The involvement of the TNF-alpha system in skeletal muscle in response to marked overuse

Lina Renström
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New Series Number 1932
ISSN 0346-6612
ISBN: 978-91-7601-802-6

Electronic version available at http://umu.diva-portal.org

Printed by: Print and Media, Umeå University
Umeå, Sweden, November, 2017

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All we have to decide is what to do with the time that has been given to us

- Gandalf the Grey

To Jocke, my parents and my sisters
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Abstract

Painful conditions having the origin within the musculoskeletal system is a common cause for people to seek medical care. Between 20-40% of all visits to the primal care in Sweden are coupled to pain from the musculoskeletal system. Muscle pain and impaired muscle function can be caused by muscles being repetitively overused and/or via heavy load. Skeletal muscle is a dynamic tissue which can undergo changes in order to fulfill what is best for optimal function. However, if the load is too heavy, morphological changes including necrosis, as well as pain can occur. The extension of the skeletal muscle is the tendon. Tendinopathy refers to illness and pain of the tendon. The peritendinous tissue is of importance in the features related to tendon pain. Common tendons/origins being afflicted by tendinopathy/pain are the Achilles tendon and the extensor origin at the elbow region.

Tumor necrosis factor alpha (TNF-alpha) is a cytokine that is involved in several biological processes. It is well-known for its involvement in the immune system and is an important target for inflammatory disorders such as rheumatoid arthritis. It is not known to what extent the TNF-alpha system is involved in the process of muscle inflammation and damage due to overuse.

Studies were conducted on rabbit and human tissue, tissues that either had undergone an excessive loading activity or tissue that was removed with surgery due to painful conditions. The tissues were evaluated via staining for morphology, in situ hybridization and immunofluorescence.

Unilateral experimental overuse of rabbit muscle (soleus muscle) led to morphological changes in the soleus muscle tissue bilaterally. The longer the experiment extended, the more was the tissue affected. This included infiltration of white blood cells in the tissue (myositis) and abnormal muscle fiber appearances. TNF-alpha mRNA was seen in white blood cells, in muscle fibers interpreted to be in a reparative stage and in white blood cells that had infiltrated into necrotic muscle fibers. There was an upregulation in expressions of TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2) in muscles that were markedly overused, with expressions in white blood cells, fibroblasts, blood vessel walls and muscle fibers. Immunoreactions for the receptors were seen in nerve fascicles of markedly overused muscles but only occasionally in normal muscles. The upregulations were seen for both experimental and contralateral sides. Overall the two receptors showed somewhat different expression patterns. Tendinopathy is associated with an increase in blood flow and infiltration of white blood cells in the tissue adjacent to the tendon. It is called the peritendinous tissue and is also richly innervated. The white blood cells and the blood vessels walls in this tissue were showing immunoreaction for TNFR1 and TNFR2. Two types of nerve fascicles were found in this tissue, one normally appearing when staining for nerve markers and one type with signs of axonal loss. The latter had clearly strong immunoreactions for TNFR1 and TNFR2.

The findings suggest that the TNF-alpha system is involved in both myopathies occurring due to overuse and in features in the peritendinous tissue in the tendinopathy situation. TNF-alpha and its receptors seem to be involved in degeneration but also in regeneration and healing of the tissue. The findings also suggest that TNF-alpha has effects on nerves showing axonal loss. The changes in the TNF-alpha system were seen both on the experimental side and contralaterally.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<td>βIII-tubulin</td>
<td>Beta-III-tubulin</td>
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<td>β-actin</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Cap</td>
<td>Captopril</td>
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<td>CK</td>
<td>Creatine kinase</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>DM</td>
<td>Dermatomyositis</td>
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<tr>
<td>DMD</td>
<td>Duchenne muscular dystrophy</td>
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<td>ECRB</td>
<td>Extensor carpi radialis brevis</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>H&amp;E</td>
<td>Haematoxylin &amp; Eosin</td>
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<td>IBM</td>
<td>Inclusion-body myositis</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IIM</td>
<td>Idiopathic inflammatory myopathies</td>
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<td>IL-1</td>
<td>Interleukin 1</td>
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<td>IL-6</td>
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<td>IR</td>
<td>Immunoreaction</td>
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<tr>
<td>KMnO₄</td>
<td>Potassium permanganate</td>
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<td>LT</td>
<td>Lymphotoxin</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NK-1R</td>
<td>Neurokinin 1 receptor (Tachykinin/Substance P receptor)</td>
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<td>NMJ</td>
<td>Neuromuscular junction</td>
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<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
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<tr>
<td>Pax-7</td>
<td>Paired box protein Pax-7</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PM</td>
<td>Polymyositis</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<td>RRX</td>
<td>Rhodamine Red X</td>
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<td>S-100β</td>
<td>S100 calcium binding protein β</td>
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<td>SP</td>
<td>Substance P</td>
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<td>Th</td>
<td>DL-Thiorphan</td>
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<td>TNF-alpha</td>
<td>Tumour Necrosis Factor alpha</td>
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<td>TNFR1</td>
<td>Tumour Necrosis Factor Receptor type 1</td>
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<td>TNFR2</td>
<td>Tumour Necrosis Factor Receptor type 2</td>
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<td>TRITC</td>
<td>Tetramethylrhodamine</td>
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<td>US</td>
<td>Ultrasound</td>
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List of original papers

1. **TNF-alpha in the Locomotor System beyond Joints: High Degree of Involvement in Myositis in a Rabbit model.**

2. **TNF-alpha in an Overuse Muscle Model – Relationship to Muscle Fiber Necrosis/Regeneration, the NK-1 Receptor and an Occurrence of Bilateral Involvement**

3. **Bilateral muscle fiber and nerve influences by TNF-alpha in response to unilateral muscle overuse – Studies on TNF receptor expressions**
   Renström L, Song Y, Ståhl P.S, Forsgren S *BMC Musculoskeletal Disorders* 2017: Accepted

4. **Marked expression of TNF receptors in human peritendinous tissues including in nerve fascicles with axonal damage – Studies on tendinopathy and tennis elbow**


TNF-alfa är en signalsubstans som är involverad i flertalet biologiska processer. Den är känt för sin del i immunförsvaret och den är ett viktigt mål för behandling av autoimmuna sjukdomar som exempelvis reumatoid artrit. Det är inte känt om TNF-alfa är inblandad i processen som uppstår vid muskelinflammation/muskelskada efter kraftig överansträngning. TNF-alfa har flera receptorer, i det här arbetet har utbredning av TNFR1 och TNFR2 analyserats.

Introduction

Muscle and tendon

Muscle tissue

Skeletal muscle has its name because it most often moves bones. Skeletal muscle tissue is striated, containing repetitive functional units of sarcomeres. It is controlled by neurons from the somatic part of the nervous system.

Muscle tissue consists of lots of muscle fibers. A muscle fiber has several nuclei and many organelles such as mitochondria and myofibrils. The myofibrils within the muscle fiber are the force generator. Within the myofibrils there are several sarcomeres which are responsible for the contraction of the muscle. The sarcomere is built of filaments, mainly myosin, actin, troponin and tropomyosin. The muscle fiber is surrounded by a thin layer of connective tissue, the sarcolemma. Outside the sarcolemma is the endomysium. A group of muscle fibers is surrounded by the perimysium and creates a muscle fascicle. Between the muscle fibers, there are satellite cells and capillaries. Several muscle fascicles create the skeletal muscle. The muscle is, usually together with other muscles, surrounded by connective tissue, called the fascia.

Skeletal muscle fibers are formed by fusion of several myoblasts (Capers, 1960, Mauro, 1961), each with one cell nucleus. That is why a myocyte (muscle fiber) contains multiple nuclei, known as myonuclei. This is one thing that distinguishes a skeletal muscle fiber from cardiac and smooth muscle fibers, the two latter having only one cell nucleus. Skeletal muscle fibers can have over hundred nuclei (Bruusgaard et al., 2003). Myoblasts that do not fuse into myocytes form satellite cells and remain quiescent until the need of muscle repair and regeneration occurs (Schultz et al., 1978). With activation, the satellite cells can re-enter the cell cycle to proliferate and differentiate into myoblasts (Moss and Leblond, 1970). Therefore, satellite cells are to be considered as monopotent myogenic stem cells (Collins et al., 2005). Almost all of the muscle cell nuclei are placed in the outer part of the muscle fiber, beneath the sarcolemma (Cadot et al., 2015). In mature normal muscle tissue, only a few percent of the nuclei are located internally; internal nuclei. What is interesting is that in muscle regeneration and several muscle disorders the nuclei become placed centrally, and the number of internal nuclei becomes increased (Cadot et al., 2015).
Figure 1. Organization of skeletal muscle. A transection of a fascicle visualize the perimysium, endomysium, muscle fiber with somatic motor neuron, capillaries and myonuclei.
The nerve supply to skeletal muscles is from myelinated motor nerves, myelinated and non-myelinated sensory nerves and non-myelinated efferent autonomic nerves. An axon of a motor fiber divides into small branches after entering the muscle and reaches several muscle fibers. The sensory nerves are responsible for sending information about body position and refine the control of muscle in normal situations, but can also signal for pain. The efferent autonomic nerves have effects on constriction and dilatation of vessels. The connection between the motor neuron and the muscle fiber is the neuromuscular junction (NMJ). The NMJ is composed of several cell types; Schwann cells, endings of motor neurons and the associated part of the muscle fiber. Between the motor neuron and the muscle fiber is the synaptic cleft, and that is where the transmitter acetylcholine (ACh) is released. ACh binds to receptors which leads to depolarization of the membrane and at the end a contraction of the muscle. The Schwann cells are important for the maintenance of the NMJ and play a role in regeneration and remodeling of impaired NMJ. NMJ-dysfunction seems to play a role in age-related muscle impairment (Gonzalez-Freire et al., 2014).

Blood vessels are responsible for transport of oxygen, carbon dioxide, nutrients and waste products between tissues. Arteries run primarily outside the muscle and branches into arterioles which enter the muscle through the epimysium. Arterioles finally branch into a network of capillaries that are embedded in the endomysium (Korthuis, 2011). Arterioles are the smallest vessels that have a smooth muscle layer, capillaries consist only of one layer of endothelium. The capillaries lie around the muscle fibers and vary in number between fibers. It has been showed that the bigger a muscle fiber is, the more capillaries is it surrounded by (Ingjer and Brodal, 1978).

There is a strong correlation between muscle strength and cross-sectional area of the muscle (Maughan et al., 1983). However, in untrained subjects who start to exercise, the first weeks of improvement in strength are not related to muscle growth. In untrained individuals, the neuromuscular adaption is instead the first event when starting the strength training (Gabriel et al., 2006, Schoenfeld, 2010). However, if the exercise continues the muscle mass will increase. That occurs after a couple of weeks to months. The fibers of mature skeletal muscle do not have the ability to undergo cell division. Muscle growth is thus hypertrophy, which is enlargement of present muscle fibers, rather than hyperplasia. The hypertrophy includes an increase in the synthesis of the myofibrillar proteins and an increased oxidative capacity.

Skeletal muscle fibers are divided into slow fibers called type 1 fibers, and fast fibers, type II fibers (Brooke and Kaiser, 1970). Type II fibers are subdivided into several undergroups. What differs them is the contraction speed, the ability to develop force and the endurance capacity.
Muscle tissue plasticity

Skeletal muscle tissue is adaptive to stimuli and has a pronounced capability of plasticity. Adaptive structural events do not only occur in the muscle fibers but also in the surroundings such as in capillaries and motor neurons. The skeletal muscles have the potential to change the composition of muscle fiber phenotypes and fiber size in response to activity and changed demands (Pette and Staron, 1997, Scott et al., 2001). Thus, physical training has an effect on fiber types in muscle (Kadi and Thornell, 1999). Changes of the neural impulse pattern to a muscle also contributes to changes in muscle fiber phenotype. Muscle plasticity refers to the fact that a muscle fiber can change its type and/or its quantity of protein production. This will benefit the muscle concerning its physiological demands. To some extent, there is a loss of skeletal muscle with aging (Lexell, 1995). Muscle tissue is, in some degree, replaced by connective tissue and fat, due to less physical activity. To prevent this tissue transformation, from muscle to connective tissue or fat, resistant exercise is effective (Peterson et al., 2011). Resistant training for elderly women is not only decreasing the muscle loss but does also increase maximal strength and explosive capacity (Edholm et al., 2017). Skeletal muscle undergoes changes that can be degenerative due to massive overuse. After passing through a degenerative stage, the muscle enters into a regenerative state in the healing process (Carlson, 1973).

