The role of apoptosis in rotator cuff tendinopathy

Thesis

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A good mind, a good heart, warm feelings- these are the most important things.

Dalai Lama

"The Dalai Lama's Little Book of Wisdom; April 1, 2010"

To
Lars, Maria and Jonas
with love

Table of contents

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Thesis at a glance

	Aim	Method	Results	Conclusion
I	To quantitate the rate of tenocyte apoptosis in torn supraspinatus tendons and in the matched intact subscapularis. To examine the potential relation between apoptotic index and tendon pathology. To explore tenocyte density, proliferation rate and p53 gene expression patterns.	Biopsies from 15 torn supraspinatus tendons with matched intact subscapularis tendon samples and 10 reference subscapularis samples were collected. Immunohistochemistry was used to define the apoptotic index (F7-26), proliferation rate (Ki67), and presence of p53 (M7001). Tendon degeneration was evaluated according to the Bonar scale. Expression of p53 and relevant genes (n=84) was examined on a subset of samples using microfluidic arrays.	The apoptotic index was significantly increased in torn supraspinatus tendon and matched subscapularis tendon (R^2=0.5742; p=0.0005). Cell density and proliferation rate were also elevated in torn supraspinatus compared to reference subscapularis tendons ($p<0.05$). A significant increase in p53 occurred specifically in torn supraspinatus tendon ($p<0.05$), and several genes encoding p53-inhibiting proteins were down-regulated in association, including HDAC1 ($p<0.05$), MDM4 ($p<0.001$) and PPM1D ($p<0.05$).	Our results suggest that tenocyte apoptosis results from more than one mechanism in the injured rotator cuff, including both intrinsic factors related specifically to the torn supraspinatus tendon, as well as a more generalized effect which also affects the matched subscapularis tendon.
II	To study partial-thickness supraspinatus tendon tears focusing on a potential role for apoptosis in early rotator cuff tendinopathy.	Biopsies were obtained from nine partially torn supraspinatus tendons, and from the matched intact subscapularis tendons, as well as from 10 reference subscapularis tendon samples. Immunohistochemistry was used to assess the density of apoptotic cells (activated caspase-3; Asp175), proliferation (Ki67) and p53 (M7001), a key protein involved in regulating cell death. The Bonar scale was used to evaluate tendon degeneration.	The density of apoptotic tendon cells and the density of cells expressing p53 were significantly increased in both the partially torn supraspinatus tendons and in the matched subscapularis tendons, compared to uninjured reference tendons. The Bonar score revealed significant tendon degeneration in the partially torn supraspinatus tendons compared to both matched and reference subscapularis tendons. Tendon cell proliferation was significantly increased in the partially torn supraspinatus tendons compared to reference subscapularis tendons.	Patients with partial thickness tears of the supraspinatus tendon demonstrated an increased density of apoptotic, p53+ tendon cells. The fact that apoptosis was accompanied by increased tendon cell proliferation suggests that apoptosis may be related to an ongoing injury-repair process. Increased tenocyte apoptosis may be a relatively early feature in rotator cuff tendinopathy and could represent a possible target for therapeutic intervention.
III	To evaluate early histological and immunohistochemical muscle changes accompanying rotator cuff tendinopathy.	Supraspinatus muscle biopsies were obtained from 24 patients undergoing arthroscopic repair of partial- or full-thickness supraspinatus tendon tears. Tissue was formalin-fixed and processed for histology (for assessment of fatty infiltration and other degenerative changes) or immunohistochemistry (to identify satellite cells [CD56+], proliferating cells [Ki67+], and myofibers containing predominantly type 1 or 2 myosin heavy chain [MHC]). Myofiber diameters and the relative content of MHC1 and MHC2 were determined morphometrically.	Degenerative changes were present in both patient groups (partial and full-thickness tears). Patients with full-thickness tears displayed reduced density of satellite cells, fewer proliferating cells, atrophy of MHC1+ and MHC2+ myofibers, and reduced MHC1 content.	Full-thickness tears demonstrate significantly reduced muscle proliferative capacity, myofiber atrophy and loss of MHC1 content compared to partial-thickness supraspinatus tendon tears.
IV	To assess the effect of smoking on the histopathology of rotator cuff tendinopathy, including apoptosis and proliferation.	Supraspinatus tendon samples of 10 smokers and 15 non-smokers with full-thickness tears were compared, focusing on the severity of tendon histopathology including apoptosis, cellularity and proliferation. Immunohistochemistry was used to assess the density of apoptotic cells (activated caspase-3; Asp175), proliferation (Ki67) and p53 (M7001). Tendon degeneration was evaluated on tissue sections stained with H&E and Alcian blue.	Supraspinatus tendons from smokers presented significantly more advanced degenerative changes accompanied by increased apoptosis, reduced tenocyte density and increased proliferation index.	Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis. This may indicate reduced tendon healing capacity in smokers.

List of papers

Paper I

Tenocyte apoptosis in the torn rotator cuff: a primary or secondary pathological event?
Lundgreen K, Lian ØB, Engebretsen L, Scott A.
Br J Sports Med. 2011 Oct;45(13):1035-9. doi: 10.1136/bjsm.2010.083188. Epub 2011 Apr 10.

Paper II

Increased levels of apoptosis and p53 in partial-thickness supraspinatus tendon tears.
Lundgreen K, Lian Ø, Scott A, Engebretsen L.
Knee Surg Sports Traumatol Arthrosc. 2013 Jul;21(7):1636-41. doi: 10.1007/s00167-012-2226-9. Epub 2012 Oct 6.

Paper III

Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears
Lundgreen K, Lian ØB, Engebretsen L, Scott A.
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Paper IV

Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis.
Kirsten Lundgreen, Øystein Bjerkestrand Lian, Alex Scott, Paulina Nassab, Angela Fearon, Lars Engebretsen.
Submitted 17.10.13

Abbreviations

AIF	Apoptosis inducing factor
CHL	Coracohumeral ligament
ECM	Extracellular matrix
FGF-2	Fibroblast growth factor-2
FTRCT	Full-thickness rotator cuff tear
GAG	Glycosaminoglycan
H&E	Hematoxylin & eosin
HIF-1α	Hypoxia inducible factor- 1α
IGF-1	Insulin growth factor -1
MHC	Myosin heavy chain
MTJ	Myotendinous junction
OTJ	Osteotendinous junction
PDGF	Platelet derived growth factor
PRP	Platelet rich plasma
PTRCT	Partial- thickness rotator cuff tear
SGHL	Superior glenohumeral ligament
TGF-β	Transforming growth factor -β
TSPCs	Tendon stem progenitor cells
VEGF	Vascular endothelial growth factor

Background

Apoptosis

Apoptosis is an active, physiologic process of programmed cell death. It is essential during development, for the preservation of tissue homeostasis and elimination of damaged cells. The process is genetically controlled, energy demanding, and associated with characteristic morphological changes. These involve chromatin condensation and fragmentation of the nucleus with internucleosomal cleavage of DNA, shrinkage of the cell membrane with bleb formation and detachment from its surroundings. These early phase changes are followed by the disassembly of the cell and its contents into membrane-enclosed vesicles (apoptotic bodies) that are removed by neighboring cells and macrophages. The process evolves without leakage of cytosol components into the intercellular medium; inflammation is not triggered. Apoptosis may follow different pathways dependent of initiating signal and tissue properties.

Mitochondria are key regulators of apoptosis; they may release a number of pro-apoptotic factors such as cytochrome c, caspase-2 and -9, and apoptosis inducing factor (AIF), from their intermembrane space. Caspases are the core component of the apoptotic machinery; constituting both key initiators and executioners (Hengartner 2000, Shi 2002). They are a family of cysteine proteases capable of cleaving numerous intracellular proteins. Two caspase-dependent apoptotic pathways have been described; one extrinsic pathway involving the cell surface death receptor and one intrinsic pathway triggered by changes in mitochondrial integrity and release of cytochrome c (Green and Amarante-Mendes 1998). Caspase-3 is the common final executioner caspase for these apoptotic pathways. Caspase-independent apoptosis involves AIF release from the mitochondria.
Dysregulation of apoptosis is associated with significant states of pathology; a decrease may lead to tumor growth, and excessive apoptosis is associated with degenerative pathologies such as Alzheimer's disease (Jellinger and Bancher 1998), osteoarthritis (Amin and Abramson 1998, Lotz et al. 1999, Chikanza and Fernandes 2000, Unglaub et al. 2009), and tendinopathy including rotator cuff tendinopathy (Yuan et al. 2002, Tuoheti et al. 2005, Lian et al. 2007, Pearce et al. 2009, Benson et al. 2010, Chen et al. 2010, Wu et al. 2011, Lundgreen et al. 2011, Lundgreen et al. 2013). Lung diseases (emphysema, chronic obstructive pulmonar disease) caused by cigarette use are associated with an increase of apoptosis (Segura-Valdez et al. 2000, Kasahara et al. 2000).
Increased apoptosis in tendinopathy has been associated with repeated mechanical strain (Barkhausen et al. 2003, Skutek et al. 2003, Scott et al. 2005, Millar et al. 2008), unloading/strain deprivation (Egerbacher et al. 2008) , electromagnetic fields (Blumenthal et al. 1997), hypoxia and oxidative stress (Yuan et al. 2003, Benson et al. 2010, Millar et al. 2012), and fluoroquinolones (Sendzik et al. 2005). Upregulation of apoptosis during tendon healing and increased tendon metabolism indicate apoptosis to be central in both normal

and pathologic matrix remodeling (Lui et al. 2007, Wu et al. 2010, Wu et al. 2012). The contribution of apoptosis to rotator cuff tendinopathy is poorly understood.

