinosis patients and controls and levels of TNF-α in tendon tissue from tendinosis patients. For details see Table 7. The assays were performed in accordance with the suppliers' instructions. The levels of BDNF and sTNFR1 are expressed as ng/mL. The levels of TNF-α were then converted to take into account the weight of the tissue samples and expressed as pg/mg of tissue.

Statistics

Statistical calculations were made in *Study III* when comparing semiquantitatively the intensities of the immunoreaction staining in samples from tendinosis subjects and in those from the controls, and in *Study IV,* for correlations and group-difference analyses of blood levels.

In general, the data in *Studies III-IV* were not normally distributed. This conclusion was based on graphic analyses including histograms, Q-Q plots, box plots and supported by skewness data and the Shaprio Wilk test. Logarithmic transformation of the data did not change the distribution to normal so, therefore, the data were analysed without transformation. Non-parametric tests were used for non-parametric data.

Between-group differences

Group differences (tendinosis vs. controls) were evaluated using the Mann–Whitney U-test for non-parametric data in *Studies III-IV*. The differences between the 75th and 25th percentiles are presented as the interquartile range (IQR). The independent t-test was used for parametric data in *Study IV (*subjects' characteristics). The data analysed in *Study IV* was the entire set for tendinosis patients and controls and the analysis was then repeated for men and women separately.

Correlation analyses

Correlation was analysed in *Study IV*. Correlations between BDNF, sTNFR1, BMI and TPA were defined with Spearman's rho (r), first for the entire data set and then repeated for men and women separately.

Inter-observer agreement

In *Study III*, the interobserver agreement concerning the staining intensity of TNF-α, TNFR1, and TNFR2 in tenocytes was evaluated using linearly weighted kappa.

Significance level

All statistical calculations were made using SPSS versions 11.0 to 20.0 for Macintosh. $P < 0.05$ was considered statistically significant.

Ethical Considerations

The studies in this thesis were approved by the Committee of Ethics at the Faculty of Medicine, Umeå University, Sweden (prior to 2004) and by the Regional Ethical Review Board in Umeå, Sweden (04-157 M). All experiments conformed to the principles expressed in the latest approved revision of the Declaration of Helsinki at the onset of the projects.

PART III

RESULTS

This part presents an overview of *StudiesI-IV*. A short introduction to each study is given and the results are discussed briefly. The general morphological findings from the first three studies are summarised initially. The results are discussed in greater depth in PART IV. The immunoexpression results are summarised in Table 8.

General Morphology

H&E stain was used to reveal tissue morphology in *Studies I-III*. Hypercellularity and hypervascularity were frequently noted in the tendinosis specimens, i.e. generally, there were more cells (tenocytes) and blood vessels than in the control specimens (Fig. 6). There was heterogeneity in tenocyte morphology within each specimen. This heterogeneity was noted in both groups, but to a greater extent in the tendinosis group. Some areas of the tendinosis specimens contained rounded, widened, or wavy tenocytes. In other areas, the tenocytes showed a more regular, slender and spindle-shaped form, which is characteristic of normal tenocytes. Some areas also contained fewer cells than normally seen — most likely due to apoptosis and necrosis. Nerve fascicles were occasionally seen in the loose peritendinous connective tissue in both tendinosis and control specimens. All these observations are consistent with previous findings for Achilles tendinosis (Bjur et al., 2005; Movin et al., 1997; Åström & Rausing, 1995).

Inflammatory cell infiltrates were not seen in the tendinosis samples. This finding is consistent with those of previous morphological studies which show that inflammation is not obvious in chronic Achilles tendinopathy (Åström & Rausing, 1995). Hypercellularity, hypervascularity and apoptosis might be signs of the tissue remodelling that occurs in chronic Achilles tendinosis.