The triceps surae muscle in humans

The gastrocnemius and soleus muscles form the triceps surae muscle. It is the most prominent muscle of the calf. The gastrocnemius muscle lies most superficially and has two muscle heads, one lateral and one medial. It originates from the distal part of the femur. The soleus muscle is a broad, flattened muscle and lies beneath the gastrocnemius. It has its origin at the superior/posterior parts of tibia and fibula and the intervening connective tissue. The two muscles converge into the Achilles tendon with insertion into the calcaneus bone. The main function of the muscle is plantar flexion of the foot, and it is activated during running and jumping. It is involved in the supination of the foot as well. Because the gastrocnemius muscle passes the knee joint it can also participate in flexion of the knee.

The plantaris muscle in humans

The plantaris muscle is a small, rudimentary and variable muscle with the origin at the lateral condyle of femur. The muscle is 5-10 cm long before it turns into a long thin tendon which continues between m. gastrocnemius and m. soleus down
on the medial side of the lower leg. The plantaris muscle contributes to flexion in the knee and the foot.

**Tendon tissue**

The extension of the muscle is the tendon and the force that muscles produces is transmitted by the tendon. Tendons are composed of dense connective tissue and connect the muscle to the bone. Most muscles pass along at least one joint, involved in the movement of that joint.

Tendon mainly consists of collagen and elastin that are embedded in a proteoglycan-water matrix, where the collagen comprises most of the dry weight (Hess et al., 1989, Jozsa et al., 1989). These extracellular components are produced by tenocytes and their premature version, the tenoblasts. Tenocytes are considered to be a subpopulation of fibroblasts (Riley, 2008) and are located between the collagen fibers (Hess et al., 1989). Proteoglycans are major components of the extracellular matrix and occur between collagen fibers. They have a high water binding ability – water stands for 70% of the tendons weight – and play an important role in structural and biochemical adaption to changes in load (Kannus, 2000, Yoon and Halper, 2005). The three-dimensional structure of the tendon is mediated by a hierarchical network of collagen fibers (Kannus, 2000).

A fine sheet of connective tissue called the endotenon encircles groups of collagen fibers and form a primary fiber bundle (subfascicle). A group of subfascicles form a secondary fiber bundle, several secondary fiber bundles create a tertiary bundle. A couple of tertiary bundles are surrounded by the epitenon, which is a sheet of connective tissue, and form the tendon. The number of subfascicles can vary between tendons. Superficially to the epitenon is the paratenon that allows free movement towards the surrounding structures (Hoffmann and Gross, 2007, Elliott, 1965). The endotenon network carries blood vessels, nerves and lymphatic vessels to the inner portion of the tendon (Hess et al., 1989). Outside the paratenon there is a partly loose connective tissue called peritendinous tissue. This will be commented on below.

**The Achilles tendon in humans**

Achilles was the son of king Peleus and the immortal goddess Thetis in the ancient Greek mythology. Thetis wanted her son to be invulnerable and for this purpose she dipped him in the river Styx. She was holding him in the right foot and because of that the right heel never touched the water. Therefore that part of him was still vulnerable. Later on in the Trojan War, Achilles was killed by a wound caused by an arrow to the right heel. The expression “Achilles heel” refers to these
legends and means the weak point of a person. Despite this, the Achilles tendon is the thickest and strongest tendon in the human body (Doral et al., 2010). As described above, it is the common tendon of the gastrocnemius and soleus muscles, thus the triceps surae muscle. The Achilles tendon inserts into the calcaneus bone and is therefore also known as the calcaneal tendon. In the distal course of the tendon the fibers make a lateral rotation. Medial fibers rotate posteriorly, fibers found posteriorly are twisted laterally etc. (Cummins et al., 1946). The rotation of Achilles tendon fibers is thought to increase tensile strength and contribute to the supination of the foot (Morimoto and Ogata, 1968). The Achilles paratenon is thick on the medial, dorsal and lateral portions but thin at the ventral side. Medially and ventrally outside the paratenon there is the loose peritendinous connective tissue.

The blood supply is principally divided into three portions, that of the musculo-tendinous junction, that of the tendon-bone junction and that occurring along the tendon (Ahmed et al., 1998). The last mentioned is the major portion. The main blood supply is thus from the peritendinous network of blood vessels which originates from the anterior and posterior tibial and peroneal arteries (Ahmed et al., 1998, Schmidt-Rohlfing et al., 1992, Chen et al., 2009). Arteries run longitudinally along the tendon and then penetrate the connective tissue sheets. Blood supply is also to some extent provided from vessels in the perimysium of the triceps surae muscle. The mid portion of the Achilles tendon is the least vascularized (Carr and Norris, 1989) and that is also the part where most of the Achilles tendon ruptures occur (Gulati et al., 2015).

The nerve supply for the Achilles tendon is primarily from nerves that innervate the triceps surae muscle and cutaneous branches of the sural nerve (Stilwell, 1957). In an animal study it was shown that most of the nerve fibers terminate in sensory nerve endings in the connective tissue around the tendon (Ackermann et al., 2003). In studies on humans it has been found that there are frequent nerve fibers in the peritendinous tissue located ventrally to the Achilles tendon in tendinopathy patients (Andersson et al., 2007). Only a few nerves pass into the tendon tissue proper, following the vascular channels in the endotenon. Different types of nerve endings are found in association with human tendons, responsible for signaling pressure and stretching which help to keep the balance in movements.

**The plantaris tendon in humans**

As described above, the plantaris muscle is a thin muscle belly which originates at the lateral condyle of femur and which turns into a long thin tendon in the posterior/superior compartment of the calf. Some claim that the plantaris is a
part of the triceps surae muscle, but most often it is described as an individual muscle. There are several anatomical variations for the plantaris muscle and its tendon (Spina, 2007, Spang et al., 2016). It can vary in size and insertion sites. Simpson and colleagues even showed that the plantaris tendon is absent in up to 20% of lower limbs (Simpson et al., 1991). Several insertion sites have been described (Nayak et al., 2010). The most common insertion variant is into the calcaneus, anteriorly to the Achilles tendon. It can also be inserted at the medial side of the calcaneus bone. A small number of plantaris tendons fuses with the distal portion of the Achilles tendon (Spang et al., 2016).

Figure 2. Schematic illustration of the insertion of the Achilles tendon at the calcaneus bone in relation to the plantaris tendon which runs ventromedially to it. In the space between these two tendons, loose connective tissue (peritendinous tissue) is located (indicated by the yellow color).

**Extensor origin of the wrist**

The common origin for the wrist extensors is the lateral epicondyle of humerus. It is a common origin for four muscles that dorsiflex the wrist and fingers. The lateral epicondyle is the origin for m. extensor carpi radialis brevis (ECRB), m. extensor carpi radialis longus, m. extensor digitorum, and m. extensor carpi ulnaris. They insert into different bones in the hand and are innervated by n. radialis.
Connective tissue in relation to tendons/muscle origins; Peritendinous tissue

The connective tissue present in relation to tendons and in regions of muscle origins such as that of elbow region/lateral epicondyle of humerus is of importance. It is the subject of studies in the present Thesis.

The Achilles tendon is especially on the dorsal side, and to a small extent, laterally and medially, surrounded by the paratenon. Ventrally to the Achilles tendon there is a fatty areolar tissue that is richly vascularized and innervated (Shaw et al., 2007). The tissue outside the tendon is often referred to as loose “peritendinous connective tissue”. The blood flow and oxygen exchange increases not only in muscles during exercise but also in the peritendinous connective tissue (Boushel et al., 2000). Of relevance for the present Thesis is the fact that the peritendinous tissue has been considered to be important when explaining the pain in tendinopathy.

Also for chronic pain conditions at regions of the muscle origins, such as tennis elbow region, it is supposed that the connective tissue is involved in the pathology (Spang and Alfredson, 2017). For matter of simplicity, the connective tissue at these regions is referred as peritendinous tissue in the present Thesis.

Rabbit muscle and tendon

Three of the papers in Thesis (I-III) are based on animal (rabbit) studies and therefore it is of importance to explain similarities and differences when compared to the human situation.

Rabbit triceps surae muscle

As in man, the triceps surae muscle in rabbits is composed of two muscles, the gastrocnemius located superficially and the soleus muscle located beneath the gastrocnemius. In rabbits, the gastrocnemius has two heads, one lateral and one medial, which originate from the condyles of femur as in humans. It is attached to the calcaneus bone by the Achilles tendon. The soleus muscle in rabbits originates from the superior posterior part of tibia and inserts into the Achilles tendon. As in humans, the triceps surae muscle is responsible for plantar flexion of the foot, and the gastrocnemius muscle is also able to flex in the knee.
There is a thin muscle, the flexor digitorium superficialis muscle, which is located parallel to the gastrocnemius and soleus muscles. The muscle continues to pass the calcaneus bone and inserts underneath the foot. The flexor digitorium superficialis muscle is not present in humans.

Although the anatomical features, for the triceps surae muscle appears in principal to be similar in humans and rabbits, there is a difference in proportion of muscle fiber phenotypes between humans and rabbits. The human soleus muscle contains approximately 80% slow type 1 muscle fibers and in gastrocnemius muscle approximately half of the fibers are slow type 1 fibers (Gollnick et al., 1974). The soleus muscle of rabbits is reported to have 96% slow type 1 muscle fibers (Peter et al., 1972) and gastrocnemius to approximately have 22% slow type 1 muscle fibers (Kost and Kost, 1982).

There seems to be a controversy concerning the existence of a rabbit plantaris muscle in the literature. The majority of publications do not mention the plantaris muscle, but rather the medially coursing flexor digitorum superficialis muscle mentioned in earlier text (Doherty et al., 2006, Huisman et al., 2014). There are, however, publications describing a plantaris muscle (Siebert et al., 2015) roughly in the position of the flexor digitorum superficialis muscle.

![Diagram of rabbit triceps surae muscle from a lateral view](image)

Figure 3. Rabbit triceps surae muscle from a lateral view. The triceps surae muscle consists of two heads of the gastrocnemius muscle and the soleus muscle. Flexor digitorium superficialis is located between the gastrocnemius and soleus muscles.
**Rabbit Achilles tendon**

The Achilles tendon in rabbits is as in humans the tendon of the triceps surae muscle. There is a similar lateral rotation of the Achilles tendon as in humans (Doherty et al., 2006). There is however a difference concerning the tendon. The tendon fibers originating from the two heads of the gastrocnemius muscle fuse together into one tendon in the end of the first quarter of their course in humans. In the rabbits they do not fuse until after reaching 93% of their course, i.e. very close to the distal end and the insertion into the calcaneus bone (Doherty et al., 2006).

The Achilles tendons relationship to other tendon structures is another aspect that differs between man and rabbit. In humans, the plantaris tendon runs along the Achilles tendon and most often does not become a part of the Achilles tendon, but has an own insertion. On the other hand, a coalescence between the tendons does often occur. In rabbits there is, as described above, a muscle which does not exists in humans; the flexor digitorium superficialis muscle. The tendon of this muscle is located anteriorly and medially to the medial gastrocnemius tendon. Along its course the tendon tracks medially and posteriorly, and inserts in the middle phalanges II-IV of the foot (Stoll et al., 2011).