Basic tendon structure and function

Tendons are a heterogeneous group of dense, fibrous connective tissue, firmly connected to muscle through the myotendinous junction (MTJ) and to bone through the osteotendinous junction (OTJ). Their principal function is to transmit muscle contraction to bone producing movement, but in addition they function as joint stabilizers and as "shock absorbers" protecting the muscle (Clegg et al. 2007). This exposes tendons to significant tensile and compressive loads and subsequently a need for strong biomechanical properties.

The tendon is composed of extracellular matrix (ECM) and a subset of cells. The quality and structure of the ECM define the biomechanical properties. It consists of protein fibers (main transmitters of tension load) composed of collagens (collagen I, III, V) and elastin embedded in a proteoglycan-water matrix (Blevins et al. 1997, Jozsa and Kannus 1997, Kannus 2000, Thornton and Hart 2011). The biomechanical role is fulfilled by a 3-dimensional structural arrangement of the ECM (Hess et al. 1989, Kannus 2000).

Collagens form up to 85% of tendon dry weight, with collagen type I being the most abundant (95% of the collagens) (Cetta et al. 1982, Birk et al. 1989, O'Brien 1997). Collagen fibrils consist of packed, cross-linked, helicoidally arranged tropocollagen molecules. The cross-links provide resistance to mechanical stress and allow energy absorption. The assembly of procollagen into mature collagen is regulated by proteoglycans (Scott and Ashe 2006). Collagen fibrils aggregate and form collagen fibers, the basic tendon unit. Fine sheaths of connective tissue surround and bind the collagen fibers together. The fibers are arranged in primary bundles (subfascicles) which aggregate progressively into secondary bundles (fascicles) and then into tertiary bundles constituting the tendon proper. The paratenon surrounds the tendon as an elastic sleeve (Hess et al. 1989, Kannus 2000). It consists mainly of collagen type I and III, and Elastin (Kvist et al. 1985). Elastin constitutes 1-2% of tendon dry mass and contributes to tendon flexibility (O'Brien 1992, Blevins et al. 1997). Underneath the paratenon the epitenon provides a dense, fibrillar collagen network which is continuous with the endotenon; a thin reticular network within the tendon. It carries blood vessels, nerves and lymphatics to the inner part of the tendon (Kannus and Jozsa 1991). Fibronectin, another glycoprotein of the ECM, mediates cell interactions with the ECM (Riley 2005). The matrix' high water content (70%) is partly due to the hydrophilic nature of proteoglycans; which supports it's resistance against shear and compression (Jozsa and Kannus 1997, Riley 2005).

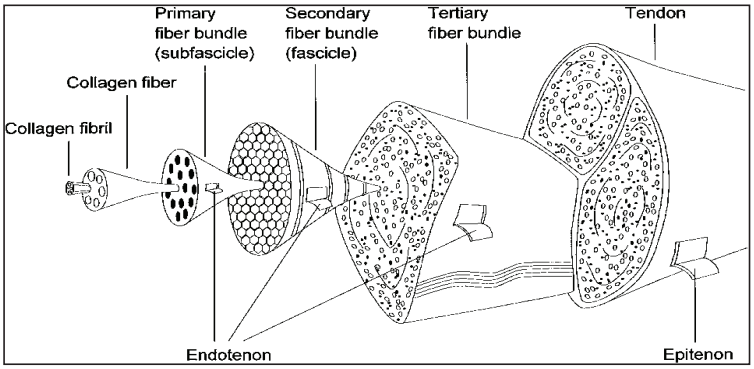


Fig 1. The organization of tendon structure from collagen fibrils to the entire tendon. Courtesy of Prof. Pekka Kannus, MD, PhD, UKK-institute, Tampere, Finland (Kannus 2000).

Different cell types reside within tendon and respond to load; among these are tenocytes, mast cells, vascular cell types and mesenchymal cells (Bi et al. 2007). Tenocytes constitute 90-95% of the cellular elements (Kannus 2000). They are mechanosensitive fibroblasts with an elongated appearance located among collagen bundles forming a 3-dimensional network linked by gap junctions (McNeilly et al. 1996). Tenocytes play a pivotal role in maintaining a biomechanically competent ECM (O'Brien 1992, Riley 2008). They are responsible for the production of ECM components, matrix degrading enzymes, growth factors and cytokines involved in the adaptive response to mechanical signals (Wall and Banes 2005, Waggett et al. 2006). The coordinated load-response is facilitated by communication between tenocytes through the gap junctions over short distances and cytokine signaling over larger distances (Schulze-Tanzil et al. 2011). Mechanical load and cell-matrix interactions are essential for the maintenance of the integrity of the tendon. Adaptation to mechanical load occurs by mechanotransduction. This describes the conversion of mechanical load into chemical signals and changes in cell behavior, and is partly due to mechanical stretching of the tendon cells, and by the effect of released cytokines. There is a dynamic association between ECM controlling the cell shape and the cell shape controlling survival and growth (Ruoslahti and Vaheri 1997).

Healthy, mature tendon typically contains scarce numbers of quiescent tenocytes (Khan and Maffulli 1998). In the young the tenocyte has a higher metabolic activity; with age the metabolic activity is slower, but the stimulation potential is maintained (Dudhia et al. 2007, Klatte-Schulz et al. 2012). Low metabolic activity and well-developed anaerobic energy production are essential prerequisites for the design and biomechanical function of tendons (Skjong et al. 2012), but as a tradeoff they hamper the healing ability after injury.

Stem cells are characterized by their ability to clone, to multipotency and self-renewal. They play a critical role in tissue repair. The existence of a tendon stem cell population in humans

was demonstrated by Bi et al in 2007 (Bi et al. 2007). They identified a mixed population of stem and progenitor cells in human hamstring tendons, and termed these tendon stem/progenitor cells (TSPCs). These constituted approximately 3-4% of the cellular elements. TSPCs possess multidifferential potential toward osteogenesis, adipogenesis and chondrogenesis (Bi et al. 2007). They recide in a unique tendon ECM niche which constitutes a specialized microenvironment composed of ECM, cells and cytokines (Scadden 2006). The niche balances quiescence, self-renewal and cell-fate commitment through modulation of bioactivities of growth factors and cytokines to which ECM proteins bind (Bi et al. 2007). The dynamic interplay between TSPCs and the niche is necessary for tissue regeneration, maintenance and repair (Bi et al. 2007, Zhang et al. 2010, Zhang and Wang 2010a, Zhang and Wang 2010c, Lui and Chan 2011, Zhang and Wang 2013). With aging there is a depletion of the TSPC pool with a significant reduction in cell number from aged tendons compared to young, and a reduction in proliferative capacity (Zhou et al. 2010, Ruzzini et al. 2013).

Basic skeletal muscle structure and function

Skeletal muscle composes 40-50% of total human body mass (Huard et al. 2002). It is the most flexible tissue in mammalian organisms with a remarkable regenerative capacity, and a broad functionality reaching from postural tonic activities to explosive contractions. The peak of functional potential in human skeletal muscles is considered to be attained around 30 years of age, followed by a gradual decline until a particularly sharp reduction in performance occurs after the age of 60 (Faulkner et al. 1990, Carlson 1992). The age-related decline in muscle mass and function may be modified by exercise training, but even in lifelong trained older adults with relatively preserved muscle mass and strength an age-related loss of muscle mass and strength occurs (Aagaard et al. 2007, Snijders et al. 2009).

Skeletal muscles are composed of muscle cells, muscle fibers and networks of nerves and blood vessels. The basic structural elements are long myofibers that generate force by contraction. They are multinucleated following the fusion of myoblasts (muscle progenitor cells). The nuclei of myofibers are postmitotic, meaning they cannot divide. The myofibers contain sarcomeres consisting of interdigitating myosin and actin filaments. The sarcomeres are the basic functional units of muscle, and are specialized to respond to neuromuscular signals. Connective tissue sheaths maintain the structural integrity of muscle fibers; the endomysium (also called basement membrane) surrounds the individual muscle fiber, bundles of muscle fibers are enveloped by the perimysium, and the epimysium ensheaths the entire muscle (Huard et al. 2002, Jarvinen et al. 2005, Nishimura 2010). These sheats contain collagen (mainly types I and III), proteoglycans, glycoproteins and cells. They constitute the extracellular matrix which is not only essential for the maintenance and regeneration of the tissue, but also for mechanotransduction and force transmission between muscle fiber and tendon (Huijing 1999, Purslow 2002, Fomovsky et al. 2010).