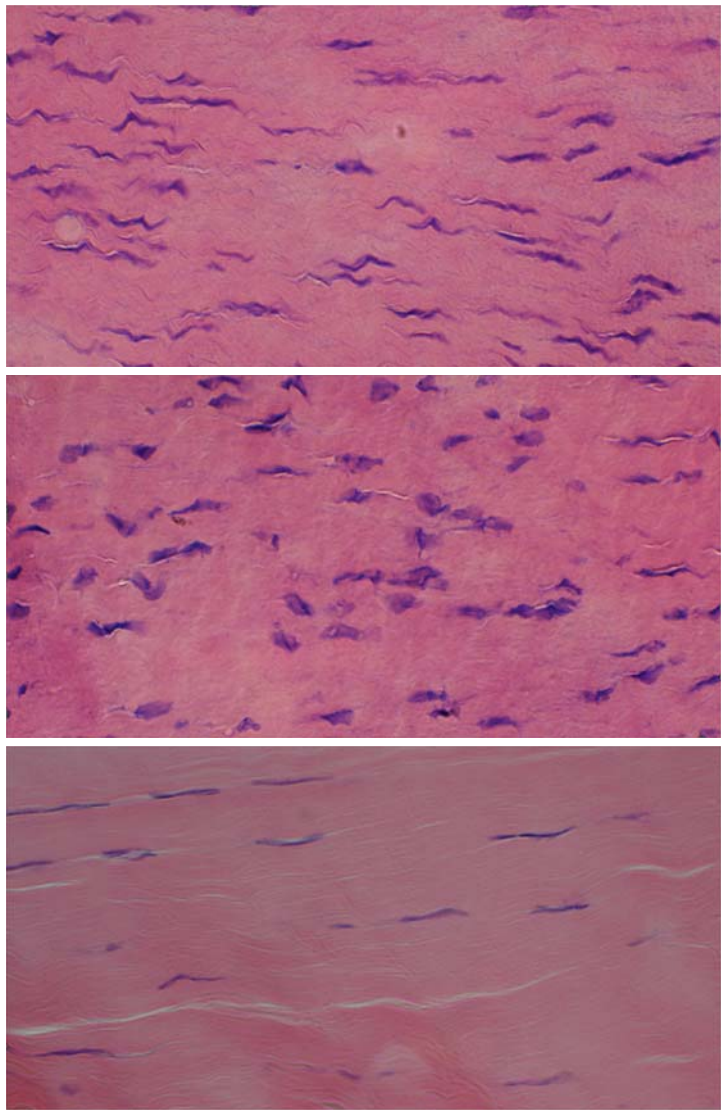

Figure 6. H&E stain of sections of tendinosis (top and middle) and normal (bottom) Achilles tendons. The tenocytes of the normal tendon are slender. In the tendinosis tendon they are rounded/wavy and more abundant.

Study I

Neurotrophins are mainly produced in neurons and are important for the development and maintenance of the central and peripheral nervous systems. They are also produced in other cell types, especially inflammatory cells. They have trophic/modulatory effets. Furthermore, neurotrophins are of importance in pain-signalling and also have apoptotic and proliferative effects via the p75 receptor. In this project, we investigated whether or not there is a neurotrophin system in the human Achilles tendon, as seen morphologically. For this purpose, samples from 15 subjects with tendinosis and from 5 controls, were evaluated. The samples were processed with immunohistochemistry, and analysed particularly for the expression patterns of the tenocytes. Blood vessel walls and nerve structures were also evaluated.

This study revealed that the tenocytes of the Achilles tendon are a possible source of NGF and BDNF production and that the p75 receptor is expressed in the tenocytes. The latter finding makes it possible for the neurotrophins to act on the tendon cells themselves or on other cells located nearby, in autocrine and paracrine fashions. The theory of possible neurotrophin production is based on the findings that the tenocytes of both tendinosis and control tendons immunoreacted strongly to NGF and BDNF. With respect to these reactions, there was heterogeneity within and between sections. The NGF and BDNF immunoreactions showed a punctuate appearance. There was a weak tendency for stronger reactions in tendinosis tendons than in the controls for BDNF, but not for NGF. BDNF, NGF and p75 immunoreactions were seen to some extent in blood vessel walls. The receptor p75, which is the common, low-affinity receptor to which both BDNF and NGF bind, was the only receptor that was detected in the tenocytes. Hence, TrkA and TrkB

immunoreactions were not seen in the tenocytes in any sample. BDNF, p75 and to some extent TrkB immunoreactions, were observed in nerve fascicles.

The findings of the expression of neurotrophins and the p75 receptor in tenocytes of the Achilles tendon make possible functions relating to apoptotic and trophic/modulatory effects in this tissue. Thus, it is possible that these substances could contribute to hyperceullarity and apoptosis — two cardinal features of tendinosis (Flodgren et al., 2005; Khan et al., 1999; Lian et al., 2007).

Study II

BDNF was considered to be the most relevant neurotrophin for further studies because it has well-known relationships with physical activity patterns and the TNF-α system (analysed in *Studies III–IV*). Based on the results of *Study I*, the aim of the present study was to prove that BDNF is produced by the tenocytes. It can be argued that the immunohistochemical detection of a substance is related to uptake, i.e., that BDNF could be absorbed from the blood, as suggested for BDNF in other peripheral tissues (Knaepen et al., 2010). Therefore, by using *in situ* hybridization, we investigated whether BDNF was expressed at the mRNA level or not.

A large number of the tenocytes in tendinosis tendons and control tendons stained strongly for BDNF mRNA. Specific mRNA reactions were black and of a granular type. The reactions were similar for slender tenocytes of normal appearance and for wavy and thickened tenocytes in the tendinosis samples. Reactions were also seen in some tenocytes of control tendons.

The results give additional proof to the theory from the *Study I* that BDNF is produced by the tenocytes in the Achilles tendon. Also in this study, marked heterogeneity was observed in the reactions, i.e.,

some of the tenocytes in both tendinosis and in control tendons did not show BDNF mRNA reactions.

BDNF can be added to the list of substances that can be produced by tenocytes. Interaction effects with other substances are likely to occur. Examples of such substances are TNF-α (*Study III*), and various neurotransmitters that our lab has previously suggested are produced by the tenocytes (Bjur et al., 2008a, 2008b; Scott et al., 2008).

Study III

Nothing is known about the TNF-α system in human Achilles tendons *in vivo*. TNF-α has effects in several diseases and there is also a relationship between this system and neurotrophins. Therefore, in this study, we sought to examine the expression pattern of TNF-α and the TNF receptors (TNFR1 and TNFR2) in the human Achilles tendon. We included a total of 23 subjects with Achilles tendinosis, and 6 controls. The same techniques were used as those used in the two first studies (immunohistochemistry and in situ hybridization). In this study, we also homogenized a small number of samples to be analysed with ELISA. Unfixed tissue samples were analysed for TNF-α with this method. In parallel with immunohistochemistry, *in situ* hybridization was done for TNF-α and TNFR1.

TNFR1 is known for its apoptotic effects. In order to further explore the aspects of apoptosis in the Achilles specimens, TUNEL staining and Caspase-3 immunofluorescence staining were done. The proliferative marker Ki-76 was also stained with immunohistochemistry.