**Myopathies and tendinopathies**

**Skeletal muscle injury**

Muscle damage due to different types of overuse do frequently occur. Repetitive muscle work is actually associated with pain and muscle injury. The extrinsic factors of importance for the work-related muscle injuries are not least the load and the overactivity duration. The intrinsic factors are the muscle fibers and the tissue surrounding the muscle. The capacity and response to a certain load is an interaction between extrinsic and intrinsic factors (Ashton-Miller, 1999). Eccentric exercise was found to cause the greatest muscle damage in an animal (rat) study (Armstrong et al., 1983).

Injury of skeletal muscle due to overuse is characterized by changes in the muscle fiber morphology, fiber degeneration, necrosis and inflammation and an increased amount of connective tissue (Hikida et al., 1983, Friden et al., 1989). The repair process is principally similar regardless of what caused the muscle damage. Muscle fiber degeneration with infiltration of white blood cells in the damaged area is the first event. Phagocytic inflammatory cells take care of
necrotic tissue and then the regeneration takes place. To avoid muscle fiber death, there is a need of obtaining extra nuclei. These are delivered by the satellite cells. At the site of the injury, growth factors are produced, several of them activating the satellite cells (Grefte et al., 2007). The satellite cells fuse with the injured fibers and contribute to the repair and regeneration process including in the protein synthesis (Hill et al., 2003). If this does not occur the muscle fiber will go through cell death. If the basal lamina of the muscle fibers is damaged, fibrin and fibronectin will form a fibrous scar. If the load is excessive and the stress continues to the muscle, proliferation of fibroblasts can occur. A dense fibrous tissue (a large scar) may be created which will interfere with the repair process and obstruct the recovery (Stauber, 2004).

**Muscle inflammation (myositis)**

Muscle inflammation (myositis) can occur due to several reasons. These can be infection, toxic events or injury. There are also idiopathic inflammatory muscle diseases leading to myositis. These diseases will be further explored below. Marked exercise overuse of untrained muscle can also lead to muscle damage with infiltration of inflammatory cells. Various studies that show the features in this process, including infiltration of white blood cells, in human muscle have been published (Dennett and Fry, 1988, Barbe and Barr, 2006). Nevertheless, a marked myositis of the type seen in idiopathic inflammatory muscle diseases were not demonstrated in these studies.

**Models for studying muscle damage/myositis**

Various types of myopathies, including those leading to myositis, have been studied experimentally. That includes myositis induced by intraperitoneal injections with lipopolysaccharide (Vitadello et al., 2010) and immunization with various muscle components (Rosenberg, 1993). Furthermore, studies have been performed whereby the development of myositis is achieved via alphavirus injections in mice (Lidbury et al., 2008) and a further model for studying myopathies including myositis is a model where hamsters are infected with leishmanial infantum (Paciello et al., 2010). These models have mainly been used in order to further help the understanding of inflammatory myopathies in man.

One model which is frequently used in studies on muscle tissue is the dystrophic (mdx) mouse model, which is a model for Duchenne’s muscular dystrophy (Radley et al., 2008). Previously, no model evaluating the myopathy/myositis features experimentally that occur after marked overuse, the aspect of muscle affection that became the goal for the present Thesis, has been presented. However, in the present laboratory a model using rabbits, which were subjected
to marked overuse experimentally, came into use. Several studies have been performed by use of this model, including a Thesis on the importance of the substance P system in the development of the muscle damage/the myositis process (Song, 2013). This model was used in the present Thesis. The morphologic features of the overuse in this model do to a large extent resemble those seen in idiopathic inflammatory diseases. Therefore, features for these diseases are described below.

Muscle damage due to exercise has also been observed (Armstrong et al., 1991, Friden and Lieber, 1992). Damaged myofibers that were in structural disorder were observed by biopsies in man after heavy eccentric exercises (Friden et al., 1983). Loss of desmin and cytoskeleton rupture were induced very short, 15 min, after eccentric exercise (Lieber et al., 1996). One model for studying repetitive contractions is by using electrical stimulation. Electrically induced eccentric contraction have been shown to induce even greater damage than voluntary contractions in humans (Crameri et al., 2007).

**Idiopathic inflammatory myopathy**

The cause of idiopathic inflammatory myopathies (IIM) are, as the name tells, not known. However, there are auto-antibodies present in these groups of patients and therefore the diseases are classified as autoimmune inflammatory myopathies (myositis) (Love et al., 1991).

Concerning autoimmune diseases, the immune system turns against its own tissue, in these cases the muscles. The reason is unknown but it is believed to be triggered by some kind of stress, virus infections or vaccination. Autoimmune myositis is not a genetic disease. However, there might be genetic factors which will makes it more or less likely that the disorder will develop.

IIM is a myopathy characterized by muscle weakness, tenderness and sometimes pain, caused by autoimmune-mediated muscle injury and inflammation. There are three major idiopathic autoimmune myopathies; Dermatomyositis, Inclusion-body myositis and polymyositis.

Dermatomyositis (DM) affects the skin with distinct rash but does also cause muscle weakness. The inflammation in DM is primary in the perimysium. Inclusion body myositis (IBM) is characterized by muscle weakness and inclusion bodies (vacuoles with deposit of abnormal proteins and filaments) in the muscle fibers. The symptoms often progress gradually and strike both proximal and distal muscles. Polymyositis (PM) is characterized by weakness and muscle
atrophy in foremost the proximal muscles. The inflammation is primary localized to the endomysium.

Extra-muscle manifestations occur in these groups of patients, the respiratory organ being the most commonly affected in the form of interstitial lung disease. Other extra-muscular manifestations are diseases in the cardiovascular, bone, endocrine, dermatological and hematological systems (Ng et al., 2009).

When introducing the IIM in Introduction of this Thesis it is relevant to somewhat discuss treatment options. It is namely a challenge to create optimal treatment regimens for these diseases because of the low incidence, the variety of complex phenotypes and the few randomized controlled trials (Gordon et al., 2012). The purpose of treatment for inflammatory myopathies is to improve muscle strength and function, to obtain remission or at least to prevent progress of the disease and prevent other organ damage (Malik et al., 2016). IBM is distinguished to PM and DM because it is resistant to standard immunomodulatory and immunosuppressive therapy (Needham and Mastaglia, 2016). The treatment response is measured by actual muscle strength and levels of circulation creatine kinase (CK), an enzyme released from damaged muscle fibers (Gazeley and Cronin, 2011). First line treatment is corticosteroids (Albayda and Christopher-Stine, 2012, Malik et al., 2016) but most patient also get a complementary immunosuppressive treatment with methotrexate, azathioprine or mycophenolate (Carstens and Schmidt, 2014, Malik et al., 2016).

Biological drugs have been a worthwhile addition in the treatment of other autoimmune diseases such as rheumatoid arthritis (RA) and morbus Crohn. In common for the biological drugs are that they are antibodies or other proteins directly targeting a specific pro-inflammatory mechanism in the disease process. One of the biologic drugs are those blocking Tumor Necrosis Factor alpha (TNF-alpha). TNF-alpha is a cytokine in the inflammatory process and there are several anti-TNF drugs on the market. As TNF-alpha is highly discussed for IIM and as the types of injury/myositis that are seen morphologically in our currently model resemble the situation for IIM, TNF-alpha is focused on in this Thesis. See further below.

**Tendinopathy and tendinosis**

Tendinopathy includes the disorders of the tendon that lead to pain. A situation with tendinopathy can be termed tendinosis when the painful condition is shown to occur together with swelling and structural changes of the tendon (Khan et al.,
The structural changes in tendon tissue in tendinosis are disorder of collagen fibers, hypercellularity and increased vascularization (Khan et al., 1999, Bjur, 2009).

**Achilles tendinosis**

Achilles tendinopathy with structural changes (tendinosis) is common among athletes, both professionals and non-professionals (Alfredson and Lorentzon, 2000). The condition is often seen in individuals between 30-60 years age (Kvist, 1991). The etiology of Achilles tendinopathy is not clear, but an interaction between intrinsic and extrinsic factors is suggested to occur (Maffulli et al., 2004). Intrinsic factors can be muscle weakness, age, gender and weight. Anatomical variations of the lower limb has also been suggested to predispose for Achilles tendinosis (Kaufman et al., 1999, Maffulli et al., 2004, Kvist, 1994). Extrinsic factors can be poor technique, poor equipment and some drugs such as corticosteroids, fluoroquinolone (antibiotics) and anabolic steroids (Jarvinen et al., 2005). These factors can predispose to Achilles tendinosis, but it remains unclear to what degree. The common opinion is that most cases with Achilles tendinosis are caused by a combination of overuse and eventually some of the intrinsic/extrinsic factors (Jarvinen et al., 2005).

Repetitive overuse of the tendon causes microtraumas. If the healing of these microtraumas is incomplete pain, oedema and tenderness will occur. The symptoms will gradually progress (Alfredson and Lorentzon, 2000). In the initial stages, there can be morning stiffness or pain of the tendon which disappears during warming up. In later stages, there is pain during exercise or even at rest.

It has been shown that there often is a very narrow coalescence between the Achilles and the plantaris tendons in Achilles tendinosis (Alfredson, 2011). Releasing and operative extirpation of the plantaris tendon coupled with a scraping technique has shown good results concerning treatment of Achilles tendinosis (Alfredson, 2011).

**Lateral epicondylitis/Tennis elbow**

Lateral epicondylitis and tennis elbow are the most common terms for diagnosis concerning pain over the lateral epicondyle of humerus, the common origin for wrist extensors. Repetitive movements of wrist extensors is the main cause of tennis elbow (Shiri et al., 2006). The term lateral epicondylitis is questioned; the suffix “-it” explains it as an inflammatory condition. Actually no inflammation is seen histologically in the tendon (Potter et al., 1995). Biopsies of the area show, as in Achilles tendinosis, a hypercellularity, neovascularization and unstructured
collagen. Furthermore, there is a pronounced innervation in the area (Ljung et al., 1999).

**Peritendinous tissue in tendinopathy**

Concerning tendinopathy, the surrounding tissue, i.e. the peritendinous tissue has been regarded to be of interest lately. This tissue has thus been found to be of importance in order to help to explain the pain that occurs in tendinosis. In situations with tendon injury due to overload, there is an increase in blood flow and local inflammation in the peritendinous tissue (Kjaer et al., 2013). In Achilles tendinosis patients, biopsies of the peritendinous tissue located ventrally showed presence of nerve fascicles and blood vessels (Andersson et al., 2007). There is an increase in blood flow which can been seen with Ultrasound (US) and Doppler (Alfredson, 2005, Ohberg et al., 2001). This is not the situation in the peritendinous tissue in situations for the healthy Achilles tendon. The peritendinous tissue has been shown to be richly innervated in situations with Achilles tendinosis when the plantaris tendon is tightly located in relation to the Achilles tendon (Spang et al., 2015). There are mainly sensory, but also some sympathetic nerves in the tissue (Spang et al., 2015).

**TNF-alpha system**

**TNF-alpha**

In the late 1960’s several researchers were investigating the possibility of an antitumoral agent in vivo. In 1968, a cytotoxic agent produced by lymphocytes was found and was named lymphotoxin (LT) (Kolb and Granger, 1968). In 1975, another cytotoxic agent was found, produced by macrophages (Carswell et al., 1975). It was named Tumor Necrosis Factor (TNF) because of the capacity to inhibit mice fibrosarcoma, an ability which was also shown for LT. In the 1980’s the DNA of LT and TNF were cloned and were seen to be quite similar (Pennica et al., 1984). Later on, TNF was renamed to TNF-alpha and LT was renamed to TNF-beta. Despite the name, TNF has not been successful in treating cancer, it has rather been the opposite. Via activating Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a pro-inflammatory transcription factor, up-regulation of carcinogenic genes as well as increased proliferation, survival and angiogenesis in tumor cells can occur (Balkwill, 2009).
TNF-alpha is a cytokine that is involved in inflammation. TNF-alpha is mainly synthesized by macrophages (Baer et al., 1998) but can also be produced by other white blood cells including mast cells and lymphocytes, as well by fibroblasts, endothelial cells, adipose tissue cells and neurons (Wajant et al., 2003). Stimulation of monocytes by TNF-alpha leads to cytotoxicity to target cells, and blocking of TNF-alpha inhibits the cytotoxicity (Philip and Epstein, 1986).