Most skeletal muscles contain a mixture of fiber types with marked differences in structure, molecular composition, metabolic profile and functional properties. The fiber type

composition affects muscle performance and is modulated throughout life to adapt to mechanical requirements (Huard et al. 2002, Charge and Rudnicki 2004, Jarvinen et al. 2005). The fiber types can be delineated according to various parameters (Pette and Staron 2001). At the present the dominating classification system is based on the presence of myosin heavy chain (MHC) isoforms (Pette and Staron 2001). In humans most skeletal muscles contain a mixture of 3 different myofibers; type 1, 2A and 2B (Ten Broek et al. 2010). Type 1 fibers (slow-twitch fibers; MHC-1) are slow, energy conserving and highly oxidative fibers optimized for prolonged low-intensity activities (tonic contraction, maintenance of posture). Type 2 fibers (fast-twitch fibers; MHC-2) are generally categorized into 2 subtypes. Type A being moderate fatigue-resistant, and type B not-fatigue resistant. Both types A and B are highly energy consuming fibers that depend mainly on anaerobic metabolism and are involved in rapid phasic contractions (Westerblad et al. 2010).

Satellite cells play a key role in the adaptive response of muscle to exercise, as well as in the maintenance of muscle's regenerative capacity. These cells are located under the basal lamina of myofibers surrounded by their own plasma membrane (MAURO 1961), and constitute approximately 2-5% of all muscle nuclei (Asakura et al. 2001). Due to their stem cell properties they are able to divide into myoblasts and in addition replenish their own cell population (Asakura et al. 2001). They possess high proliferative potential and are activated in response to several stimuli, such as muscle damage, testosterone, muscle stretch and exercise. Several studies have demonstrated that a loss of mechanical stimulus (i.e. unloading) reduces the number of satellite cells in muscle. This reduction in satellite cell number is thought to result from an impairment of proliferation and/or an increase in the level of satellite cell apoptosis, leading to decreased muscle mass and protein content (Darr and Schultz 1989, Schultz et al. 1994, Mozdziak et al. 1998, Matsuba et al. 2009). In a recent review Relaix et al conclude that skeletal muscle does not regenerate without satellite cells (Relaix and Zammit 2012). The potential association of satellite cells with rotator cuff muscle atrophy has not been studied

Rotator cuff anatomy and function

The rotator cuff is a myotendinous complex formed by four muscle-tendon units which fuse and form a common aponeurosis that blends with the joint capsule before inserting onto the tuberosities of the humerus (Clark and Harryman 1992). The contributing muscles are the subscapularis anteriorly, supraspinatus superiorly, infraspinatus and teres minor posteriorly. They arise from separate origins at the anterior and posterior parts of the scapula. The area of the rotator cuff insertion is termed the rotator cuff footprint. Recent anatomic investigations have indicated the joint capsule to constitute a significant part of the insertion; reinforcing and completing the insertion area (Nimura et al. 2012). The primary role of the rotator cuff complex is to stabilize the glenohumeral joint, in particular during mid-ranges of motion where the capsular ligaments are lax.

Subscapularis

The subscapularis is essential for normal glenohumeral biomechanics and function (Denard et al. 2011). It is the largest and most powerful muscle of the rotator cuff. The muscle functions as an internal rotator, and contributes significantly to anterior stability of the glenohumeral joint (Keating et al. 1993).

From its origin at the anterior surface of the scapula, the muscle courses laterally underneath the coracoid, converges into its tendinous part at the level of the glenoid, and inserts on the lesser tuberosity adjacent to the bicipital groove. The insertion extends distally to the proximal humeral metaphysis. The footprint of the subscapularis tendon is roughly comma-shaped with a dimension of approximately 40mm (length) x 16-20mm (width) (Curtis et al. 2006, Ide et al. 2008). The upper two-thirds are tendinous, the lower one-third is muscular (Clark and Harryman 1992). Fibers of the subscapularis tendon extend over the bicipital groove and interdigitate with supraspinatus fibers at the greater tuberosity (Clark and Harryman 1992, Boon et al. 2004, Gleason et al. 2006, MacDonald 2006). The subscapularis is innervated by the upper and lower subscapular nerves (C5-C7).

Supraspinatus

The supraspinatus is activated during all elevation activities, applying compression of the humeral head against the concave glenoid fossa, stabilizing the glenohumeral joint. Together with the deltoid the supraspinatus is considered the primary elevator of the glenohumeral joint (Hess 2000).

The muscle origins with an anterior and posterior muscle belly from the supraspinatus fossa, passes beneath the acromion and inserts at the anterior part of the major tubercle (Clark and Harryman 1992). Approximately 15mm before its insertion the tendon fuses with the infraspinatus tendon. A complex composite structure of heterogenous layers of collagen reinforced with the coracohumeral ligament (CHL) and capsule reduces stress concentration on the aponeurosis (Clark and Harryman 1992). The footprint of the supraspinatus has been focus for several studies (Dugas et al. 2002, Ruotolo et al. 2004, Curtis et al. 2006, Mochizuki et al. 2008, Nimura et al. 2012). Mochizuki et al in a reinvestigation of the anatomy reported the supraspinatus footprint to be triangular and smaller than previously reported; describing a hitherto unrecognized significant anterior extension of the infraspinatus insertion (Mochizuki et al. 2008). The dimension of the supraspinatus footprint was estimated to be 6.9mm (width) x 12.6mm (length). In 21% of the specimens the insertion extended anteriorly to the superior part of the lesser tuberosity. The suprascapular nerve (C4-C6) innervates the supraspinatus muscle.

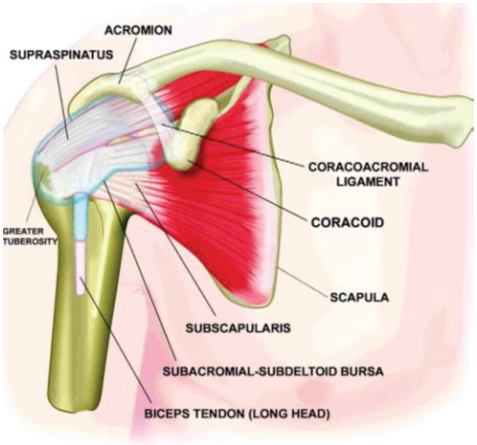


Fig 2. Drawing of the right shoulder from anterior, showing the relationship of the coracoacromial arch, rotator cuff and intervening subacromial–subdeltoid bursa, biceps tendon (long head) and bony structures. Courtesy of M.J.Rutten (Rutten et al. 2007)

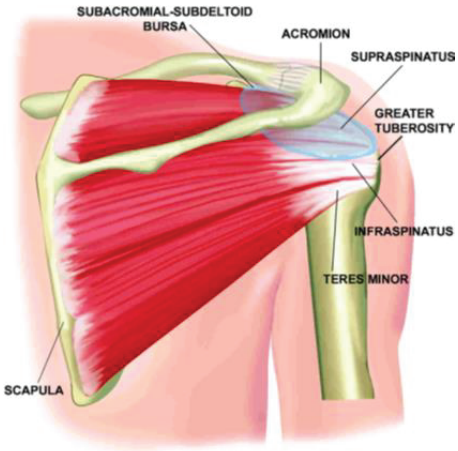


Fig3. Drawing of the right shoulder from posterior showing the various rotator cuff constituents in relation to the subacromial–subdeltoid bursa, humeral head and other bony structures.
Courtesy of M.J. Rutten (Rutten et al. 2007)

Infraspinatus

The infraspinatus in conjunction with teres minor applies a posteroinferior force to the glenohumeral joint resisting superior and anterior translation of the humeral head. Both muscles are active during external rotation and abduction. Studies indicate a position dependent effect on external rotation (Escamilla et al. 2009)**.**

The infraspinatus constitutes the second largest muscle of the rotator cuff (Curtis et al. 2006). It arises from the infraspinatus fossa including the inferior surface of the scapular spine. The tendon inserts at the major tubercle in a trapezoidal manner extending to the anterolateral part of the tubercle overlapping and extending the supraspinatus footprint (Mochizuki et al. 2008). Before the insertion it merges with the supraspinatus tendon superiorly; posteriorly it merges with the teres minor just proximal to the MTJ (Clark and Harryman 1992).

The suprascapular nerve (C4-C6) supplies the infraspinatus muscle. The footprint according to Mochizuki et al constitutes approximately 10.2mm (width) x 32.7mm (length) (Mochizuki

et al. 2008). The innervation of the infraspinatus muscle is supplied by the suprascapular nerve (C4-C6).