The immunoreaction patterns and overall immunoreaction intensity in the tenocytes were assessed. Two investigators independently rated the overall intensity of the tenocyte

immunoreactions of TNF-α and TNF receptors in each section using a five-point rating scale (0 = no staining, 1 = barely detectable overall staining, 2 = clearly detectable but not strong overall staining intensity, 3 = strong overall staining intensity, and 4 = very strong overall staining intensity). When making these evaluations, the reaction within the section of one specimen was judged in relation to those that were seen within the sections of the other specimens, i.e. relative scoring was done. The average of the two investigators' scores was used to give an estimation of the overall reaction pattern.

On the whole, strong tenocyte immunoreactions were detected in the tenocytes for TNF-α, TNFR1 and TNFR2 (Fig. 7). But, the widened/rounded tenocytes exhibited the most intense immunoreactions. The receptor immunoreactions were seen both intracellularly and in the cell membrane. The immunoreactions intensities are presented below. Here, the IQR is presented as a range (i.e. the difference between the 75th and 25th percentiles) in contrast to how the IQR was presented in the original paper.

TNFR1 immunoreactions were significantly stronger in tendinosis (median = 3.0, IQR 1.0) than in the control (median = 1.5, IQR 1.4, P = 0.018) groups. There was no difference between the TNF-α tenocyte immunoreaction intensity of the tendinosis (median = 2.0, IQR 1.3) and that of the control (median = 1.75, IQR 1.4, P = 0.37) groups. Similarly, there was no difference between the intensity of the tenocyte TNFR2 immunoreaction of the tendinosis (median = 3.5, IQR 1.8) and that of the control (median = 2.5, IQR 1.6, P = 0.11) groups. Immunoreactions for TNFR2 were seen in the blood vessel walls; this was not the case for the other antigens examined. On the whole, the tenocytes were the cell type in which the reactions dominated for all three antigens.

Apoptosis and proliferation are cardinal features in late stage tendinosis and, therefore, are interesting aspects to consider. In this study, only some of the tenocytes were positive for the apoptosis markers TUNEL staining and Caspase-3. Some tenocytes were positive for the proliferative marker Ki-67. In contrast, TNFR1 was detected throughout the specimens.

The reactions for Caspase-3, TUNEL-staining and Ki-67 were seen to a larger extent in tendinosis tendons than in the control tendons. The existence of more apoptotic and proliferative events in tendinosis than in normal tendons is expected. The immunohistochemical findings in this study suggest that the TNF-α system is involved in both types of events in Achilles tendinosis.

Furthermore, mRNA expression was evaluated for TNF-α and one of the receptors, TNFR1. TNFR1 was chosen in preference to TNFR2 because TNFR1 showed more variability in the overall magnitude of immunoreactions between samples and because it is related to apoptosis. Tenocytes in biopsies from both tendinosis and control tendons showed marked TNF-α mRNA reactions. The intensity of the reactions varied from tenocyte to tenocyte, and both wavy/rounded and slender tenocytes expressed TNF-α mRNA. TNFR1 mRNA was also detected in tenocytes in biopsies from both tendinosis and control tendons.

The detection of TNF-α mRNA gives further evidence to the hypothesis that TNF-α is produced by the tenocytes. The results on TNFR1 mRNA and TNFR1 immunoreactions also indicate that TNF-α produces effects in the tendon tissue, and that the cells that are mainly influenced are the tenocytes. Additional evidence of TNF-α production in the Achilles tendon was the detection of TNF-α as seen in analysis of homogenised Achilles tendon tissue from subjects with tendinosis (the median concentration was 0.268, IQR 0.115, pg/mg tissue).

We have here shown that there is a TNF-α system in Achilles tendons. The situation is similar to that for BDNF (*Studies I-II*) in that TNF-α and BDNF are detectable both at the protein and mRNA levels and that there are receptor expressions for both substances. The immunoexpression of TNFR2 in the blood vessels is interesting because TNFR2 enhances angiogenesis in conditions with low oxygen levels (Luo et al., 2006). A summary of the results for *studies I-III* are shown in Table 8.

Table 8. Summary of results *(Studies I-III)*

Compound	Controls	Tendinosis	mRNA
BDNF	++++	++++ *	yes
NGF	++++	++++	NA
TrkA	-	-	NA
TrkB	-	-	NA
p75	+++	+++	NA
TNF-α	++++	++++	yes
TNFR1	+++	++++	yes
TNFR2	++++	++++	NA
Ki-67	+	+	NA
Caspase-3	+	++	NA
TUNEL	+	++	NA

This table show the magnitude of expression in tenocyte as analysed with immunohistochemistry for ligands and their receptors, and concerning TUNEL staining, in human subjects with Achilles tendinosis, and in controls without pain and structural changes in their Achilles tendon. For TNF-α, TNFR1 and TNFR2, quantifications of the immunoreactions staining intensity were conducted in *Study III*. Presence of mRNA expressions is indicated with yes or no. Note that this scale is not the same as the scale used in *Study III*. This scale was set up retrospectively in order to summarise the results of this thesis.

Scale of immunoreaction intensity:

++++	very strong and reactions seen in most cells.
+++	strong and reactions seen in most cells.
++	intermediate and reactions seen in a significant number of the cells.
+	weak or reactions infrequently seen.
-	no expression.