TNF-alpha is primary produced as a transmembrane protein (Kriegler et al., 1988). From the transmembrane stage, TNF-alpha can be cleaved to a soluble form, sTNF-alpha (Black et al., 1997).

**TNF receptors**

In 1990, two receptors were found to bind TNF-alpha (Brockhaus et al., 1990). Their sizes were approximately 55kD and 75kD, and therefore they are sometimes referred to as TNFp55 and TNFp75. The most commonly used names today however are TNF Receptor type 1 (TNFR1) for p55 and TNF Receptor type 2 (TNFR2) for p75. TNFR1 is found to be expressed in all kinds of cell types, whereas TNFR2 mainly has been found in haematopoietic cells and other cells of the immune system (Van Herreweghe et al., 2010).

**The actions of TNF-alpha via TNFR1 and TNFR2**

TNF-alpha can bind to TNFR1 and thereafter via pathways activate NF-κB. The transcription factor NF-κB mediates transcription of several proteins involved in cell survival and proliferation, inflammatory responses and protection against apoptosis. This includes other cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6), growth factors, adhesive molecules and other proteins contributing to synthesis of prostaglandins, leukotrienes and nitrogen oxide. TNF-alpha can thus induce apoptosis and necrosis as well as anti-apoptotic effects by signalling via NF-κB through TNFR1 (Van Herreweghe et al., 2010). Binding of TNF-alpha to TNFR1 can also activate the mitogen-activated protein kinase (MAPK) pathway which also leads to activation of transcription factors, involved in cell differentiation, proliferation but which also lead to pro-apoptotic effects. Beyond TNFR1 and TNFR2, TNF-alpha can bind to several other receptors. There is a group of in total 27 receptors in this group and they are together named the TNF Receptor Superfamily. Some of them, one being TNFR1, have a death domain (Wilson et al., 2009).

The role of TNFR2 is much more unclear than that of TNFR1. Unlike TNFR1, TNFR2 has no intracellular death domain, but is still able to contribute to apoptosis (Declercq et al., 1998, Wang and Al-Lamki, 2013). TNF-alpha signaling
via TNFR2 can activate NF-κB, and it seems that this signaling is more longstanding (Rothe et al., 1995) than the signaling via TNFR1. TNFR2 is reported to play a major role in the lymphoid system (Wajant et al., 2003).

TNFR1 binds to both membrane bound and soluble TNF-alpha. TNFR2 is mainly activated via membrane bound TNF-alpha. Not only can the two receptors have independent signaling but they can also influence each other via crosstalks. A crosstalk between TNFR1 and TNFR2 occurs at several levels (Naude et al., 2011). These crosstalks can have both agonistic and antagonistic effects. An example is that stimulation of TNFR2 can enhance TNFR1-induced apoptosis by inhibiting NF-κB anti-apoptotic signaling (Fotin-Mleczek et al., 2002).

TNF-alpha can be of importance for blood regulation via having effects on angiogenesis (Fajardo et al., 1992) and via being a mediator driving blood vessel remodeling in inflammation (Baluk et al., 2009) and TNF-alpha is on the whole reported to have an effect on the proliferation of vascular smooth muscle cells (Qi et al., 2015). The TNF-alpha system is furthermore reported to be upregulated in response to ischemia (Gesslein et al., 2010). However, TNFR1 and TNFR2 appear to play different roles in ischemia-mediated angiogenesis, as well as arteriogenesis, (Luo et al., 2006). Thus, they have opposite effects on the endothelial cells, as seen in a study on a femoral artery ligation model in mice. Thus, endothelial cell survival and migration occurred in response to activation of TNFR2 but not TNFR1 (Luo et al., 2006).

**TNF-alpha and muscle and tendon tissue**

Expressions for TNF-alpha and TNF receptors have been noted for skeletal muscle fibers of patients suffering from idiopathic inflammatory myopathies and Duchenne muscular dystrophy (DMD) (Kuru et al., 2003, De Bleecker et al., 1999, Fedczyna et al., 2001). The levels of TNF-alpha as seen biochemically were on the whole increased in the muscle of these myopathies as well as in the muscle of mdx mice compared to controls (Grounds et al., 2008). Changed TNF-alpha levels in muscle tissue are reported in disease situations. Examination of muscle samples (vastus lateralis) from patients who had suffered from stroke e.g. showed that TNF-alpha mRNA levels were clearly higher in paretic as compared to control leg muscle (Hafer-Macko et al., 2005). Cell culture studies showed that stimulation of myoblasts increased cytokine (IL-6) production (Tseng et al., 2010). The findings of various studies further imply that TNF-alpha has effects for muscle tissue. Results of cell culture studies on muscle cells thus suggest that pro-inflammatory cytokines such as TNF-alpha enhance Fas-mediated apoptosis of these cells (Kondo et al., 2009). Such a proposal was also presented by Efthimiou and collaborators (Efthimiou et al., 2006). Early studies on muscle cells in culture
furthermore led to a suggestion that TNF-alpha can play an important role in the pathogenesis of the muscle destruction that occurs in myositis (Kalovidouris and Plotkin, 1995). On the other hand, TNF-alpha may be important in myogenesis. Myogenesis was decreased when blocking TNF-alpha was performed, and stimulated after adding it (Chen et al., 2007) in injured muscle in mice.

Studies in our department have shown that the tenocytes of tendons, especially those of tendinosis tendons, show marked expression of TNF-alpha and TNF receptors (Gaida et al., 2012). The peritendinous tissue was not examined in these studies.

**TNF-alpha in relation to nerve tissue**

It is since long known that TNF-alpha and TNF-alpha mRNA are upregulated in non-neuronal cells early after nerve injury (Sommer and Schafers, 1998, La Fleur et al., 1996). There is also an upregulation of TNF-alpha in neurons after ischemia (Liu et al., 1994) and an upregulation of TNF-alpha mRNA in dorsal root ganglion neurons after injury (Murphy et al., 1995). Injury of sciatic nerve leads to a marked increase in the anterograde transport of TNF-alpha to the injury site (Schafers et al., 2002) leading to the hypothesis that TNF-alpha is involved in pain sensations after injury and/or degeneration and regeneration after injury. Via performing fMRI, Hess and colleagues (Hess et al., 2011) found that RA patients experienced quick pain relief in response to anti-TNF-alpha treatment. The effect on the inflammation could not be that fast, suggesting that anti-TNF-alpha treatment instead rapidly had an effect on the nervous system.

TNF-alpha and its receptors seem to play a role in neurodegenerative diseases. Whilst signalling by TNFR1 leads to neuronal destruction, binding of TNF-alpha to TNFR2 has a proliferative and neuronal protective function (Fontaine et al., 2002). This means that TNF-alpha can participate in nerve degeneration as well as nerve regeneration (Camara-Lemarroy et al., 2010).

**Rheumatoid Arthritis and TNF-alpha**

Rheumatoid arthritis (RA) is an autoimmune chronic symmetric polyarthritis. There is an inflammation in the synovial membrane, the inner layer of connective tissue in the capsule of joints. Granulation tissue is created (pannus). Pannus is an abnormal tissue which invades and destroys joint structures. Pannus is a vascularized tissue of fibroblasts and several types of white blood cells. The synovial membrane is otherwise rather acellular.
Measuring of cytokines in the inflammatory cells of the synovial fluid showed presence of IL-6, IL-1 and TNF-alpha (Firestein et al., 1990). Expressions of TNF-alpha was then seen in the synovial membrane (Chu et al., 1991) and TNF-alpha was also seen in the serum of RA patients (Tetta et al., 1990). There has been a lot of studies on blocking cytokines since the early 1990s, TNF-alpha not being the obvious target in the beginning. However, Fong and colleagues showed that blocking of TNF-alpha decreased the expression of IL-6 and IL-1 in an animal (baboon) study (Fong et al., 1989). The effect of blocking TNF-alpha was repeated in rheumatoid synovial cultures in vitro, showing decreased expression of several pro-inflammatory cytokines (Haworth et al., 1991, Brennan et al., 1989). RA synovial tissue was in the present Thesis used as a reference tissue concerning visualization of TNF-alpha expressions.

Use of TNF-alpha inhibitors is today a well-established complementary treatment to those RA patients who not respond to other disease modifying drugs such as methotrexate.

Other signal substances in parallel to TNF-alpha

TNF-alpha is known to have inter-relationships with other signal substances. That includes neurotrophins and neuropeptides. In a previous Thesis presented in our Department it was shown that the substance P (SP)/neurokinin 1 receptor (NK-1R) system was upregulated in the myositis process for rabbits (Song, 2013). Of particular interest for the present Thesis is the known fact that there are interactions between TNF-alpha and SP. Therefore a possibility of relationship between TNF-alpha and the SP/NK-1R system was evaluated. There are also numerous other cytokines and further signal substances than TNF-alpha that can have effects in relation to injury/inflammation but which are not in focus in the present Thesis. It is since long known that there are marked interactions between cytokines, neuropeptides, classical nerve transmitters, hormones and other factors in various situations (Hokfelt et al., 1992, Kawamura et al., 1998, Ekblad et al., 2000).

Why study the TNF-alpha system

It is obvious that the features in myositis developing in response to marked overuse as well as in tendinopathy, especially the peritendinous tissue, are not
fully understood. The details in the expressions of the TNF-alpha system for these situations is unclear. As TNF-alpha has such marked effects in various pathological situations and as targeting TNF-alpha is much discussed concerning IIM in man, which shows morphological changes which appear similar to those in our myositis model, further information on the system for these conditions is welcome.
Aim

The aim of the study was to examine the importance of the TNF-alpha system in relation to the myositis that occurs due to marked muscle overuse and in the situation of tendinopathy/tendinosis.

More precisely it was evaluated to what extent the TNF-alpha system is involved in:

1. The evolving inflammatory process
2. The affection of the muscular system (the muscle fibers)
3. The affection of the nerves innervating the myositis and tendinopathy areas

An animal model was used, enabling to evaluate whether muscle overuse ipsilaterally also leads to influences on the TNF-alpha system in the contralateral muscle. Evaluations of human tissue from painful areas in situations with tendinopathy were made with particular focus on the innervation.
Materials and Methods

Obtaining of rabbit muscle tissue

Animals

46 female rabbits were used in the studies. They were 6-9 months old and had an average weight of 4 kg. The animals were divided into eight groups, see table 1.

40 of the animals underwent an exercise experiment leading to marked muscle overuse. The right leg of the animal was exposed to the experiment for two hours every second day for 1, 3 or 6 weeks. Six of the animals were not included in the exercise protocol.

22 of the animals were in the exercise experiment for 1 week and were also given injections. For 17 of these, the purpose of the injections was to achieve muscle inflammation. The injections were given shortly after each experiment session in the loose connective tissue around the Achilles tendon (on the experimental side) of the animals. Substances injected were Sodium Chloride (NaCl), Substance P (SP) (S6883, Sigma), DL-Thiorphan (Th) (T6031, Sigma) and Captopril (Cap) (C4042, Sigma) in different combinations (see table 1). NaCl is a salt solution (given as a control substance), SP is a neuropeptide/neurotransmitter, Th is a neutral endopeptidase inhibitor and Cap is an angiotensin-converting enzyme inhibitor.