Teres minor

The teres minor arises from the middle portion of the lateral border of the scapula and inserts onto the inferior facet of the greater tuberosity with a tendinous portion that extends to a distal muscular insertion. It is the smallest of the rotator cuff muscles, but provides approximately 45% of external rotational force (Gerber et al. 2007a). The medial-lateral dimension is reported to be 13.9mm (Dugas et al. 2002). The teres minor is usually innervated by the axillary nerve (C4-C6), but innervation by the suprascapular nerve may also occur (Von Lanz and Wachsmuth 1959).

The rotator interval and the long head of the biceps tendon

The rotator cuff aponeurosis is interrupted anterosuperiorly by the coracoid and the long head of the biceps tendon, which exits the joint through a complex pulley system at the entry of the bicipital sulcus. The triangular area between the superior border of the subscapularis, the anterior border of the supraspinatus and the coracoid process medially constitutes the “rotator interval” of the rotator cuff. This area contains the coracohumeral (CHL) and the superior glenohumeral ligaments (SGHL), tendon fibers from supraspinatus and subscapularis, the long head of the biceps tendon and the anterior joint capsule. According to a cadaveric study by Jost et al the interval tissue presents a unique medial and lateral configuration (Jost et al. 2000a). The medial part of the rotator interval is constituted by two layers; the CHL superficially, the joint capsule and the SGHL at the articular side. Laterally the rotator interval contains four tissue layers. The first layer contains a superior portion of the CHL covering the anterosuperior insertions of the subscapularis and supraspinatus, the second layer contains CHL merging with tendon fibers of subscapularis and supraspinatus. The deep portion of the CHL extends through the third layer. The fourth layer contains the SGHL and joint capsule. The SGHL origins at the superior glenoid tubercle, and inserts on the fovea capitis near the lesser tuberosity (Harryman et al. 1992). The CHL arises from the lateral coracoid base, and demonstrates a great variability in insertion points (Yang et al. 2009a). These ligaments and the upper subscapularis tendon form the reflection pulley which stabilizes the long head of the biceps tendon at its entry point to the bicipital groove.

The rotator interval has been attributed a “check-rein” function against excessive motion of the glenohumeral joint (Harryman et al. 1992). Through its anatomical configuration it limits external rotation and inferior translation (Harryman et al. 1992, Jost et al. 2000a, Gaskill et al. 2011).

Vascular supply

The arterial supply of the rotator cuff occurs mainly through the axillary artery which supplies muscular branches, tendon branches and branches ascending through the OTJ

(Determe et al. 1996, Papakonstantinou et al. 2012). The contributing branches supply a vast anastomotic network (Determe et al. 1996). The muscular parts receive vessels mainly from the subscapular and suprascapular arteries; the tendinous parts are vascularized by the anterior and posterior circumflex arteries, the thoracoacromial (which gives rise to the acromial artery) and suprahumeral arteries (Rothman and Parke 1965, Determe et al. 1996). In a recent study Papakonstantinou et al reported consistent crossover of vessels at the OTJ of the rotator cuff tendons, in particular at the supraspinatus insertion (Papakonstantinou et al. 2012).
A critical zone of hypovascularity approximately 1cm proximal to the supraspinatus insertion has been described (Rothman and Parke 1965, Ling et al. 1990, Determe et al. 1996). This area correlates with the convergence zone between branches ascending from the anterior and posterior circumflex arteries and the acromial artery. Whether this area truly is a hypovascular region or a region prone to position dependent hypoperfusion is still a matter of controversy (Rothman and Parke 1965, Rathbun and Macnab 1970, Lohr and Uhthoff 1990, Ling et al. 1990, Chansky and Iannotti 1991, Brooks et al. 1992, Determe et al. 1996, Rudzki et al. 2008, Adler et al. 2008, Levy et al. 2008). This so called “critical zone” is prone to tendinopathic changes which has initiated a debate regarding the role of hypoxia in the development of rotator cuff tendinopathy.

Morphology of the rotator cuff enthesis

The enthesis defines the attachment site of tendon to bone. The rotator cuff tendons interdigitate near the insertion. The morphology of this phenomenon has been studied in detail, revealing the common supraspinatus and infraspinatus tendon insertion to constitute of five layers (Clark and Harryman 1992). The superficial layer contains the superior part of the CHL. This is followed by a layer of uniformly arranged tendon bundles. The interdigitation of tendon fibers occurs primarily in the third layer which presents smaller tendon fascicles without a uniform orientation. In the fourth layer the deep portion of the CHL and collagen fibres reinforce loose connective tissue. The articular side is lined with the capsule. The dimension of the vascular supply diminishes from superior to inferior with only scarce capillaries present in the fourth layer.
At the enthesis the tendon gradually changes its character from tendon to fibrocartilage to lamellar bone(Clark and Harryman 1992). This encompasses a complex transition in cell phenotype, ECM components and a change of biomechanical properties. The transition optimizes the tendon’s ability to resist and endure compressive and shear forces. The change into a cartilage-like matrix reduces the tensile properties, which has been suggested to predispose this region to injury (Almekinders et al. 2003)**.**

The concept of balanced force couples

This concept describes how the intact rotator cuff balances the glenohumeral articulation counteracting other shoulder muscles, in particular the deltoid, coordinating its anterior and posterior force vectors adapting to required biomechanics. The superior deltoid moment is

balanced by the inferomedial moment of the inferior rotator cuff in the coronal plane preventing superior migration of the humeral head. The anterior force of the subscapularis tendon is balanced with the infraspinatus and teres minor posteriorly in the transvers plane. A disruption of the supraspinatus is balanced as long as the infraspinatus and subscapularis tendons are preserved, but involves a significant increase in force requirements on these tendons (Thompson et al. 1996, Hansen et al. 2008). With the establishment of a massive rotator cuff tear involving the subscapularis and/or the posteroinferior rotator cuff the ability to balance the joint is lost and the humeral head experiences anterosuperior migration (Burkhart 1992) (fig 4).

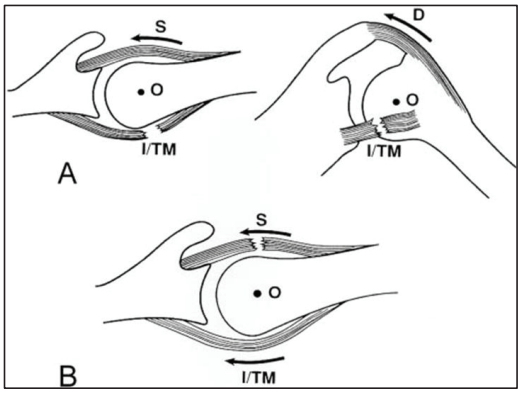


Fig 4. A: The transverse plane force couple and the coronal plane force couple are disrupted because of a massive rotator cuff tear involving the posterior rotator cuff (infraspinatus and teres minor tendons). **B:** An alternative pattern of disruption of the transverse plane force couple, because of a massive tear involving the anterior rotator cuff (that is, the subscapularis tendon). I, infraspinatus; TM, teres minor; O, center of rotation; S, subscapularis; D, deltoid. Courtesy of S.S. Burkhart (Lo and Burkhart 2003).

Tendinopathy

Tendinopathy is a *clinical* diagnosis describing tendon pain and impaired performance accompanied by pathology in or around the tendon (Khan and Maffulli 1998). It commonly affects athletes and sedentary populations. Among tendons prone to this condition are the patellar tendon of the knee, the achilles tendon of the ankle, and the supraspinatus tendon of the shoulder. Tendinosis describes a tendon with verified structural changes diagnosed by ultrasound, MRI or histological evaluation of biopsies independent of clinical symptoms (Maffulli et al. 1998, Khan et al. 1999, Alfredson et al. 2003). The structural changes are primarily of degenerative nature, and associated with a failed healing response. These changes include collagen disorganization, increased proteoglycan content, cellular changes, and neural and vascular ingrowths (Khan et al. 1999, Alfredson et al. 2003, Kongsgaard and Langberg 2011). Signs of inflammation are scarce or missing (Alfredson et al. 2001,

Hashimoto et al. 2003, Almekinders et al. 2003, Longo et al. 2007, Longo et al. 2008). Tendinosis precedes the onset of symptoms and establishment of tendinopathy (Khan et al. 1996, Maffulli et al. 2000). This represents a challenge in studies on human tendinopathy samples. They represent tissue with established disease but with no information on the initial time course of the condition. This complicates the establishment of a staged pathophysiology of tendinopathy in humans, and makes the use of valid animal models an important source to gain knowledge. Animal models have indicated that inflammation may play a role in the initiation of tendinopathy, but does not affect propagation and progression of the disease process (Rees et al. 2009). Recent human studies indicate that an inflammatory reaction is central in the onset of tendinopathy (Millar et al. 2009, Millar et al. 2010). Evidence points towards an association between increased tendon blood flow (through neoangiogenesis or increased blood flow in already existing vessels), increased mast cell density and inflammation in tendinosis (Scott 2013). Mast cells interact with tenocytes through cell receptors for ligands produced by tenocytes (eg substance P, a nociceptive neurotransmitter and proinflammatory mediator), and through secretion of inflammatory mediators which influence the tenocytes (ao VEGF, vascular endothelial growth factor) (Scott 2013).