* tendency for stronger BDNF immunoexpresssion in tendinosis vs controls
NA = not available

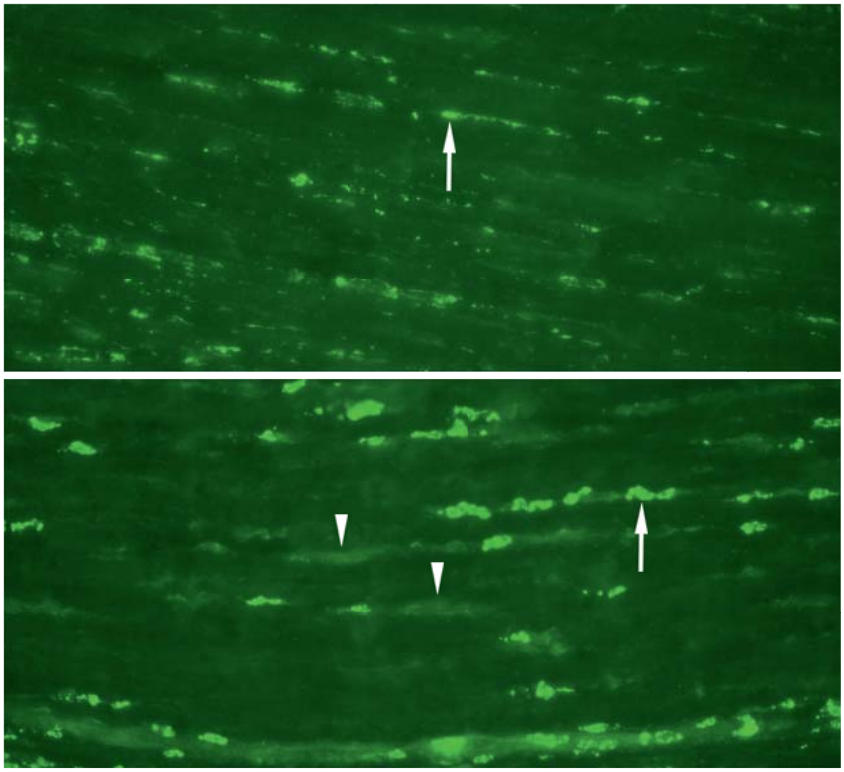

Figure 7. Sections of tendinosis specimens processed for TNFR1 (top) and TNFR2 (bottom). Most of the tenocytes are immunoreactive. Arrows point at immunoreactive tenocytes. Arrowheads point at non-reactive tenocytes.

Study IV

In the fourth study, we evaluated whether the levels of substances related to the TNF-α and neurotrophin systems (sTNFR1 and BDNF) in the blood are different in patients with tendinosis compared with those in controls.

The concentrations of sTNFR1 and BDNF in blood serum from 19 controls and 22 subjects with tendinosis were analysed using ELISA. sTNFR1 was chosen because TNF-α, but not TNFR1, has a fast turnover rate in the blood and because TNFR1 was found to be elevated in tendinosis in *Study III*. It has also been suggested that sTNFR1 is a better marker than TNF-α in various pathological conditions (Blann & McCollum, 1998). Furthermore, as PA can aggravate tendinopathy symptoms, we also analysed how this parameter is related to the TNF-α and BDNF systems. Total PA (TPA) was measured using a questionnaire (PYTPAQ). It is known that BMI influences both BDNF and sTNFR1, which is why this factor was also analysed.

There was no difference in the median TPA of the tendinosis and control groups (135.2 MET-h/week, IQR 52.8 vs 143.1 MET-h/week, IQR 45.8, P = 0.40). Similarly, there were no differences in the median levels of sTNFR1 (1.13 ng/mL, IQR 0.20; 1.11 ng/mL, IQR 0.18, respectively, P = 0.79) and BDNF (14.6 ng/mL, IQR 5.5; 14.6 ng/mL, IQR 3.8, respectively, P = 0.66) in subjects with tendinosis and in the controls. This finding could be expected because it is known that most of the circulating BDNF is produced in the brain (Knaepen et al., 2010).

Although we did not detect any difference in the blood concentrations, several interesting correlations were noted. TPA was

associated with sTNFR1 in patients with tendinosis ($r = 0.67$, $P < 0.01$, $n = 22$) but not in the controls ($r = 0.32$, $P = 0.18$, $n = 19$). No statistically significant correlations for BDNF and TPA were noted for the entire data set (tendinosis: $r = 0.32$, $P = 0.15$, $n = 22$; controls: $r = -0.01$, $P = 0.97$, $n = 19$). In the data analysis, we discovered a gender-dependent difference in both sTNFR1 and BDNF. It was found that, in women with tendinosis, BDNF and TPA were strongly correlated ($r = 0.9$, $P < 0.01$, $n = 8$), but not in men with tendinosis or non of the control groups (men tendinosis: $r = 0.04$, $P = 0.89$, $n = 14$; women control: $r = -0.08$, $P = 0.83$, $n = 9$; men control: -0.07, $P = 0.85$, $n = 10$). sTNFR1 was correlated with TPA in the male tendinosis group ($r = 0.65$, $P = 0.01$, $n = 14$) but no statistically significant correlation was found between sTNFR1 and TPA either in the women tendinosis group ($r = 0.67$, $P = 0.07$, $n = 8$) or in the two control groups (men: $r = 0.04$, $P = 0.91$, $n = 10$; women: $r = 0.40$, $P = 0.29$, $n = 9$).

It was also noted that a moderate, but not statistically significant correlation was seen between BMI and BDNF ($r = 0.60$, $P = 0.12$) and between BMI and TPA ($r = 0.69$, $P = 0.06$) in women with tendinosis. Thus, BMI could possibly be a confounding factor for the association between BDNF and TPA in women with tendinosis.

The major finding in this study was that physical activity level seems to influence the blood levels of sTNFR1 and BDNF in patients with tendinosis, but not in controls, and that apparently there was a gender-dependent difference. These findings could be of interest with respect to pain in relation to PA in Achilles tendinosis; this is discussed in more detail in PART IV.