<table>
<thead>
<tr>
<th>Group</th>
<th>Exercise</th>
<th>Injection</th>
<th>No. of animals</th>
<th>Papers</th>
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<td>-</td>
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<td>6</td>
<td>I, II, III</td>
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<tr>
<td>2</td>
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<td>1 week</td>
<td>NaCl</td>
<td>5</td>
<td>I, III</td>
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<tr>
<td>4a</td>
<td>1 week</td>
<td>SP+Th+ Cap</td>
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<td>Cap</td>
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<td>6</td>
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Table 1. Groups of animals; experiment period and given injections, having pro-inflammatory effects, or not.
Between experiment sessions the animals were kept in cages allowing movement.

**Experimental design**

Animals were anaesthetized during the exercise experiment. Intramuscular injections of fentanyl-fluanisone (0.095mg/kg fentanyl citrate and 3mg/kg fluanisone) and diazepam (1mg/kg) were given at the start. Further fentanyl-fluanisone (0.03mg/kg fentanyl citrate and 1 mg/kg fluanisone) injections were given every 30-45 min to sustain anaesthesia.

Backman and collaborators (Backman et al., 1990) originally developed a kicking machine. This was used in the study. The right foot was attached to a pedal leading to passive moving of the foot by a pneumatic piston. The size of the movement was set to 9.5 cm, allowing a movement in the range of motion in the ankle of 55-65° with 35-40° plantarflexion and 20-25° dorsiflexion (extension). The flexion-extension movements over the ankle were held at a speed of 150 movements per minute. Simultaneously during the plantar flexion, an active contraction of the m. triceps surae was induced through electrical stimulation. Two surface electrodes (Pediatric electrodes 40 426A, Hewlett Packard, Andover, MA, USA) were placed 2 cm apart from each other over the m. triceps surae. The electrical stimulation of m. triceps surae was synchronized with the plantar flexion movement of the pneumatic piston by a microswitch, which triggered the stimulator unit (Disa stimulator Type 14E, Disa Elektronik A/S, Herlev, Denmark). 85 ms after the initiation of the plantar flexion an impulse with the

Figure 4. Rabbit in the kicking machine during the overuse experiments.
duration of 0.2 ms was delivered at an amplitude of 35-50V. The left foot/left leg were not attached to the kicking machine. The pelvis was strapped down with band to restrict movements to the right foot.

After every training session, the rabbits were given buprenorphine (0.01-0.05 mg/kg) for analgesia.

The day after the last exercise experiment session, the animals were euthanized with an excessive amount of sodium pentobarbital. The triceps surae muscle with tendon was excised from both sides. After the excision of the m. triceps surae from both experimental and non-experimental sides the soleus parts were dissected and cut into pieces.

**Obtaining of human tissue**

**Patients**

Human tissue samples were taken from patients suffering from plantaris-associated Achilles tendinopathy or tennis elbow (pain in the area of the common extensor origin at the elbow region) for at least 3 months. 34 plantaris tissue samples including peritendinous tissue were obtained from 30 patients; four patients had bilateral symptoms (23 men, 7 women, mean age 47 years). Tissue samples from the tennis elbow area were obtained from 4 patients (2 men, 2 women, mean age 46 years). Clinical examinations and tissue collecting were all done by the well-experienced surgeon Håkan Alfredson.

**Surgery for Achilles/plantaris tendinopathy**

The patients with plantaris-associated Achilles tendinopathy/tendinosis were diagnosed by anamnesis of pain and stiffness in combination with signs of midportion Achilles tendinopathy with involvement of the plantaris tendon as seen by UC with Color Doppler. As a treatment for this condition, the plantaris tendon and peritendinous tissue between the Achilles and plantaris tendons were removed (Alfredson, 2011, Masci et al., 2015).

The surgical removing was assessed by a short longitudinal incision at the medial side of the Achilles tendon midportion. The plantaris tendon was then visible and removed together with the fatty richly vascularized loose connective tissue located inbetween the Achilles and plantaris tendons.
**Surgery for tennis elbow**

Pain at palpation of the common extensor origin, pain from the elbow when doing wrist extension to resistance and positive 3rd test gave the diagnosis tennis elbow. The Ultrasound with Color Doppler evaluation showed a high blood flow in the area and structural changes. Skin markers were placed where the Ultrasound with Color Doppler detected high blood flow outside the extensor origin. Local anaesthesia was given by 3-4 ml of Xylocaine-adrenaline (10mg Xylocaine and 5mg adrenaline per ml) and the connective tissue from the region with thickened fibrous tissue was removed via minimal invasive procedure.

**Reference tissue from RA synovium**

In paper I, RA synovial tissue was used as a reference tissue in the studies on animal tissue. Synovial tissue was collected during surgery for knee prosthesis. The tissue was fixed and stained in the same way as was the Achilles/plantaris and animal tissue, see below.

**Fixation and sectioning**

**Rabbit muscle tissue**

Pieces of soleus muscle of an approximate size of 5-8x10mm were taken care of in two different ways. They were either directly mounted in an optimal cutting temperature (OCT) compound (Tissue Mek, Miles Laboratory, Naperville, IL, USA) on a cardboard and frozen in propane chilled with liquid nitrogen in -80°C or fixed by immersion overnight in 4°C in 4% formaldehyde in 0.1 M phosphate buffer pH 7.0. The latter were then washed in Tyrode’s solution (10% sucrose) at 4°C overnight and then mounted and frozen as described above.

The samples were cut by a cryostat (Leica Microsystem CM 300, Heidelberg, Germany) into 5-8µm thick sections and mounted on glass precoated with chrome-alum gelatin.

**Human tissue**

Directly after surgery, the tissues samples were put in a fixative solution (4% formaldehyde in 0.1 M phosphate buffer, pH 7.0) at 4°C overnight. Then the tissue was washed three times in Tyrode’s solution (10% sucrose), the first
washing step being performed at 4°C overnight. The tissue samples were cut into smaller pieces and were then mounted on a thin cardboard hooped by OCT embedding medium (Tissue Mek, Miles Laboratory, Naperville, IL, USA). The last step involved freezing which was performed as described above.

The tissue samples were cut by into 7 µm thick sections by a cryostat (Leica Microsystem CM 300, Heidelberg, Germany) and then mounted on superfrost plus slides (Thermo Scientific, Braunschweig, Germany).

**Staining for morphology**

One section of all of animal and human tissue samples was stained with Hematoxylin & Eosin (H&E) for demonstration and investigation of morphology. The sections were put onto slides and put in Harris hematoxylin for 2 min. They were then rinsed in distilled water and then dipped in acetic acid for 15 seconds. A new rinsing in 37°C tap water followed before the sections were stained in 1% eosin for 1 min. Then the sections were dehydrated in ethanol 2 min three times. Finally, the cover glass was placed on top of the sections.

**In situ hybridization**

In situ hybridization (ISH) was used for detection of TNF-alpha and TNFR1 mRNA in animal tissue (I-III). Representative specimens from experimental and control animals were selected, in total 25. Both experimental and non-experimental sides were included. The tissue specimens were cut into 10 µm thick sections with a cryostat and mounted onto Super Frost Plus slides (nr.041200, Menzel Gläser, Braunschweig, Germany). Digoxigenin (DIG)-hyperlabeled oligonucleotide probes (ssDNA) were used to evaluate the mRNA. The antisense probe sequences are described below (table 2).

The procedures were carried out according to an established protocol (Panoskaltsis-Mortari and Bucy, 1995). The dilution was 50 ng in 15µl hybridization solution and an alkaline phosphatase (AP)-labelled anti-D16 antibody (Roche Germany, 11 093 274 910) was used for detection. Corresponding sense DIG-hyperlabeled ssDNA probes were used as negative controls and a β-actin antisense probe was used as a positive control.
Sections were then mounted in Pertex mounting medium. For further details, see paper I and III.

<table>
<thead>
<tr>
<th>Table 2.</th>
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</thead>
<tbody>
<tr>
<td><strong>Probe</strong></td>
<td><strong>Code</strong></td>
<td><strong>Source</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>GD1001-DS</td>
<td>Gene Detect, New Zealand</td>
<td>CGGCGAAGCGGCTGACAGTG TGAGTGAGGAGCAGTGAGGA GCGGCAGC</td>
</tr>
<tr>
<td>TNFR1</td>
<td>GD1001-DS</td>
<td>Gene Detect, New Zealand</td>
<td>TCCTCGATGTCCCTCCAGGCA GCCCAGCAGGTCCATGTCGC GGAGCAGC</td>
</tr>
</tbody>
</table>

Table 2. Sequences for antisense probes for detection of TNF-alpha mRNA and TNFR1 mRNA.

**Immunohistochemistry**

Immunohistochemical procedures were performed to detect TNF-alpha, TNFR1 and TNFR2. Antibodies were goat polyclonal IgG antibodies. Mainly fixed but to some degree unfixed tissue were investigated. Some of the sections were dipped in potassium permanganate for 2 min with the purpose to enhance the immunofluorescence reactions. Most sections did not undergo this step. Firstly, the sections were defrosted and dried before being rinsed in 0.01 M phosphate buffer saline (PBS), pH 7.2 with sodium azide, three times for 5 min. The sections were then put in PBS with 1 % Triton X-100 for 20 min (Kebo lab, Stockholm) and rinsed in PBS 3x5 min. An incubation in 5% normal donkey serum (code no: 017-000-121, Jackson Immune Research Lab. Inc) diluted in PBS for 15 min followed. Then the sections were incubated with the primary antibody diluted in PBS for 60 min in 37°C. The sections were washed in PBS 3x5 min and then another incubation in normal donkey serum followed. After this, the sections were incubated with the secondary antibody diluted in PBS. For labelling with goat antibodies FITC-conjugated donkey-antigoat (I-IV) or Alexa FluorO 488 donkey-antigoat (I, II) (secondary antibody) were used. Finally the sections were rinsed in PBS 3x5 min and mounted with Vectashield Mounting Medium (H-1000, Vector Laboratories, Burlingame, CA, USA) (I, II, IV) or Vectashield Antifade Mounting Medium (III). In some sections Vectashield Antifade Mounting Medium with DAPI (H-1500, Vector Laboratories, Burlingame, USA) was used for marking of cell nuclei (III).
Also other substances were stained for. The primary antibodies used were mouse monoclonal antibodies (antibodies against CD68, neutrophils/T-cells, mast cells, fibroblasts, eosinophil peroxidase, βIII-tubulin, neurofilament and Schwann cells). In this case no incubation with potassium permanganate was performed. Rabbit normal serum was used (code no: X0902, DakoCytomation, Glostrup, Denmark) and dilutions were diluted in PBS with bovine serum albumin (BSA). The staining procedures were otherwise as described above.

Double staining related to stainings using various combinations were also performed (see below). That included stainings for CD68, T-cell/neutrophil marker, NK-1R, desmin, Pax7, CD 31, Schwann cells and S-100β. A list of all used antibodies is shown in table 3.

<table>
<thead>
<tr>
<th>Table 3. List of primary antibodies.</th>
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</thead>
<tbody>
<tr>
<td><strong>Antigen</strong></td>
</tr>
<tr>
<td>TNF-alpha</td>
</tr>
<tr>
<td>TNF-alpha</td>
</tr>
<tr>
<td>TNFR1</td>
</tr>
<tr>
<td>TNFR2</td>
</tr>
<tr>
<td>CD68</td>
</tr>
<tr>
<td>T-cell/Neutrophil marker</td>
</tr>
<tr>
<td>NK-1R</td>
</tr>
<tr>
<td>Desmin</td>
</tr>
<tr>
<td>Pax7</td>
</tr>
<tr>
<td>CD31</td>
</tr>
<tr>
<td>S-100β</td>
</tr>
<tr>
<td>βIII-tubulin</td>
</tr>
<tr>
<td>Neurofilament</td>
</tr>
<tr>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>Mastcells</td>
</tr>
<tr>
<td>Fibroblasts</td>
</tr>
</tbody>
</table>
**Control stainings**

Control stainings with preabsorbed antibody was made in parallel with ordinary stainings for the elements in the TNF-alpha system. Peptides used for the preabsorption were thus TNFR1, and TNFR2 antigens (peptides provided by Santa Cruz Biotechnology, Dallas, TX, USA) (III). Furthermore, the TNF-alpha antibody used in I and II was preabsorbed with TNF-alpha antigen T6674 (Sigma). The antibodies were incubated with the peptides 4°C overnight, and then the same staining procedure as above followed. Controls also included stainings when the primary antibody was eliminated. All the antibodies used have been previously utilized and tested in the laboratory.