Several theories on the etiology of tendinosis have been proposed. The main theories are the mechanical, the vascular and the neural theory. These are not mutual exclusive, and may coexist (Rees et al. 2006). The mechanical theory focuses on tendon “overload” or “overuse”. Repeated loading leads to an accumulation of repetitive micro-damage over time (Riley 2004, Rees et al. 2006)**.** The tenocytes fail to adapt to the increased matrix turnover; the matrix remodeling process switches to a degenerative process (Pufe et al. 2005). Overuse is often involved in the establishment of tendinopathy, but this theory is challenged by the fact that tendinopathy also occurs among sedentary individuals (Rolf and Movin 1997, Alfredson and Lorentzon 2000). The vascular theory remains controversial. It focuses on the poor vascularity of tendons in general and in particular in areas vulnerable to vascular compromise like the controversial “critical zone” in the supraspinatus tendon. Hypoperfusion is hypothesized to reduce the healing potential by obstructing the metabolic activity. The discovery that human tenocytes may produce classic neuronal signal substances (eg. acetylcholine, catecholamines, substance P, glutamate) founds the basis for the neural theory (Danielson 2009). This theory suggests neural signal substances contribute to tendon degeneration, tenocyte proliferation, apoptosis, angiogenesis and pain (Andersson et al. 2011a, Fong et al. 2012, Fong et al. 2013). Recent studies have indicated a possible central neuronal component (Andersson et al. 2011b, Alfredson et al. 2012).

As previously mentioned tenocytes modulate the production of the ECM directly and indirectly by the secretion of signal substances. Reports on alterations of the tenocyte cell population preceding tendinosis, and an increase of apoptosis (programmed cell death) in tendinopathy imply a possible cellular insufficiency (Yuan et al. 2002, Cook et al. 2004, Tuoheti et al. 2005, Lian et al. 2007, Pearce et al. 2009, Benson et al. 2010, Lundgreen et al.

2011, Lundgreen et al. 2013). Another issue in tendinopathy is genetic predisposition, rendering some individuals more susceptible to this condition (Magra and Maffulli 2008, Posthumus et al. 2010). Lifestyle diseases like hyperlipidemia, diabetes and smoking are associated with an increased risk of tendinopathy (Ozgurtas et al. 2003, Gaida et al. 2008, Ramchurn et al. 2009, Baumgarten et al. 2010, Abboud and Kim 2010, Pineda et al. 2011, Abate et al. 2013, Beason et al. 2013). This indicates a possible need to differentiate between load-induced and metabolic-induced conditions.

Tendon healing

Tendon injury triggers a repair process, which depends on the ability of tenocytes to respond to injury induced changes of the surrounding matrix (Sharma and Maffulli 2005). The repair process involves several overlapping steps; the essential factors are tenocyte proliferation and synthesis of neo-matrix. Information regarding tendon healing is largely based on animal studies, but healing is believed to follow the same pathway in humans. The process can be summarized in three phases: the inflammatory phase, the proliferative phase, and the remodeling phase. During the inflammatory phase cellular migration and activities dominate; inflammatory cells are recruited, necrotic material is removed, angiogenesis is initiated, and tenocyte proliferation is stimulated. The production of collagen type III, which is deficient in number of cross links, is initiated. Collagen III synthesis peaks during the proliferative phase which begins after a few days and lasts approximately 6 weeks. The remodeling phase involves consolidation from cellular to fibrous tissue, a shift towards type I vs type III collagen synthesis, and gradual maturation of the fibrous tissue to scar tissue. The process may last up to a year; in the end tenocyte metabolism and vascularity decline (Sharma and Maffulli 2006). The healing process culminates in scar tissue characterized by reduced elasticity, mobility and increased propencity for recurrence of injury (Liu et al. 2011a).

Recent publications indicate a central role for TSPCs in the regulation of tissue repair following injury and/or overload; these demonstrate TSPCs to respond with alteration of differentiation, proliferation and collagen production to biomechanical and biochemical signals (Zhang et al. 2010, Zhang and Wang 2010c, Zhang et al. 2011).

Rotator cuff tendinopathy

Rotator cuff tendinopathy includes a spectrum of pathology ranging from tendinosis and partial-thickness tears, to full-thickness tears and rotator cuff arthropathy. The prevalence of rotator cuff tears reported by sonography, MRI and cadaver studies varies from 6% to 34%, and increases with age (Sher et al. 1995a, Reilly et al. 2006a, Moosmayer et al. 2009, Yamamoto et al. 2010). In individuals between 60 and 80 years of age up to 30% may be affected; beyond the age of 80 up to 50% are reported to present rotator cuff tears. (Jerosch et al. 1991, Milgrom et al. 1995, Sher et al. 1995b). Individuals under the age of 40 rarely present full-thickness tears.

According to Yamamoto et al approximately 1/3 of tears are symptomatic (Yamamoto et al. 2011). The conversion from an asymptomatic to a symptomatic tear is correlated with an increase in tear size (Yamaguchi et al. 2001, Moosmayer et al. 2010, Mall et al. 2010). The natural history of rotator cuff tendinopathy is unclear; earlier studies have indicated a progressive course of the condition (Neer 1983, Yamanaka and Matsumoto 1994, Yamaguchi et al. 2001, Kartus et al. 2006). This is challenged by a recent report of minimal tear progression in small, symptomatic, full-thickness supraspinatus tears; 25% tear progression was shown during an average of 3.5 years following non-operative treatment (Fucentese et al. 2012).

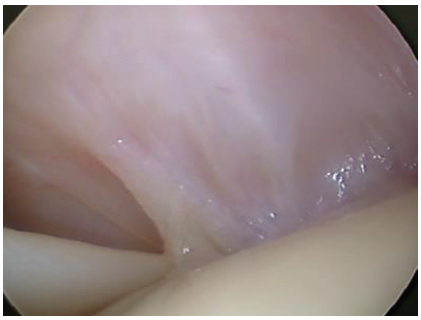
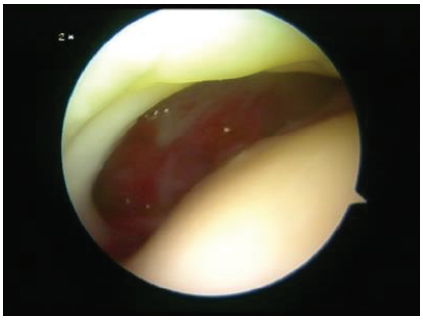
A  B 

Fig 5. A: Normal articular side supraspinatus insertion with the long biceps tendon entering the bicipital groove to the left; right shoulder. B: Supraspinatus tendon tear; right shoulder**.**

Etiology

The etiology of rotator cuff tendinopathy is multifactorial; the combination of extrinsic, intrinsic and environmental factors affects the homeostasis of the rotator cuff tendon (Lewis et al. 2009).

Extrinsic causes rely on structures outside the tendon impinging on the cuff externally (the coracoacromial arch) or internally (posterosuperior and anterosuperior glenoid edge), resulting in injury by friction and rubbing of the rotator cuff (Neer 1972, Walch et al. 1991, Habermeyer et al. 2004). Neer introduced the concept of subacromial impingement (Neer 1972). He suggested a progressive spectrum of rotator cuff tendinopathy resulted from compression of the rotator cuff from the coracoacromial arch. This theory has not been conclusively confirmed (Ozaki et al. 1988, Ogata and Uhthoff 1990, Sano et al. 1999, Budoff et al. 2003).

There is a growing body of evidence to support intrinsic tendon degeneration and a failed healing response to be central in rotator cuff tendinopathy. Tendon degeneration is attributed to overuse, poor vascularity and the influence of genetic, systemic and environmental factors (Ogata and Uhthoff 1990, Fukuda et al. 1990, Fukuda et al. 1994). The intrinsic theory is supported by studies describing initiation of tendinosis at the articular side

of the supraspinatus tendon, and the evidence of general, degenerative changes prior to rupture (Sano et al. 1999, Hashimoto et al. 2003, Benson et al. 2009).
There are conflicting theories regarding the vascularity of the rotator cuff (please see rotator cuff anatomy section for more information). Recent studies indicate increased vascularity in smaller tears, and reduced vascularity associated with increasing tear size (Matthews et al. 2006, Hegedus et al. 2010).
Endocrine and metabolic disorders (eg. hypercholesterolemia, diabetes, hypertension), and genetic make-up are associated with progressive tendon degeneration, increased susceptibility to rotator cuff tears, and poorer prognosis (Harryman et al. 2003, Harvie et al. 2004, Wendelboe et al. 2004, Tashjian et al. 2006, Gwilym et al. 2009, Abboud and Kim 2010, Gumina et al. 2012, Gumina et al. 2013, Beason et al. 2013). Indications suggest these factors reduce the tensile strength altering the quality of the extracellular matrix, predisposing the tendon to rupture (Beason et al. 2011).