PART IV

DISCUSSION

Here I discuss the methods and the results, interpret the findings in a bigger context, and make suggestions for future research.

Strengths and limitations

The immunohistochemistry methods used in this thesis have been standardized through several decades of research, not least of all in our own lab. Thus, these methods are expected to be optimized, and their strengths and limitations well known.

The specificity of antibody staining is always an issue. Although manufacturers state that the antibodies are specific, cross-reactivity may occur. Therefore, in all the immunohistochemistry studies, two approaches were used to test for specificity. These were: (1) omission of the primary antibody to test whether there are cross-reactions with the secondary antibody, and (2) letting the primary antibody be preabsorbed with the antigen before adding it to the section. After this procedure, the antibody should not bind to the tissue because the antibody is saturated and washed away. To test for specificity, we also did additional positive control staining on tissues that are well known for expressing the antigens.

The procedure for quantifying the immunoreaction intensities has limitations, because of the subjective aspect of the assessment. Nevertheless, this procedure is commonly used, and two investigators always quantified the intensity independently.

The reliability of our assertion that the tenocytes do produce the substances examined is high because we used methods for detection at both protein and mRNA levels. If the mRNA had not been detected, production elsewhere could not have been excluded.

An additional strength of the studies in this thesis was that they were performed exclusively on humans.

Another important methodological aspect to discuss is the heterogeneity of age and gender of the population studied. The group studied was heterogeneous because patients were both men and women, both young and old, and they had had symptoms for a wide range of durations (3 months to several years). Nevertheless, the principal results (the expressions for TNF-α and neurotrophins, and

their receptors) applied to all the subjects. Variation in the intensity of expression was seen between samples, but variation was seen to be particularly pronounced within specimens, i.e, within one specimen from the same subject. The value of studying a heterogeneous group is that the results can be generalised to a large population. In general, the controls were recruited to match the patients, i.e., this group was also heterogeneous with respect to age and sex.

For ethical reasons, it is controversial to collect biopsies from normal tendons. Therefore control biopsies were few, and these were taken from the superficial, dorsal side of the Achilles tendon. This location differed from the location from which where biopsies were taken in the patients. It is unclear whether this discrepancy influenced the results or not. Nevertheless, generally, similar results were found for immunoexpression in patients and controls. Biopsies were taken only once. It was not possible to assess the expression patterns over time.

An interesting question is whether or not the results can also be applied to other tendons. The tendons analysed were exclusively Achilles tendons, which means that the results cannot automatically be applied to other tendons. However, tendons from different locations do share fundamental properties. In our group, we have considerable experience of studying numerous other signal substances and their receptors both in the Achilles and patellar tendons. The expression patterns for these signal substances and receptors are similar in Achilles and patellar tendinosis and normal tendons (Bjur et al., 2008a, 2008b; Danielson et al., 2006, 2007). There are also similar morphological features in the tendons in patellar and Achilles tendinosis (Khan et al., 1999).

We compared blood serum concentrations of sTNFR1 and BDNF with the TPA score (*Study IV*). There is no perfect tool for measuring physical activity. We decided that it was most relevant to measure TPA for the preceding year, because chronic tendinopathy develops over a long time. Therefore, a questionnaire was used to measure TPA. Limitations of questionnaires include the fact that they

are partly subjective and that they are related to recall bias (i.e. the individual might not correctly recall their PA level.)

The samples in Studies I–IV were small. However, the groups were large enough to answer the questions that were set up in *aims*. In *Study IV*, with regard to the separation of subjects into men and women groups, additional significant results might have been obtained if larger populations had been used.

Comments on the general findings

Trophic/modulatory and apoptotic events are key events in tendinosis. These events do also occur in normal tendons, though to a lesser extent. The presence of BDNF and TNF-α and their receptors suggests that these substances are involved in such events in the Achilles tendon. Presumably, they act both on the same cell that they are produced in (autocrine function), on neighbouring cells (paracrine function) or have more peripheral effects inside and outside the tendon tissue, i.e. not only effects on the tendon cells. As expected, both apoptotic and proliferation markers were more expressed in tendon tissue from patients with tendinosis than in tissue from controls. Nevertheless, for both groups, only a subpopulation of the tenocytes was in a stage of apoptosis or proliferation. Thus, apoptosis and proliferation occur simultaneously in a tendon. These are events that indicate matrix remodelling.

In *Studies I-III*, it was found that both the immunoexpression intensity and the tenocyte morphology varied both between and within samples. Such variability is also seen concerning morphology (Khan et al., 1999) and other signal substances (Bjur et al., 2008b) for tenocytes of the Achilles tendon. The observation that the immunoreaction pattern was associated with tenocyte morphology (*Study III*), i.e. the most rounded/wavy tenocytes expressed strongest immunoreactions, is interesting because such tenocyte morphology is predominantly a sign of a pathological/reorganisation condition. Hence, tenocyte rounding is a key feature of tendinosis tendons (Bjur

et al., 2008a; Maffulli et al., 2004; Åström & Rausing, 1995). Tenocyte rounding can indicate that the tenocytes are activated increasingly and that they produce proteoclycans rather than collagen (Cook et al., 2004).

The TNFR1 tenocyte immunoreaction appeared to be upregulated in patients with tendinosis compared with in controls. The implication of this is not readily explained. Increased TNFR1 may relate to increased activation of the death pathway, and may also relate to tenocyte rounding. Stimulation with TNF-α leads to a rounding of cultured tenocytes (John et al., 2010).