**Double staining**

For interpreting of the structures expressing TNF-alpha, TNFR1 and TNFR2 immunoreactions double stainings were made (I, III, IV). These stainings were performed on fixed and unfixed tissue with different combinations of the antibodies. The sections were incubated with the primary antibody (polyclonal antibody against TNF-alpha, TNFR1 or TNFR2) in 4°C overnight and then incubated with FITC-conjugated donkey antigoat secondary antibody for 30 min in 37°C. Then followed a new incubation with another primary antibody (monoclonal antibody), use of normal donkey serum and wash in PBS 3x5 before incubation with a different secondary antibody (TRITC-conjugated, Red-X-conjugated or Alexa-fluor conjugated antibody).

In order to help analyzing the cell stage (regeneration/degeneration) in abnormal muscle fibers expressing TNF-alpha and/or TNFR1 mRNA double stainings with NK-1R and desmin were performed (II, III). Parallel sections to sections processed with in situ hybridization for TNF-alpha and TNFR1 mRNA were thus processed with immunohistochemistry. These parallel sections were double stained with NK-1R (sc-5220, Santa Cruz Biotechnology, Dallas, TX, USA) and desmin (M0760, Dako Cytomation, Glostrup, Denmark). Firstly the sections were immunohisto-chemically processed with the NK-1R antibody, in the same way as described above including use of the same normal serum and secondary antibody. Staining for desmin followed. Rabbit normal serum was then utilized and the secondary antibody used was an anti-mouse immunoglobulin/TRITC (R0276, Dako, Denmark)

For further descriptions of the procedures for single and double stainings, see I-IV. List of all secondary antibodies used is shown below (table 4).
Table 4. List of secondary antibodies.

<table>
<thead>
<tr>
<th>Secondary ab</th>
<th>Code</th>
<th>Source</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-conjugated Affini Pure Donkey Antigoat</td>
<td>705-095-147</td>
<td>Jackson ImmunoResearch</td>
<td>I-IV</td>
</tr>
<tr>
<td>Alexa FluorO 488 Donkey Antigoat</td>
<td>A-11055</td>
<td>Invitrogen</td>
<td>I</td>
</tr>
<tr>
<td>TRITC-conjugated Rabbit Antimouse</td>
<td>R0276</td>
<td>DakoCytomation</td>
<td>I</td>
</tr>
<tr>
<td>Alexa FluorO 568 Donkey Antigoat</td>
<td>A-11057</td>
<td>Invitrogen</td>
<td>II</td>
</tr>
<tr>
<td>TRITC Rabbit Antimouse</td>
<td>R0276</td>
<td>DakoCytomation</td>
<td>II, IV</td>
</tr>
<tr>
<td>Rhodamine Red-X-conjugated</td>
<td>713-295-003</td>
<td>Jackson ImmunoResearch</td>
<td>III</td>
</tr>
<tr>
<td>Alexa Fluor 647-conjugated antiserum</td>
<td>S21374</td>
<td>Invitrogen</td>
<td>III</td>
</tr>
</tbody>
</table>

Identification of neuromuscular junctions and cell nuclei

Some of the sections were labelled with α-bungarotoxin for demarcation of neuromuscular junctions (NMJ). Some sections processed for double staining were mounted in Vectashield Antifade Mounting Medium with DAPI (H-1500, Vector Laboratories, Burlingame, USA) for marking of cell nuclei.

Visualizing of the results

The single stainings were evaluated in a Zeiss Axioscope 2 plus microscope equipped with epifluorescence technique and an Olympus DP70 digital camera. The results of double stainings were examined by a Leica DM600B fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). Photos were then taken by a color CCD camera (Leica DFC 490) and a digital high-speed fluorescence CCD camera (Leica DFC360 FX).

Quantification

It was found relevant to quantitatively evaluate data in paper III. For this purpose, animals were divided into two groups; animals that had a normal
morphology and those that had developed a clear myositis (irrespective of group/treatment). Then the degrees of immunoreactions for TNFR1 and TNF2 were evaluated semi-quantitively for the most relevant structures.

**Ethics for rabbit studies**

The study protocol was approved by the local ethical committee at Umeå University (A 34/07, A95/07). The approval was obtained before the start of the study. A licensed breeder had bred all animals for the sole purpose of being used in animal experiments. All efforts were made to minimize animal suffering.

**Ethics for human studies**

The study on tendinopathy/tennis elbow materials was approved by the Regional Ethical Board in Umeå (dnr 04-157M; 2011-83-32M). The use of control synovial material is in accordance with previous approval (dnr 2011-318-32M; 05-016M) (for RA). The experiments were performed according to the principles expressed in the Declaration of Helsinki. All patients included had given an informed consent.
Results

Rabbit muscle tissue (I-III)

Morphology

The animals which did not participate in the muscle overuse experiment showed a normal morphology. There was a diminutive variation in muscle fiber size, the fibers were tightly packed and there was no visible muscle fiber necrosis. The 1-week group given no injection treatment or injections with NaCl showed a comparable morphology with the exception of occasional abnormal muscle fiber appearances. Morphological changes were on the other hand seen in the 1-week group given injections having pro-inflammatory effects, and in the 3, and 6, week groups (I-III) (Fig. 5). The changes were most obvious for the animals in the 6-week group (group 6). There was an increase in loose connective tissue, variations in muscle fiber sizes and an increase in number of internal nuclei within muscle fibers. Areas of myositis (areas with marked invasion of white blood cells coupled to muscle fiber changes) were also regularly seen. There was

Figure 5. Normal muscle morphology in (a) (control animal), myositis areas in (b) and (c) (experimental, 6 week group) in rabbit muscle tissue. White arrows at abnormal muscle fibers with an increase in internal nuclei. Black arrows at muscle fibers totally invaded by white blood cells.
thus a distinct infiltration of white blood cells in the loose connective tissue and in muscle fibers showing features of necrosis. Abnormal muscle fibers which were not undergoing necrosis could also been seen in the areas of myositis.

**Bilateral involvement as seen morphologically**

Morphological changes were seen for both experimental and non-experimental sides (II, III). The morphological features described above could thus be seen in both sides. They occurred to a similar extent on both sides. The 1-week groups not given injection or being injected with NaCl had only occasional abnormal muscle fibers bilaterally and all other experimental groups showed the above described changes, including myositis, bilaterally.

**In situ hybridization (ISH)**

Sections of specimens were investigated for detection of TNF-alpha and TNFR1 mRNA (I-III). Both sides of experimental animals and specimens of nonexperimental animals were evaluated. There was no mRNA for TNF-alpha and TNFR1 in the control group (group 1). The abnormal muscle fibers occasionally seen in the 1 week group were found to express both TNF-alpha and TNFR1 mRNA (the TNFR1 mRNA reactions for necrotic fibers were localized for infiltrating white blood cells). In the 1-week group given injections of pro-inflammatory character (group 4a-c) and in 3- and 6 week groups (groups 5, 6) TNF-alpha and TNFR1 mRNA were seen to a large extent. That included reactions in the white blood cells of inflammatory infiltrates (Fig. 6) and in the white blood cells invading necrotic muscle fibers. The abnormal muscle fibers in the myositis areas that were not necrotic had a patchy and widespread reaction for TNF-alpha and TNFR1 mRNA. TNF-alpha mRNA was also seen in some of the vessels and fibroblasts of

![Figure 6. Dispersed cells in rabbit muscle tissue (experimental group with pro-inflammatory injection, group 4a). Staining for demonstration of TNF-alpha mRNA. Cells in the loose connective tissue show reactions (arrowheads).](image)
the myositis animals. TNFR1 mRNA reactions were seen in some of the fibroblasts in the myositis group but never in the control animals.

The abnormal non-necrotic muscle fibers showing TNF-alpha mRNA (II) and TNFR1 mRNA (III) were compared concerning immunoreactions (IR) for desmin and NK-1R via stainings of parallel sections. The stainings showed that these muscle fibers had a strong and generalized desmin IR and a point-like NK-1R IR. The interpretation was that the fibers are in a regenerative/reparative state (II, III). Muscle fibers with normal morphology expressed a striated desmin IR pattern and no NK-1R IR.

The same ISH results were seen for both experimental and non-experimental sides.

*Immunohistochemistry (IHC)*

**Fibroblasts**

In the 1 week groups given injections with pro-inflammatory effect (group 4a-c), and the 3 and 6 week groups (groups 5, 6) fibroblasts in the connective tissue areas were seen to express TNFR2 immunoreactions (IR), most clearly so in the 6 week group (III). A similar pattern was seen bilaterally. Fibroblasts in the
control group or 1 week group without injections did not show TNFR2 IR. TNF-alpha and TNFR1 IR were only seen in some fibroblasts in the 6 week group.

**White blood cells**

Myositis areas with infiltration of white blood cells were especially seen in the 6 week group but were also seen in the 1 week groups with injections and 3 week group. IR for TNF-alpha and TNF receptors were seen in white blood cells on both sides. TNFR2 IR was seen in most white blood cells, TNF-alpha and TNFR1 IR were seen to a lesser extent.

**Muscle fibers**

There was no IR for TNF-alpha in muscle fibers. In comparison, TNF-alpha mRNA could be seen in muscle fibers (see above). However, IR for both receptors were seen in muscle fibers. TNFR1 and TNFR2 showed different expression patterns. TNFR2 IR was seen in small rounded structures in the outer part of the muscle fibers for all groups bilaterally including in control animals (Fig. 8). Double stainings verified that these rounded small structures corresponded to myonuclei of the muscle fibers. TNFR2 IR was also seen in invading white blood cells in muscle fibers showing a necrotic appearance.

TNFR1 IR was never seen in muscle fibers in control animals. TNFR1 IR was on the other hand found to be spread diffusely in the cytoplasm, especially seen close to the cell membrane (Fig. 9), in certain muscle fibers of the myositis animals (corresponding to fibers classified as abnormal non-necrotic muscle fibers). This expression pattern was

![IHC - TNFR2](image)

**Figure 8.** Sample from control group. Small rounded structures in the outer parts of the muscle fibers are showing TNFR2 IR in (a). Fig (b) is a control staining with antibody being preabsorbed with antigen. Parallel stainings showed that these small structures correspond to myonuclei.
seen on both experimental and contralateral sides and was most obvious for the 6 week group. These fibers were not invaded by white blood cells.

![IHC - TNFR1](image1.png) ![IHC - TNFR1 - Preabsorbed antibody](image2.png)

Figure 9. Sample from 6 week group. White small dots in the outer parts of muscle fibers are showing TNFR1 IR in (a) (asterisks). Fig (b) is a control staining with preabsorbed antibody.

**Blood vessel walls**

TNFR2 IR was seen in some of the blood vessel walls in all groups (III), but more often and exhibiting a stronger IR in animals in the 6-week group than other groups. The TNFR2 IR was localized to the smooth muscle layer of the vessels. There was no TNFR2 IR in capillaries.

TNFR1 IR was occasionally seen in blood vessel walls of the myositis animals. The immunoreactivity was located to the nuclei of the cells in the smooth muscle layer.

TNF-alpha IR was not seen in blood vessel walls.

**Nerve fascicles**

There was no clear TNF-alpha IR in nerve structures. However, reactions for both receptors were seen in nerve fascicles (III). TNFR IR was however only very occasionally seen in nerve fascicles in the control group and 1 week group with no pro-inflammatory injections. In animals with longer experiment periods (3 and especially 6 weeks) or with pro-inflammatory injections for 1 week animals
TNFR1 and TNFR2 IR were more clearly seen. Double stainings showed that TNFR1 IR was localized in Schwann cells and axons, whilst TNFR2 was only seen in Schwann cells.