Smoking is a commonly recognized cause of morbidity and mortality affecting multiple organ systems. 26% of Norwegian adults smoke; 16% smoke on a daily basis (*ref http://www.fhi.no/artikler/?id=70823)*. Smoking is associated with increased incidence of musculoskeletal injury and pain (Altarac et al. 2000, Holmen et al. 2000, McRae et al. 2011, Titchener et al. 2013). There is a growing amount of literature demonstrating the negative impact of smoking on wound healing, fracture healing and tissue repair (Mosely and Finseth 1977, Cobb et al. 1994, Daftari et al. 1994, Carpenter et al. 1996, Jorgensen et al. 1998, Haverstock and Mandracchia 1998, Ueng et al. 1999, Knuutinen et al. 2002, Kuri et al. 2005, Lee et al. 2013). The inhalation of cigarette smoke by non-smokers (so called second-hand smoke) is also associated with major adverse effects including impaired wound healing (Pirkle et al. 1996, Ueng et al. 1997, Torok et al. 2000, Wong et al. 2004).
Cigarette smoke constitutes an aerosol of more than 4000 chemicals, including nicotine, aromatic hydrocarbons, carbon monoxide, N-nitrosamines, hydrogen cyanide, and aldehydes (Roemer et al. 2004). Nicotine and carbon monoxide decrease micro perfusion and tissue oxygenation leading to tissue hypoxia (Mosely and Finseth 1977, Jensen et al. 1991, Leow and Maibach 1998). This alters cellular metabolism and healing potential. Cigarette smoke induced increased apoptosis of human endothelial cells in a study performed by Wang J et al 2001 (Wang et al. 2001). Animal studies on the effects of smoking and nicotine reveal negative effects on the biomechanical properties of mesenchymal stem cells and tendon tissue (Ichinose et al. 2010, Ruiz et al. 2012), reduced fibroblast migration and survival(Wong and Martins-Green 2004), and impaired tendon healing (Duygulu et al. 2006, Galatz et al. 2006). There are studies indicating other tobacco agents than nicotine to be responsible for the negative effects of smoking on the musculoskeletal system (Akhter et al. 2003, Yang and Liu 2004, Benatti et al. 2005, Skott et al. 2006, Dahl and Toksvig-Larsen 2007).
Human studies including a study on cadavers indicate smoking to be a risk factor for early development of rotator cuff tears, and associated with reduced post-operative functional

outcome (Mallon et al. 2004, Kane et al. 2006, Baumgarten et al. 2010, Kukkonen et al. 2012, Carbone et al. 2012a). Carbone et al and Baumgarten et al have indicated smoking to be a risk factor for rotator cuff tear with a dose- and time-dependent relationship (Baumgarten et al. 2010, Carbone et al. 2012b). The specific range of dose and duration of exposure needed to harm the tissue is not known. There are indications that the effects of smoking on the skeleton are at least partially reversible, but the time frame is unknown (Lee et al. 2013).

Classification

Rotator cuff tears are classified according to symptomatic status and onset. Tear onset is described as acute (traumatic) or chronic (atraumatic, degenerative), or as a combination of both (acute on chronic). Tear extension (partial- versus full-thickness tears), tear size, location and muscle quality are evaluated on imaging findings and intraoperatively.

Pain free tears are commonly termed asymptomatic, but are associated with muscle weakness in active shoulder elevation motions (Moosmayer et al. 2009, Kim et al. 2009, Nakajima et al. 2012).

Traumatic tears usually result from a significant fall or trauma to an abducted, externally rotated arm. These tears are more likely full-thickness tears, and commonly affect relatively young, middle-aged men (Mall et al. 2013). The subscapularis tendon is often involved in these tears which tend to be larger than degenerative (atraumatic) tears (Mall et al. 2013). True traumatic tears are rare; scientific evidence indicates that most rotator cuff tears occur in aged or diseased tendons (Sorensen et al. 2007, Mall et al. 2010).

Chronic tears evolve from tendinosis and partial-thickness tears (Mall et al. 2010). Most rotator cuff tears originate in the supraspinatus tendon which is more prone to degenerative changes (Codman and Akerson 1931, Tuite et al. 1997, Tuite et al. 1998, Kim et al. 2010b). These superior tears may propagate anteriorly into the rotator interval and the subscapularis tendon (termed anterosuperior tears). The progression into the infraspinatus tendon is more common probably because of their common enthesis; these tears are termed posterosuperior tears.

The anterior to posterior and medial to lateral distances are measured to classify tear size. There are several classification systems. In this thesis we use the classification introduced by Post et al measuring the anterior to posterior dimension (Post et al. 1983): small tears <1 cm, medium tears <3 cm, large tears <5 cm and massive tears >5 cm.

Partial thickness rotator cuff tears are the most common tear configuration (Fukuda et al. 1990, Fukuda et al. 1994, Breazeale and Craig 1997, Fukuda 2000). In a review of rotator cuff tear prevalence reported in cadaveric and radiological studies, Reilly et al found the incidence of partial-thickness tears in asymptomatic shoulders to be approximately 16-22% (Reilly et al. 2006b). A progression of tear size has been reported to occur within 2 years in up to 53% of partial-thickness rotator cuff tears; 28% evolve to full-thickness tears

(Yamanaka and Matsumoto 1994, Maman et al. 2009, Mall et al. 2010). Partial-thickness tears are classified into three subtypes: bursal-side, intratendinous, articular-side. Articular-side partial tears are 2-3 times more common than bursal-side tears (Ellman 1990, Gartsman and Milne 1995), and the majority affects the supraspinatus tendon (McConville and Iannotti 1999). Intratendinous tears are harder to detect, but indications are that these may be common and precede articular- and bursal-side partial tears (Uchiyama et al. 2010). In the younger population, especially among overhead throwing athletes, the tears tend to occur more posteriorly at the supraspinatus-infraspinatus interval (Strauss et al. 2011). Partial thickness tears alter the strain pattern on the remaining intact cuff and predispose the tissue to tear propagation (Reilly et al. 2003a, Reilly et al. 2003b, Mazzocca et al. 2008, Yang et al. 2009b).

Reports on the natural history of symptomatic full-thickness tears indicate a progressive time course; 50% of symptomatic tears increased within 2 years (Maman et al. 2009, Safran et al. 2011). A slower progression rate has been indicated in small supraspinatus tears (Fucentese et al. 2012) and asymptomatic tears (Yamaguchi et al. 2001, Mall et al. 2010); 25% of small, symptomatic supraspinatus tears progressed during 3.7 years; 51% asymptomatic tears developed symptoms within 2.8 years, 50% of these progressed in size; 20 % of asymptomatic tears progressed without developing symptoms.

Secondary muscle changes

Rotator cuff tendon tears are accompanied by secondary changes in the rotator cuff muscles including muscle atrophy and fatty infiltration (Goutallier et al. 1994, Thomazeau et al. 1996, Thomazeau et al. 1997, Zanetti et al. 1998, Fuchs et al. 2008). These muscular changes are especially pronounced in patients with large and massive tears, and are associated with reduced healing potential following rotator cuff repair and poor functional outcome (Goutallier et al. 1994, Jost et al. 2000b, Fuchs et al. 2006, Gladstone et al. 2007, Liem et al. 2007, Kim et al. 2010a). Whether rotator cuff muscle atrophy may partially or completely reverse following tendon repair is a matter of controversy, but fatty infiltration is undisputedly regarded as a later, irreversible change (Goutallier et al. 1994, Thomazeau et al. 1997, Gerber et al. 2000, Jost et al. 2000b, Goutallier et al. 2003, Gerber et al. 2004, Gladstone et al. 2007, Liem et al. 2007, Gerber et al. 2007b, Zumstein et al. 2008, Melis et al. 2009). Atrophy of the supraspinatus muscle is often classified by calculating the occupational ratio of the scapular fossa on MRI according to Thomazeau (Thomazeau et al. 1997). Fatty infiltration can be evaluated on MRI and CT according to the classification of Goutallier (Goutallier et al. 1994, Fuchs et al. 1999).

Little information exists regarding early histological and immunohistochemical muscular changes accompanying rotator cuff tears in humans. Available data are mainly confined to animal studies (Gerber et al. 2004, Safran et al. 2005, Rubino et al. 2007, Rubino et al. 2008, Itoigawa et al. 2011, Liu et al. 2011b, Kim et al. 2012a) and a few cadaveric and biopsy studies (Nakagaki et al. 1996, Irlenbusch and Gansen 2003, Srinivasan et al. 2007, Lovering and Russ 2008, Steinbacher et al. 2010). These describe muscle fiber atrophy, altered fiber type distribution and composition, and increased interfascicular fatty and fibrotic tissue.