The median blood concentrations of sTNFR1 and BDNF were similar in patients and in controls. Substance concentration is influenced by production in many peripheral tissues, including the Achilles tendon. Moreover, in mice the major site of BDNF production is not in the peripheral parts of the body, but in the brain (Rasmussen et al., 2009). In rat, it has been suggested that BDNF easily crosses the blood-brain barrier (Sartorius et al., 2009), which means that the blood concentration of BDNF might reflect the BDNF production in the brain.

One hypothesis was that pain could provoke the release of BDNF and lead to a high level of sTNFR1 in the blood. This was not supported by the results (*Study IV*), or the model might not be appropriate to this aspect. The blood concentration may be upregulated when there are marked pain sensations, but return to normal when a patient does not have pain at the time of sampling. Therefore, it is possible that the patients did not have pain at the time of sampling. It is also possible that the controls had pain in areas other than the Achilles tendon (this was not checked), at the time of blood sampling. Future work should study the effect of acute tendon pain on BDNF and sTNFR1 blood concentrations.

TNF-α and neurotrophins have interaction effects. The field of interaction effects is a complex one. Presumably the TNF-α and neurotrophin interaction effects occur in tenocytes in both healthy and diseased tendons. Further consideration is given to the interaction possibilities in the pain and interference sections below.

Inflammation reconsidered

Both TNF-α and BDNF are inflammatory-modulating substances. TNF-α is a pro-inflammatory cytokine that plays important roles in several inflammatory diseases (Aggarwal et al., 2012). TNF-α is expressed highly in inflammatory cells but has several pathways — it may not always activate the pro-inflammatory pathways (Aggarwal et al., 2012). This can be the case in tendinosis. Furthermore, BDNF is expressed by inflammatory cells in other tissues (Johansson et al., 2008). Also in this case, the substance may not be related to pro-inflammatory effects.

For a long time, chronic Achilles tendon pain was described as "tendinitis", indicating that there is inflammation. There are, however, no inflammatory cells in tendon tissue in chronic Achilles tendinosis (Bjur et al., 2005; Åström, 1997) and there is no increase of PGE_2 concentration in this condition (Alfredson et al., 1999). In this thesis inflammation was not seen in the tendon tissue proper (*Studies I–III*). This fact does not rule out the occurrence of inflammatory events at some stages in the disease process. Non-steroidal anti-inflammatory drugs (NSAIDS) are sometimes used in early-stage tendinopathy (Fallon et al., 2008). In rat model of a repetitive task, TNF-α and other cytokines increased in serum and tendon and were related to macrophage infiltrate in this tissue (Barbe et al., 2008).

Inflammation is essentially a beneficial event and is the body's primary way of initiating the healing of injured tissues. Thus the upregulation of pro-inflammatory substances is not necessarily related to harmful effects, but can be related to the initiation of healing processes. This is noted for healing skeletal muscles in which case the inflammatory phase is followed by proliferation and differentiation of myogenic cells (Chargé & Rudnicki, 2004).

It has been speculated that the chronic pain in tendinosis tendons is chronic because the tissue itself is unable to start an

inflammatory process (Fenwick et al., 2001). Induction of inflammation may actually be a step in beneficial treatment (Fenwick et al., 2001). As both mini-invasive scraping and eccentric training, two commonly used treatments, affect the tendon, initiation of inflammation may occur. The upregulation of TNFR1 in chronically painful tendons can be an attempt to start a pro-inflammatory cascade that initiates healing. However, we could not detect infiltration of TNFR1-positive inflammatory cells into the tendon tissue, and TNFR2 was not upregulated in tendinosis tendons (*Study III*).

The plantaris tendon is implicated in the pathogenesis of Achilles tendinosis (Alfredson, 2011a; van Sterkenburg et al., 2011) and its involvement in Achilles tendinosis is interesting from an inflammatory perspective. In Achilles tendinosis, these two tendons are sometimes coalesced by loose connective tissue. In this coalescing tissue, there is a mass of fibroblasts but also a presence of inflammatory cells (Spang et al., in press). The extirpation of the plantaris tendon, combined with scraping of the Achilles tendon, is a promising treatment for Achilles tendinosis (Alfredson, 2011a). It is likely that several substances that are related to inflammatory functions are produced in the fibroblasts of this loose connective tissue. It is interesting that one such substance is acetylcholine (ACh), which has anti-inflammatory functions (Kawashima & Fujii, 2003). Its synthesising enzyme, choline acetyltransferase (ChAt), is expressed not only by the fibroblasts in the loose connective tissue between the tendons, but also by the tenocytes of the Achilles tendon (Bjur et al., 2008b) and the plantaris tendon in chronic painful mid-portion Achilles tendinosis (Spang et al., in press).

Aspects on pain

Pain can be modulated both at peripheral and central levels (Woolf, 1983). Thus, both neurotrophins and TNF-α have pain-modulating effects at both peripheral and central levels. Both TNF-α (Fordyce et al., 2008) and BDNF (Pezet & McMahon, 2006) are involved in

conditions of chronic pain. Whether they exert pain-modulating effects in tendinopathy or not was not studied in this thesis but a discussion of this issue is needed.

There is a relationship between TNF-α and BDNF in relation to pain. It has been shown that anti-TNF treatment for patients with rheumatoid arthritis, a treatment that reduces their pain, leads to a decrease in BDNF levels in the blood (Grimsholm et al., 2008). Also, anti-TNF treatment attenuates the BDNF levels in the dorsal root ganglia and spinal cord, as seen in studies on a rat model (Onda et al., 2004). It was recently described that blocking of TNF-α markedly lowers the nociceptive central nervous system activity in the brain in patients with rheumatism (Hess et al., 2011). This reduction occurred rapidly, i.e. before joint swelling was affected, which suggests that anti-TNF treatment has brain-related effects, which could be even more important than the anti-inflammatory effect in the joints.