Figure 10. Nerve fascicles showing IR for TNFR1 in myositis animal. Parts of large nerve fascicle (N) in (a) and small nerve fascicles in (b, c). Immunoreactions occur in the form of whitish reactions. Asterisks at perineurium. Samples from 3 and 6 week.

Neuromuscular junctions

TNFR2 IR was seen in neuromuscular junctions (NMJ) for all tissue samples including those of non-experimental animals. In order to localize NMJ in sections labelling with α-bungarotoxin for demarcation of NMJ was made. TNF-alpha and TNFR1 IR was never seen for NMJ.

Figure 11. Stainings for TNFR2 (a) and staining with α-bungarotoxin in a parallel section (b). Arrows at neuromuscular junction, where TNFR2 is observed. Arrowheads at small vessels with IR for TNFR2. Control animal.
For summary of the TNF receptors expression patterns, see study (III).

**Human tissue samples (IV)**

Nerve fascicles and fine nerve fibers were seen in the peritendinous tissue (loose connective tissue) of both tennis elbow and Achilles/plantaris specimens. Close to the nerve fascicles small vessels were frequently observed. Arterioles and venules were also seen. There were numerous dispersed cells in the peritendinous tissue as well.

**Dispersed cells**

The dispersed cells in the peritendinous tissue represented white blood cells and fibroblasts. Most of the white blood cells were macrophages. TNF-alpha IR and TNFR1 IR were frequently seen in fibroblasts whilst TNFR2 IR was usually not seen in these cells. Macrophages frequently exhibited TNFR1 IR and to some extent TNFR2 IR. TNF-alpha IR was never seen in macrophages. The mast cells showed TNF-alpha IR, and to some extent TNFR1 and TNFR2 IR.

![Figure 12. Dispersed cells in the peritendinous tissue, immunoreaction for TNFR1. Achilles/plantaris peritendinous tissue.](image)
**Nerve fascicles**

The nerve structures were visualized via staining for neurofilament and βIII-tubulin. There was a difference in the pattern of neurofilament/βIII-tubulin IR between different nerve fascicles. Some of them were homogenously stained for neurofilament/βIII-tubulin whilst others were not. These homogenously stained (for neurofilament/βIII-tubulin) did not express TNF-alpha IR at all, showed only very limited reaction for TNFR1 IR and TNFR2 IR to some degree. Nerve fascicles with non-homogenous IR for neurofilament/βIII-tubulin had in comparison a distinct increase in magnitude of TNFR1 and TNFR2 IR compared to the homogenously stained nerves fascicles (figure 13 and 14).

![Figure 13](image13.png)

**Figure 13.** Parallel sections of a nerve fascicle in the connective tissue from a tennis elbow patient. Asterisks indicate corresponding parts of perineurium. The nerve fascicle is homogenously stained for neurofilament (a) and βIII-tubulin (b). There is no TNF-alpha IR (c), limited TNFR1 IR (d) and to some extent TNFR2 IR (e). Arrowheads at immunoreactions.

![Figure 14](image14.png)

**Figure 14.** Parallel sections of a nerve fascicle in the connective tissue of an elbow patient. Asterisks at corresponding parts of perineurium. The nerve fascicle is not homogenously stained for neurofilament (b) and βIII-tubulin (b) indicating loss of axons. Arrowheads indicate occurrence of some TNF-alpha IR in (c), strong TNFR1 IR in (d) and a particularly distinct TNFR2 IR in (e).
Blood vessel walls

Reactions for TNFR2 were seen for the blood vessel walls in the peritendinous tissue (arterioles, venules and capillaries). The TNFR2 IR was primary seen in the smooth muscle layer of arterioles, but also larger blood vessels and capillaries did to some extent exhibit TNFR2. TNFR1 IR were also seen in blood vessels walls but to a lesser extent. There were also blood vessels walls with no immunoreaction for either TNFR2 or TNFR1 in the peritendinous tissue. Weak TNF-alpha IR were seen for some of the small blood vessel walls.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>TNF-alpha</th>
<th>TNFR1</th>
<th>TNFR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Normal nerve fascicles</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>Nerve fascicles with axonal loss</td>
<td>-/+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 6. Summary of results for the most frequently occurring cell types and the nerve fascicles in the human peritendinous tissue. (-) is for no IR, (-/+): occasionally seen/weak IR, (+): moderately seen IR, (++): frequently seen IR.
Discussion

Major findings

A major finding in this Thesis is that the TNF-alpha system is found to be highly expressed in the myositis process that occurs after experimental muscle overuse. That includes occurrence of TNF receptor reactions in white blood cells, fibroblasts and blood vessel walls and most interestingly also in abnormal muscle fibers; TNFR1 reactions were seen in the interior of non-necrotic muscle fibers and TNFR2 reactions were noted for the white blood cells that invaded the necrotic muscle fibers. Furthermore, the changes that were noted concerning the TNF-alpha system occurred not only on the experimental side but also contralaterally. Thus, upregulations in expressions of TNF-alpha and the TNF receptor reactions were noted bilaterally.

The studies on painful areas in humans, namely peritendinous tissue of patients with Achilles tendinosis and tennis elbow, complemented the experimental studies. TNF receptor reactions were also frequently noted for the cells in the human peritendinous tissue, the cells mainly corresponding to fibroblasts and macrophages. Reactions were also seen for blood vessel walls.

An especially important finding was related to the findings for the nerve structures in both the areas of the myositis process in the experimental situation and those of the painful areas of humans. Thus, there was a clear increase in TNF receptor expressions in the experimental situation. With respect to Achilles/plantaris tendinosis and tennis elbow it was noted that the nerve fascicles that exhibited features of axonal loss showed clearly more distinct TNF receptor reactions than the nerve fascicles that were normally appearing.

In total, it is apparent that the TNF-alpha system seems to be markedly involved in the processes that occur in tissues of the locomotor system that is under influence of marked overuse and that is affected by chronic pain. Via examinations of the experimental model and the painful areas of humans, a picture of the importance of the TNF-alpha system could thus be depicted for the evolving inflammatory process, the muscle tissue (the muscle fibers) and the nerve structures.
Strengths, limitations and methodological considerations

Via the use of a model in rabbits, the features of the TNF-alpha system could be followed in response to development of myositis and muscle fiber and nerve influences in a situation with overuse. One should have in mind that the use of a special apparatus (the kicking machine), the use of electrical stimulation and the injecting of substances having pro-inflammatory effects are things that to different extents can contribute to the outcome of the experiment. Furthermore, obligatory parts were the giving of anesthesia during the exercise and the analgesic substance afterwards. Nevertheless, although the overuse experiment with rabbits indeed is an experimental situation, valuable information can be obtained via the model and which can not be directly obtained from studies on human beings. The development of morphological features and the changes in expressions for the elements in the TNF-alpha system could be followed via evaluations at different time points. Furthermore, a main aspect is that comparisons with the human situation could be obtained via evaluations of painful areas (peritendinous tissue in association with painful tendons and tissue from tennis elbow). It was therefore logic to combine both experimental studies with studies on human tissue.

In future studies using the experimental model still other muscles should be evaluated, including muscles from both sides. Furthermore, evaluations at the level of the spinal cord/spinal ganglia would be worthwhile. In future studies it would also be of interest to explore the muscle fiber changes in relation to fiber type.

An aspect that could be looked upon as a limitation is that control tissue for humans could not be analyzed. That is completely related to ethical considerations. It would thus not be ethically correct to do the kind of operations that were made on the tendinosis/tennis elbow patients on completely healthy individuals. However, due to the fact that marked infiltrations of dispersed cells (white blood cells and fibroblasts) are likely not to occur for healthy persons and due to the fact that abnormal nerve fascicles could be compared with normal such ones important information could be obtained.

Another limitation might be methodologically related to the staining procedures. Thus, it is well-known that variable results can be obtained with various antibodies, although these by the companies are reported to detect a certain substance. Nevertheless, control stainings including preabsorption stainings for IHC and stainings using positive and negative controls for IHS were made. Furthermore, the results from IHC could be compared with those from ISH. It is also a fact that two different TNF-alpha antibodies were used in the present
studies. Furthermore, in parallel with stainings using one of the currently used TNF-alpha antibodies, still another TNF-alpha antibody from another source was utilized in our previous studies on the TNF-alpha system for tendon tissue proper (Gaida et al., 2012). These previous studies showed that the reaction patterns were similar with both antibodies.

**TNF-alpha in relation to the inflammatory process**

It is well-known that the TNF-alpha system is involved in inflammation (Vassalli, 1992, Munro et al., 1989, Feldmann et al., 1996). Accordingly, it was observed that TNF-alpha mRNA and TNF-alpha IR (I, II), TNFR1 mRNA (III), and immunoreactions for both TNF receptors (III) were detected in the infiltrating white blood cells. Double-stainings in (I) showed that a coexistence between TNF-alpha and CD68 (macrophage marker) occurred. The situation was different for the human peritendinous tissue where macrophages did not display TNF-alpha IR. On the other hand, the macrophages in the latter tissue very frequently displayed TNFR1 IR (IV). Overall it is apparent that the TNF-alpha system has a relation to the infiltrating white blood cells in the tissues evaluated in the present Thesis. That included a relationship to the white blood cell infiltration into the muscle fibers that became necrotic.

**TNF-alpha in relation to damage and reparation of muscle fibers**

It is well-known that TNF-alpha can have detrimental effects, including a role in development of the injury that occurs in ischemia (Gesslein et al., 2010). Concerning the situation in inflammatory myopathies, it has been suggested that TNF-alpha can be of significance for the myositis development (Efthimiou et al., 2006) and to be involved in the damage of the muscle fibers (Tews and Goebel, 1998). The results of the present Thesis showed that TNF-alpha mRNA was detected in the white blood cells (I, II) and TNFR1 mRNA and TNFR2 IR in those that had infiltrated into necrotic muscle fibers (III). Thus, TNF-alpha can be considered to contribute to necrotic processes via acting on the infiltration of white blood cells. Nevertheless, a degeneration/necrosis of the muscle fibers is necessary in order to give place for the forthcoming reparation of the tissue.
TNF-alpha can also have reparative/regenerative functions (Inoue et al., 2000), including for muscle tissue (Karalaki et al., 2009) and for tendons (Schulze-Tanzil et al., 2011). The observations on desmin immunoreactions in the present Thesis are hereby of interest. It is namely shown that overexpression of desmin occurs during regenerative phases of skeletal muscle (Gallanti et al., 1992), a feature that we noted for the abnormal non-necrotic muscle fibers displaying TNF-alpha mRNA (II) and TNFR1 mRNA (III). In the immunohistochemical analysis it was furthermore noted that such abnormal muscle fibers displayed TNFR1 IR (III). It was also observed that TNFR2 IR was detected in internal nuclei of muscle fibers in the experimental groups (III), a feature that also can indicate a reparative capacity.

**TNF-alpha in relation to nerve influences**

It is known that TNF-alpha can be a mediator of pain (Boettger et al., 2008) and that TNFR1 and TNFR2 can be found in nociceptors in pain situations (Schaible, 2010, Hess et al., 2011). TNF-alpha neutralization in animals in a rat model of antigen-induced arthritis had a pronounced antinociceptive effect (Boettger et al., 2008). The release of TNF-alpha from activated glia cells can cause pain by acting on spinal cord dorsal horn neurons (Suter et al., 2007). Expressions for the elements in the TNF-alpha system were extensively analyzed for in the nerve structures in the present Thesis. Study III showed that immunoreactions for TNFR1 and TNFR2 became clearly more evident in nerve fascicles with increasing experimental time for the rabbits and study IV showed that the abnormal nerve fascicles in human peritendinous tissue displayed clearly more evident receptor reactions than normal nerve fascicles. Both findings suggest that the TNF-alpha system is involved in damaging/painful situations. To what extent the findings relate to nerve fiber degeneration or attempts for regeneration remains to be answered. One possibility is that both types of functions occur. In any case, these types of findings of TNF receptor reactions in nerve fascicles have not previously been made for skeletal muscle nor tendons.