Histopathology of rotator cuff tendinopathy

Rotator cuff tendinopathy is associated with a progressive reduction of healing potential as tendon degeneration and tear size increases (Matthews et al. 2006). The degenerative changes encompass progressive thinning of the collagen fibres with loss of structure, myxoid degeneration, hyaline degeneration, chondroid metaplasia and fatty infiltration (Hashimoto et al. 2003). Small tears show evidence of inflammation, hypercellularity and increased angiogenesis whereas massive tears present decreased proliferative activity and hypocellularity in a biopsy study including histological and immunohistochemical examinations conducted by Matthews et al (Matthews et al. 2006). The presence of inflammation had previously not been revealed in histological studies of the rotator cuff. Later studies have reinforced a role for inflammation in the early pathogenesis of rotator cuff tendinopathy (Millar et al. 2009, Millar et al. 2010).

There is no apparent correlation between the amount of degenerative changes and clinical symptomatology (Dean et al. 2012). The association of rotator cuff tendinopathy with hypoxia, upregulation of HIF-1α and VEGF and inflammatory markers, increased proliferation and load alterations illustrate an ongoing, chronic repair process amenable to biologic interventions (Yuan et al. 2002, Yuan et al. 2003, Tuoheti et al. 2005, Millar et al. 2008, Millar et al. 2009, Benson et al. 2010, Millar et al. 2010, Lundgreen et al. 2011, Millar et al. 2012, Lundgreen et al. 2013).

Treatment

Surgical treatment of rotator cuff tendinopathy is applied after failed conservative treatment except in the case of a traumatic tendon tear where early surgical intervention is advocated (Bassett and Cofield 1983, Petersen and Murphy 2011, Bjornsson et al. 2011). The interventions reach from subacromial decompression in the case of therapy-resistant impingement and partial rotator cuff tears involving less than 50% of the tendon thickness, to tendon repair in the case of rotator cuff tears, and tendon transfers when facing an irreparable tear; reverse shoulder prosthesis with or without tendon transfer may be applied in the case of rotator cuff arthropathy. In addition the biceps tendon needs to be addressed with tenodesis or tenotomy when the biceps and its pulley system are affected.

Rotator cuff repair is a clinically very successful procedure. However, healing occurs through scar tissue and is often incomplete. Despite the introduction of several innovative surgical techniques (eg. single row with several suture modifications, single row with triple loaded suture anchors, double row, triple row, suture bridge, transosseous and transosseous equivalent repair) aiming to optimize the mechanical construct and improve healing of rotator cuff tears, failure rates remain high, especially among large and massive tears (Harryman et al. 1991, Jost et al. 2000b, Galatz et al. 2004, Boileau et al. 2005, Anderson et al. 2006, Bishop et al. 2006, Park et al. 2006, Huijsmans et al. 2007, Liem et al. 2007, Lafosse et al. 2007, Zumstein et al. 2008, Park et al. 2010, Koh et al. 2011, Ma et al. 2012). Recent literature does not support an advantage by the use of one specific technique based on

clinical outcome scores (Charousset et al. 2007, Franceschi et al. 2007, Burks et al. 2009, Grasso et al. 2009, Aydin et al. 2010, Koh et al. 2011, Ma et al. 2012, Lapner et al. 2012), or healing rate analyzed by CT-arthrogram or MRI (Franceschi et al. 2007, Burks et al. 2009, Koh et al. 2012, Ma et al. 2012, Lapner et al. 2012). The tendon remains the weak link of the repair. Whereas some advocate the use of single row repair constructs in small rotator cuff tears and double-row repair in medium and large tears, others are concerned by reports of medial repair construct failure following double-row techniques and increased costs associated with longer surgical time and higher implant costs (Franceschi et al. 2007, Aydin et al. 2010, Voigt et al. 2010, Saridakis and Jones 2010, Koh et al. 2011, Cho et al. 2011, Lorbach and Tompkins 2012, Bisson et al. 2012).

Present and emerging treatment modalities are driven by factors that target the biochemical signaling of the repair site to encourage healing and remodeling of the tendon-to-bone insertion of the rotator cuff. Among techniques being explored mainly in animal studies are the applications of exogenous growth factors and stem cells (bone-marrow derived or tendon derived), alone or in combination with scaffolds of different characteristics (Rodeo et al. 2007, Rodeo 2007, Li et al. 2009, Gulotta and Rodeo 2009, Derwin et al. 2010, Gulotta et al. 2010, Zhang and Wang 2010b, Gulotta and Rodeo 2011, Kovacevic et al. 2011, Manning et al. 2011, Gulotta et al. 2011a, Gulotta et al. 2011b, Gulotta et al. 2011c, Kim et al. 2012b).

In the search for the “perfect cocktail” of growth factors to obtain healing great interest has been given to platelet-rich-plasma (PRP). PRP provides a significant increase of platelets and growth factors including PDGF, VEGF, TGF-β, FGF-2 and IGF-1, above the “normal” physiologic level found in whole blood (Angeline and Rodeo 2012). This may potentially enhance the recruitment and proliferation of stem cells, tenocytes, and endothelial cells. Studies on the use of PRP in rotator cuff repair use different delivery systems resulting in different composition and biological characteristics of PRP. Consequentially this discrepancy makes the comparison between findings difficult as illustrated by their conflicting results (Barber et al. 2011, Castricini et al. 2011, Randelli et al. 2011, Jo et al. 2011, Bergeson et al. 2012, Rodeo et al. 2012, Chahal et al. 2012). Accordingly the evidence base for clinical support of the efficacy of PRP in rotator cuff repair is limited.

Aims of the thesis

The contribution of apoptosis to rotator cuff tendinopathy is poorly understood. The aim of the present studies has been to elucidate the extent of apoptosis in rotator cuff tendinopathy in relation to progression of tendon pathology, accompanying muscle changes and smoking.

The particular aim and hypothesis of each study were:

1. Aim: Quantitate the rate of tenocyte apoptosis in torn supraspinatus tendons and in the matched intact subscapularis, and examine a potential relation between apoptotic index (AI) and tendon pathology.
    a. Hypothesis: torn supraspinatus tendons demonstrate increased apoptosis and severe tendinosis

2. Aim: Clarify if increased apoptosis precedes the establishment of full-thickness tears by examining the extent of tenocyte apoptosis in partially torn supraspinatus tendons and matched subscapularis tendons.
    a. Hypothesis: apoptosis is increased in partial-thickness supraspinatus tendon tears

3. Aim: Examine changes in muscle morphology and fibre type associated with the progression of rotator cuff tendinopathy and apoptosis.
    a. Hypothesis: rotator cuff muscle accompanying full-thickness tears show increased degeneration and apoptosis

4. Aim: Explore the effects of smoking on the histopathology of rotator cuff tendinosis including the extent of tenocyte apoptosis and proliferation.
    a. Hypothesis: smoking decreases tendon quality and increases apoptosis

Summary of papers

Paper I

Tenocyte apoptosis in the torn rotator cuff: a primary or secondary pathological event?

Lundgreen K, Lian ØB, Engebretsen L, Scott A.
Br J Sports Med. 2011 Oct;45(13):1035-9. doi: 10.1136/bjsm.2010.083188. Epub 2011 Apr 10.

Purpose

To quantitate the rate of tenocyte apoptosis in torn supraspinatus tendons and in the matched intact subscapularis. To examine the potential relation between apoptotic index and tendon pathology. To explore tenocyte density, proliferation rate and p53 gene expression patterns.

Methods

15 torn supraspinatus tendons with matched intact subscapularis tendon samples and 10 reference subscapularis samples were collected. Immunohistochemistry was used to define the apoptotic index (F7-26), proliferation rate (Ki67), and presence of p53 (M7001). Tendon degeneration was evaluated according to the Bonar scale. Expression of p53 and relevant genes (n=84) was examined on a subset of samples using microfluidic arrays.

Results

The apoptotic index was significantly increased in torn supraspinatus tendon and matched subscapularis tendon (R^2=0.5742; p=0.0005). Cell density and proliferation rate were also elevated in torn supraspinatus compared to reference subscapularis tendons ($p<0.05$). A significant increase in p53 occurred specifically in torn supraspinatus tendon ($p<0.05$), and several genes encoding p53-inhibiting proteins were down-regulated in association, including HDAC1 ($p<0.05$), MDM4 ($p<0.001$) and PPM1D ($p<0.05$).

Conclusion

Our results suggest that tenocyte apoptosis results from more than one mechanism in the injured rotator cuff, including both intrinsic factors related specifically to the torn supraspinatus tendon, as well as a more generalized effect which also affects the matched subscapularis tendon.

Paper II

Increased levels of apoptosis and p53 in partial-thickness supraspinatus tendon tears.

Lundgreen K, Lian Ø, Scott A, Engebretsen L.
Knee Surg Sports Traumatol Arthrosc. 2013 Jul;21(7):1636-41. doi: 10.1007/s00167-012-2226-9. Epub 2012 Oct 6.

Purpose

To study partial-thickness supraspinatus tendon tears focusing on a potential role for apoptosis in early rotator cuff tendinopathy.