The results of this thesis show that the neurotrophin and TNF-α systems were mainly confined to the tenocytes, although expression of BDNF and the receptors p75 and TrkB was also found in nerves located in the peritendinous tissue. Thus, even though the main part of the neurotrophin system is related to the tenocytes, TNF-α and the neurophins may also exert effects on nerves. Nerves in tendons are mostly found in the peritendinous tissue. Although some specimens in our studies contained parts of this tissue, we did not focus specifically on this area (focus was on the tendon tissue proper). Therefore it is an important task for future research to analyse the peritendinous tissue with respect to the neurotrophin and TNF-α systems. Also, it would be interesting to investigate in more depth the role of these systems at central levels (in glia cells, in the dorsal horn and in the brain), in relation to chronic Achilles tendon pain, and, in parallel, to study pain perception. Interestingly, it has been suggested that activation of glial cells by inflammatory mediators, e.g. TNF-α, that are produced locally in the tissue is related to chronic pain (Willis et al., 2008).

Aspects on physical activity

In *Study IV,* it was found that there was an association between TPA during the preceding year and the sTNFR1 concentration in patients with tendinosis, but not in the controls. That is to say, a high level of PA (on a one-year basis) is related to higher levels of sTNFR1 in the blood. Thus, in general, patients that were more physically active had higher levels of sTNFR1. Whether or not PA elevates the levels of sTNFR1 in patients with tendinosis should be investigated in future studies.

We found that TPA was associated in different ways with the TNF-α and BDNF systems in men with tendinosis compared with in women with tendinosis. In women, TPA was associated with BDNF, whereas in men TPA was associated with sTNFR1. No such associations were found in the controls. These findings suggest that there is a gender difference in how these substances are related to TPA in tendinosis. As the sample sizes were small, these associations should be clarified in future studies.

Gaida and collaborators suggested that there is a gender difference in the pathobiology of tendinopathy. They discovered that men and women have differently distributed fat and that this is related to the production of cytokines (Gaida, 2009). Also, normal tendons do respond differently to exercise in men and women (Heinemeier & Kjaer, 2011).

An additional finding was that TPA was similar in patients with tendinosis and in the controls. Apparently this opposes the theory that high PA is a risk factor for tendinosis. But the patients concerned had had pain for a long time and therefore may have lowered their PA. It should be kept in mind that causal-relationships cannot be proved using retrospective studies.

Different types of PA may alter the blood levels of BDNF in different ways. These aspects have been reviewed by Knaepen and collaborators (2010). In this review, both effects in relation to the duration and the type of exercise have been discussed. Therefore, it would be interesting in future studies to analyse blood samples after

heavy exercise, as well as after treatments for tendinosis, such as eccentric exercise.

Tendons constantly encounter loaded and unloaded phases in everyday life. Various peripheral tissues are influence by PA (i.e. show responses to loading). Mechantransduction is a theory of how living tissues maintain normal structure through exercise (Khan & Scott, 2009). TNF-α is involved in mechanotransduction for several cell types (Wang et al., 2003; Yang et al., 1998; Zhan et al., 2007). As expression of TNF-α and its receptors was found in the tencoytes, the communication between the tenocytes can be influenced via TNF-α with respect to tendon loading. Fibroblasts from the rat heart increase their TNF-α release when stretched (Yokoyama et al., 1999). Immobilisation also changes the expression of TNF-α (Uchida et al., 2005). However, cultures of human tendon fibroblasts do not release TNF-α when stretched (Skutek et al., 2001).

Future research is needed to explain the manner in which TNF-α, as well as neurotrophins, are involved in mechantransduction in healthy and diseased tendons.

Interference with TNF-α and neurotrophins

Different kinds of treatments can interfere with the effect of TNF-α and neurotrophins. Aspects of this are discussed here.

Anti-TNF treatment has revolutionised the situation for patients with inflammatory diseases, in particular rheumatoid arthritis, but also diseases like Crohn's disease (Feldmann & Maini, 2003). However, a great disadvantage of anti-TNF treatment is that it is associated with numerous adverse effects, including infections, blood diseases, and cancer (Aggarwal et al., 2012).

In one pilot study, the effect of the TNF-α blocker adalimumab was tested in Achilles tendinopathy (Fredberg & Ostgaard, 2009). The results from this study do not support the use of this TNF blocker as treatment. The use of TNF blockers has been tested in closely related

conditions, such as ankylosing spondylitis (Braun et al., 2005) and refractory heel enthesitis (D'Agostino et al., 2002) and promising results have been claimed. Here, it should here be underscored that the latter conditions are scientifically verified inflammatory conditions. Further human studies are needed to clarify the importance of anti-TNF treatment in Achilles tendinopathy.

Another question is whether treatment that interferes with neurotrophins is worthwhile or not. Both BDNF and NGF are related to pain-modulating effects in other diseases. Little is known about the effect of BDNF treatment on pain. Much more is known about NGF. NGF treatment can reduce pain in, e.g. patients with osteoarthritis (Lane et al., 2010). However, this treatment leads to adverse effects and, because of this, Phase III trials were put on hold (Lane et al., 2010; Wood, 2010).

Bearing in mind this information, neurotrophin-related treatment for the Achilles tendon seems far-fetched, but more research may shed new light on it.