The results of a recent study suggest that TNF-alpha has a function in relation to neurite outgrowth (Pozniak et al., 2016) and treatment with TNF-alpha inhibitor in a rat model showed a protective effect for axons, but did not recover the demyelination that occurred in the model used (Buyukakilli et al., 2014). Furthermore, attenuation of TNFR expression has been shown to be associated with recovery from nerve injury (Andrade et al., 2014). Nevertheless, TNF-alpha is known to not only participate in nerve regeneration but also nerve degeneration (Camara-Lemarroy et al., 2010).
TNF-alpha in relation to substance P

It is previously known for various tissues that there is an interrelationship between the SP system and the TNF-alpha system (Brunelleschi et al., 1998, Denadai-Souza et al., 2009). Neuropeptides can on the whole influence the production of cytokines (Kawamura et al., 1998), SP for example enhancing the secretion of TNF-alpha from neuroglial cells stimulated with lipopolysaccharide (Luber-Narod et al., 1994). Furthermore, SP is shown to selectively activate TNF-alpha gene expression in murine mast cells (Ansel et al., 1993). In the present Thesis therefore comparisons between the systems were made, via stainings for the NK-1R in the case of the SP system (II, III). It was found that the systems occur in parallel for the muscle fibers undergoing necrosis as well as those undergoing presumable reparation. One possibility is that NK-1R activation via SP is involved in the activation of the TNF-alpha system. Thus, both systems can be related to the attempts for reparation of the muscular tissue. Presumably, also other signal substance systems are co-operating.

TNFR2 at neuromuscular junctions

In the present Thesis, immunoreaction for TNFR2 but not TNFR1 was noted for the NMJ (III). That was the case for all animal group. Such a diversity between TNFR1 and TNFR2 has not previously been shown. The findings show that the TNF-alpha effects that occur at the NMJ are related to TNFR2 effects. Very little is described in the literature concerning the NMJ in relation to TNF-alpha. What is known is that TNF-alpha transiently increases the frequency of miniature endplate potentials in rats (Caratsch et al., 1994) and that deletion of TNFR2 impairs motor performance in mice (Probert, 2015).

Findings of nerve influences concerning the TNF-alpha system bilaterally

Bilateral effects in muscle strength has been seen after unilateral exercise experiments (Slater-Hammel, 1950, Komi et al., 1978). In our studies, experimental unilateral muscle overuse led to morphological muscle changes bilaterally. There was no difference in the expression of the TNF-alpha system between experimental and non-experimental sides, including the expression patterns in nerves. One possibility is that effects via the nervous system are
involved in the morphological and TNF-alpha related changes that occur bilaterally. Effects via the circulatory system can not completely be ruled out. Further studies are needed to solve the aspect concerning bilateral features.

Earlier research in our group showed the occurrence of the bilateral involvement of the SP-system after unilateral muscle overuse using the presently used model (Song et al., 2013). Bilateral upregulation of TNF-alpha and IL-10 in dorsal root ganglia has been seen previously after unilateral sciatic nerve injury in rats (Jancalek et al., 2010). Immune activation close to a peripheral nerve leads to allodynia not only in the ipsilateral side but also contralaterally (Chacur et al., 2001). It is also shown that TNF-alpha can trigger and maintain bilateral inflammatory pain after unilateral treatment of TNF-alpha (Russell et al., 2009).

**TNF-alpha in relation to the blood vessels**

The TNF-alpha system can be involved in effects on the vasculature, e.g. having a stimulation effect on angiogenesis (Gesslein et al., 2010). There is also evidence which suggests that there is a vascular involvement in the pathogenesis of idiopathic inflammatory myopathies, there being a role of vascular cell dysfunction and hypoxia in this pathogenesis (Grundtman and Lundberg, 2009). In the current Thesis, it was found that there was a marked expression of TNFR1 and especially TNFR2 in blood vessel walls in the human peritendinous tissue (IV). In (III), it was noted that TNFR2 IR was frequently seen in the blood vessels walls of experimental animals. The findings suggest that effects via the TNF-alpha system can be of importance in remodeling processes in the painful peritendinous tissue as well as in the myositis process. An importance in relation to remodeling has also been suggested for TNF-alpha in inflammation processes in the airways (Baluk et al., 2009).

**What about anti-TNF treatment? Should instead substances be given with TNF-alpha agonistic effects?**

TNF inhibitors are indicated in the treatment of e.g. RA, morbus Crohn and ulcerative colitis. The use of anti-TNF treatment has greatly improved the situations for RA patients. It has also been shown that patients with RA that are treated with TNF inhibitors have a significantly decreased risk of cardiovascular events (Roubille et al., 2015)
The use of anti-TNF treatment (etanercept) has been noted to reduce the breakdown of muscle tissue in dystrophic mdx mice, the model for Duchenne Muscular Dystrophy (Hodgetts et al., 2006). Preliminary studies were also previously presented which on the whole suggested that TNF-alpha may be a target for myositis development (Chevrel et al., 2005, Baer, 2006). A few case reports has shown improvement for the IIM patients (Hengstman et al., 2003, Anandacoomarasamy et al., 2005). Efthimiou and colleagues showed an improvement in muscle strength and small decrease in CK (Efthimiou et al., 2006). Another study showed no improvement in muscle strength but a corticosteroid-sparing efficiency (Amato, 2011). Nevertheless, researches also early concluded that more research was needed in order to clarify if this type of treatment is beneficial or not in this condition (Mastaglia, 2008).

It has also been argued that TNF-alpha may provide a target for treating tendinopathy/tendinosis (Millar et al., 2009, Hosaka et al., 2005). It is actually a fact that anti-TNF treatment has been tested concerning closely related conditions such as ankylosing spondylitis (Henderson and Davis, 2006, Braun et al., 2005). Concerning the most frequent pain-related condition for the Achilles tendon (mid-portion Achilles tendinosis) the situation has all the time been unclear.

There are some serious side effects to consider concerning TNF-alpha inhibitor treatment. It is well-known that anti-TNF treatment can have adverse effects. A systemic review and meta-analysis concluded that anti-TNF treatment increased the risk for both serious infections and malignancies, with the number needed to harm being 59 and 154 (Bongartz et al., 2006).

During the most recent years there are several reports showing that anti-TNF treatment actually can induce inflammatory myopathies (Coudere et al., 2014, Brunasso et al., 2014, Liu et al., 2013). This means that anti-TNF-alpha treatment rather is contradictory than useful for muscle affection with myositis. In our study (III) it was also concluded that TNF-alpha blocking might have negative effects in the phase of reparation after the myositis. Thus, we noted TNF receptor expressions in fibers that were interpreted to be in a regenerative stage.

Nevertheless, next-generation TNFR-selective TNF therapeutics are available. These are considered to be an effective approach in treating certain diseases (neurodegenerative diseases) (Dong et al., 2016). In the study by Dong et al, a new type of treatment in a mouse model was given and which correspond to a TNFR1-selective antagonistic antibody and an agonistic TNFR2-selective TNF. In this way, the neuroprotection functions of TNFR2 came into account. It would be interesting to explore how such a TNF therapy influences myositis processes and reparative capacity for muscle tissue.
In total, it is apparent that TNF-alpha apparently is highly involved in reparative features. The role of TNF can vary with the type, severity and stage of the injury (Tidball, 2005).

**Concluding remarks**

It is apparent that myositis and the painful situations for humans investigated (peritendinous tissue) can be added to the list of situations where the TNF-alpha system is upregulated. Of particular interest from an original point of view are the different findings for TNFR1 and TNFR2 for the muscle fibers and the comparatively marked receptor reactivities for the nerve fascicles. The differences in the expression patterns for the two TNF receptors in the animal experiment favour a hypothesis that they have diverse actions in both healthy and damaged muscle tissue. The findings for the receptors for the nerve fascicles, including on the contralateral side, indeed show that the TNF-alpha system has effect on the nervous system.
Acknowledgements

There are several people that I am grateful to. I want to thank you all for support and patience, guidance and encouragement over the years. Without you this Thesis would never have been completed. In particular warmly thanks to:

**Sture Forsgren**, my fantastic supervisor and friend. Thank you for the tremendous work you have laid on me, for my development during the time as a PhD student. You are funny, kind and caring and always have the time to help. Thank you for your guidance and patience. I could never had a better supervisor and for that I am forever grateful.

**Per Stål**, my co-supervisor. Thank you for all that you have taught me about muscles. You have always been there to help and encourage me.

**Håkan Alfredson**, my co-supervisor. Thank you for your enthusiasm and teaching. You are an inspiration.

**Anna-Karin Olofsson** and **Ulla Hedlund**, thank you for all the teaching and help in the lab. You are great mentors and were always there to support me. You have taught me everything I know about the lab. Also thank you for your inspiring good mood and patience, even though I sometimes failed with what I was doing in the lab.

**Christoph Spang**, my “extra” supervisor and friend, which I shared the smallest office in the Anatomy Department with. Thank you for your encouragement and a great collaboration. I would also like to thank you for the fun times we have had outside the lab.

**Yafeng Song**, my co-worker and friend. Thank you for great collaboration in article II, III and all the laughs we have had together.

**Gustav Andersson**, my co-worker and friend. Thank you for all that you have learned me! You are inspiring in the way you are; hard working, successful and always happy.

**Ludvig Backman**, my co-worker and friend. Even though we never had worked together in a project, I know that you are very good at what you do and are a great teacher. Thank you for the interesting discussions and laughs in the lab.
To Anita Dreyer-Perkiömäki, Anna-Lena Tallander and Selamit Kefala for your administrative assistance. Thank you Göran Dahlgren who allowed me to work with a PC when everyone else wanted a Macintosh. You were always there to fix any kind of technical problems.

To Lotta Alfredson for the help with the biopsies.

To Jamie Gaida and Craig Purdam for collaboration in article I

To Ronny Lorentzon, Clas Backman, Adrian Lamaroux and Fellon Robson-Long for the work with the experimental rabbit model. That includes thanks to Ronny for the injection experiments.

And thank you to all others former or present co-workers at the Department of Integrative Medical Biology. You all contribute to the pleasant atmosphere at the department. Special thanks to; Mona Lidström, Paul Kingham, Peyman Kelk, Farhan Shah, Patrik Danielson, Vahid Harandi, Sandrine LeRoux, Johan Bagge, Anton Tjust, Gloria Fong, Gunnel Folkesson, Jinxia Liu, Fatima Pedrosa-Domellöf, Lev Novikov, Ludmila Novikova and Eva Carlsson. Paul also for borrowing of antibodies.

And finally but not the least, thank you to my loved ones. Thanks to my love, Jocke Lindström who is always there for me. Thank you for all the times you have picked me up after a late train back from Umeå, thank you for all the support at home. Thanks to my parents, Bo Renström and Stina Renström. You are my idols and I will always be inspired of you. Thank you to my beloved sisters and best friends, Ida Renström and Hanna Renström. You have been a great support.

Thank you Sabina Renström and Oskar Johansson, my dear friends. You have the absolutely best hotel in town. Thank you for all the times I have slept on your cozy cough, thank you for all the times you have cooked me dinner and thank you for all the times you have picked me up at the train station. It would never have been possible without you. Thanks also to Margaretha Fahlström for letting me stay at your place in Berghem, and Frida Fahlström and Anton Petterson who also had me as a guest several times in Vännfors.
Funding

Financial support was obtained from the Faculty of Medicine, Umeå University, Idrottshögskolan, Umeå University, the J.C. Kempe and Seth M. Kempe Memorial Foundations, Örnsköldsvik, Magn Bergvalls Stiftelse, The Swedish National Centre for Research in Sports (CIF) and Margareta, Kjell and Håkan Alfredsons Stiftelsen.

The founders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.
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