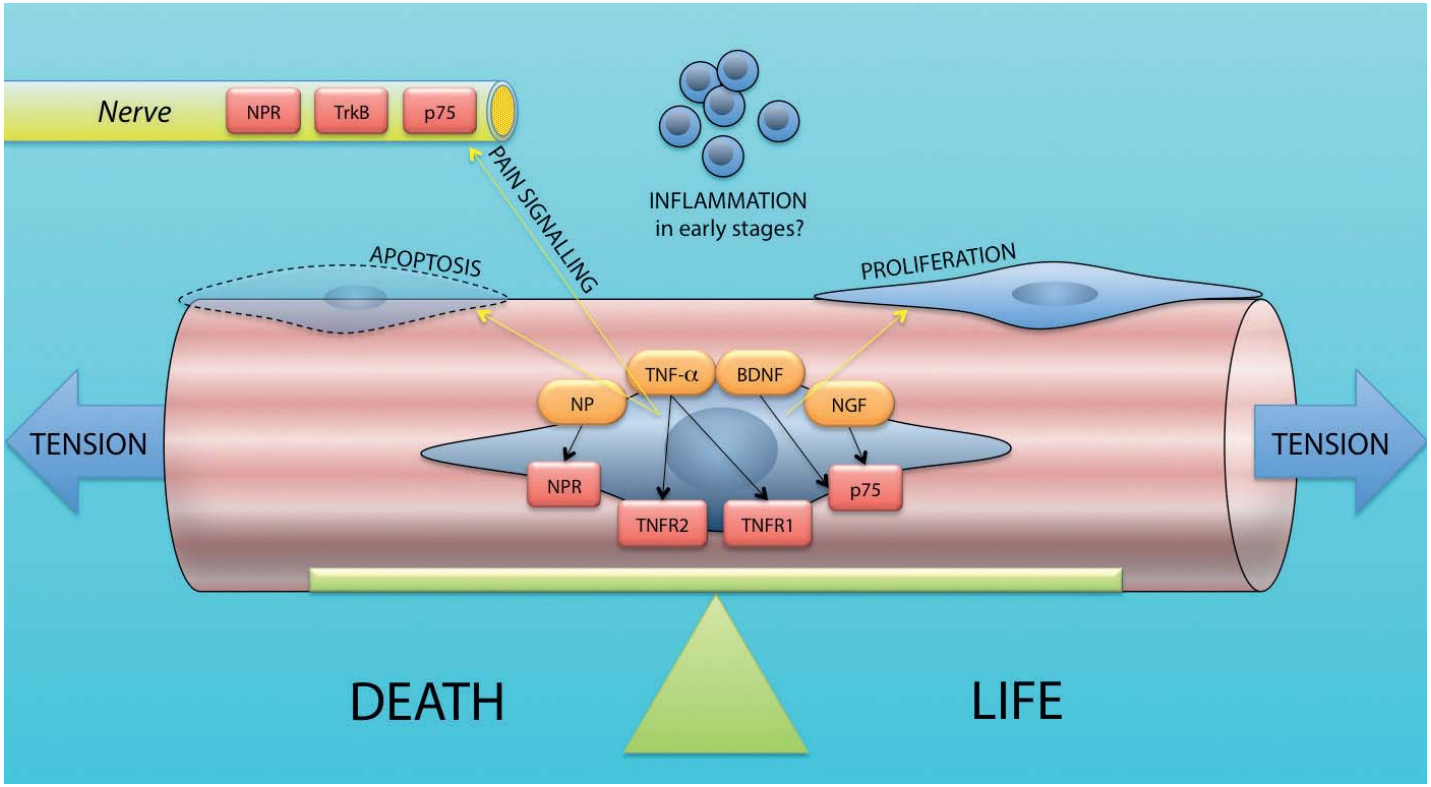

Figure 8. Schematic theory of TNF-α and neurotrophin signalling in the Achilles tendon, based on their function in other tissues and the findings presented in this thesis of how they and their receptors are expressed. The homeostasis of the tenocytes may be controlled by both TNF-α and neurotrophins. Interaction effects may occur between the two substance families as well as with other substances, for example neuropeptides (NP) and their receptors (NPR), as well as neurotransmitters/neurotransmitter receptors, which previously have been shown to be expressed in tendon tissue. There may also be a link to pain signalling, and possibly pro-inflammatory effects are of importance in early stages of tendinosis. Loading of a tendon may via mechanotransduction lead to altered signalling.

Future directions

So far, the neurotrophin and TNF-α systems are unexplored with respect to tendons. These systems are of great importance in other tissues. Ultimately, future research on these substances will help us understand what their importance is in tendinosis.

One important task would be to study the response of these substances to load/immobilisation, preferably by intra-tendinous, *in vivo* measurements. Also, research on the different stages of tendinopathy, and the interaction effects between TNF-α and neurotrophins, as well as with other signal substances, would be of importance to gain further understanding of the role of the TNF-α and BDNF systems in human Achilles tendons, as well as in other tendons. Specifically, the metabolic effects related to proliferation and apoptosis should be elucidated. Such research could be done in studies of cell cultures and animal models. The relationship between TNF-α/neurotrophins and pain should also be investigated further. Hopefully, such studies can lead to new treatments.

Conclusions

I have discussed the findings of two potent substance families in the human Achilles tendon. Their essential roles in other tissues, and their impact in various diseases, indicate that they are substances with great potential for the Achilles tendon as well. The findings of the three ligands (NGF, BDNF, TNF-α) and their receptors will not solve all the questions concerning tendinosis, but they should be added to the great list of beneficial and harmful biochemical substances that are likely to control cell metabolism in normal and diseased tendons. It is hoped that the function of the network of these related biochemical substances will be established in forthcoming research.

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Department of Integrative Medical Biology, Anatomy, and
Department of Surgical and Perioperative Sciences, Sports Medicine, Umeå University
Umeå, Sweden, 2013