Methods

Biopsies were obtained from nine partially torn supraspinatus tendons, from the matched intact subscapularis tendons, and also from 10 reference subscapularis tendon samples. Immunohistochemistry was used to assess the density of apoptotic cells (activated caspase-3; Asp175), proliferation (Ki67) and p53 (M7001), a key protein involved in regulating cell death. The Bonar scale was used to evaluate tendon degeneration.

Results

The density of apoptotic tendon cells and the density of cells expressing p53 were significantly increased in both the partially torn supraspinatus tendons and in the matched subscapularis tendons, compared to uninjured reference tendons. The Bonar score revealed significant tendon degeneration in the partially torn supraspinatus tendons compared to both matched and reference subscapularis tendons. Tendon cell proliferation was significantly increased in the partially torn supraspinatus tendons compared to reference subscapularis tendons.

Conclusion

Patients with partial thickness tears of the supraspinatus tendon demonstrated an increased density of apoptotic, p53+ tendon cells. The fact that apoptosis was accompanied by increased tendon cell proliferation suggests that apoptosis may be related to an ongoing injury-repair process. Increased tenocyte apoptosis may be a relatively early feature in rotator cuff tendinopathy and could represent a possible target for therapeutic intervention.

Paper III

Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears

Lundgreen K, Lian ØB, Engebretsen L, Scott A.
Submitted 12-12-17. Accepted 13-09-02 by ACTA.

Purpose

To evaluate early histological and immunohistochemical muscle changes accompanying rotator cuff tendinopathy.

Methods

Supraspinatus muscle biopsies were obtained from 24 patients undergoing arthroscopic repair of partial- or full-thickness supraspinatus tendon tears. Tissue was formalin-fixed and processed for histology (for assessment of fatty infiltration and other degenerative changes) or immunohistochemistry (to identify satellite cells [CD56+], proliferating cells [Ki67+], and myofibers containing predominantly type 1 or 2 myosin heavy chain [MHC]). Myofiber diameters and the relative content of MHC1 and MHC2 were determined morphometrically.

Results

Degenerative changes were present in both patient groups (partial and full-thickness tears). Patients with full-thickness tears displayed reduced density of satellite cells, fewer proliferating cells, atrophy of MHC1+ and MHC2+ myofibers, and reduced MHC1 content.

Conclusion

Full-thickness tears show significantly reduced muscle proliferative capacity, myofiber atrophy, and loss of MHC1 content compared to partial-thickness supraspinatus tendon tears.

Paper IV

Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis.

Kirsten Lundgreen, Øystein Bjerkestrand Lian, Alex Scott, Paulina Nassab, Angela Fearon, Lars Engebretsen
Submitted 17.10.13.

Purpose

The purpose of this study was to assess the effect of smoking on the histopathology of rotator cuff tendinopathy, including apoptosis and proliferation.

Methods

Supraspinatus samples of 10 smokers and 15 non-smokers with full-thickness tears were compared focusing on severity of tendinopathy including apoptosis, cellularity and proliferation. Immunohistochemistry was used to assess the density of apoptotic cells (activated caspase-3; Asp175), proliferation (Ki67) and p53 (M7001). Tendon degeneration was evaluated on tissue sections stained with H&E and Alcian blue.

Results

Supraspinatus tendons from smokers presented significantly more advanced degenerative changes accompanied by increased apoptosis, reduced tenocyte density, and increased proliferation index ($P < 0.05$).

Conclusion

Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis. This may indicate reduced healing capacity.

General discussion

Material and methods

Material

Patients were recruited from the department of orthopaedic surgery at Oslo University Hospital Ullevål (May 2007-September 2009) and from the department of orthopaedic surgery at Lovisenberg diaconal hospital (September 2009- April 2012).
A total of 44 patients scheduled for arthroscopic surgery were recruited. They received oral and written information about the study and the biopsy-procedure, before signing the consent form. The patients were categorized into three groups:

1. References; 10 patients with labral tears
2. Patients with partial- or full-thickness supraspinatus tears; 9/15 patients
3. Smokers with full-thickness supraspinatus tears; 10 patients

1. Reference patients (Papers I and II):

Inclusion criteria: symptomatic labral lesion.
Exclusion criteria: age<30 years, any pathology of the rotator cuff on MRI and/or at arthroscopy, systemic inflammatory disorders, diabetes mellitus, use of tobacco products.
Characteristics of included reference patients: 10 patients, mean age 43.9 years (range 32-51), 5 women, 5 men.

2. Partial- and full-thickness tear patients (Papers I, II, III, IV)

Inclusion criteria: symptomatic partial- or full-thickness supraspinatus tear accompanied by intact matched subscapularis tendon on MRI and at arthroscopy.
Exclusion criteria: age<40 years, fatty infiltration of rotator cuff muscle on MRI exceeding grade 2 according to Goutallier (Goutallier et al. 1994), systemic inflammatory disorders, diabetes mellitus, use of tobacco products.
Characteristics of included patients:
Partial-thickness tears: 9 patients; 5 men, 4 women; mean age 54 years (range 45-60); 5 bursal- and 4 articular-side tears. Median symptom duration was 13 months (range 6-24 months). 2/9 reported traumatic onset; 7/9 were chronic tears.
Full-thickness tears: 15 patients; 10 men, 5 women; mean age 57.7 years (range 49-69). 13 tears were size medium, 2 tears size small. Median symptom duration was 11 months (range 1-72 months). 4/15 reported traumatic onset, 2/15 acute on chronic onset; 9/15 were chronic tears.

3. Smokers (Paper IV)

Inclusion criteria: active smoker (≥10 cigarettes/day) with a symptomatic full-thickness supraspinatus tear without subscapularis tendon pathology at arthroscopy.
Exclusion criteria: non-smoker, age<40 years, fatty infiltration of rotator cuff muscle on MRI exceeding grade 2 according to Goutallier (Goutallier et al. 1994), systemic inflammatory disorders, diabetes mellitus.
Characteristics of included patients: 10 patients smoking 17 cigarettes/day (mean value; range 10-30); 8 men, 2 women; mean age 52 years (range 42-64). 6 tears were medium, 4 small. 7/10 patients had a tear of the dominant shoulder. Median symptom duration was 19 months (range 6-36). 3/10 reported traumatic onset, 1/10 acute on chronic onset; 6/10 were chronic tears.

Methods

Surgical technique; tissue collection

The surgery was completely arthroscopic in all patients. Surgery was performed under general anaesthesia in the lateral decubitus position. The tears were classified intraoperatively by the operating surgeon (K.L. in 33/34 cuff tear patients). Partial-thickness tears (PTRCT) were defined as articular- or bursal-side tears depending on the location of tendon defect. Full-thickness tears (FTRCT) were classified according to Post et al defining small tears<1cm, medium tears <3cm, large tears<5cm, and massive tears >5cm (Post et al. 1983). A biopsy punch was used to harvest samples of subscapularis and supraspinatus tendon, and supraspinatus muscle. Estimated size of each sample was 3x3x3mm^3.
Subscapularis biopsies were collected from the reference patients and from the rotator cuff tear patients. These were harvested at the superior part of the tendon approximately 1,5cm from its insertion on the lesser tuberosity.
Biopsies from the supraspinatus tendon and muscle were collected from the rotator cuff patients. These were collected from the tear edge in full-thickness tears and from the tear edge and the residual, macroscopic intact part of the tendon in partial-thickness tears.
Muscle biopsies of the supraspinatus were harvested arthroscopically from the fascial side of the supraspinatus muscle belly with a 3mm biopsy punch passing through the subacromial space after completion of the rotator cuff repair (single row technique).
Apart from one early postoperative infection among the smokers no complications occurred. This postoperative infection responded to two arthroscopic revisions and antibiotics. The implants were not removed. The tear healed with a residual defect smaller than the original tear.
For histology and immunohistochemistry, tissue samples were fixed in fresh 10% buffered formalin for 16-24 hours at 4ºC and then subsequently dehydrated and paraffin-embedded. Tendon samples were mounted with fibers oriented longitudinally, muscle samples were mounted with fibers oriented transversely. The dehydration, fiber orientation and paraffin embedding procedures were performed at the department of pathology at Oslo University Hospital Ullevål by Ingebjørg Løstegård Goverud. Thereafter the samples were shipped to

Alex Scott at the University of British Columbia in Vancouver, Canada for staining and analyses. The staining was performed at the Centre for Translational and Applied Genomics in Vancouver, Canada. The evaluation of the tissue samples were mainly performed in collaboration by Alex Scott and Kirsten Lundgreen. At UBC technical assistance was given by Chris Duronio, Ashwairiya Sharma, Gloria Fong and Paulina Nassab. In the fourth paper Angie Fearon contributed with the revised Bonar score evaluation.
In our first paper we also performed gene expression analysis on a subset of the patients; tissue samples were placed directly in RNA preservative (RNAlater, Ambion) immediately after dissection, incubated at 4°C for 24 hours, then stored at -20° C.
In papers III and IV the stained samples were scanned and evaluated using an Aperio Scanscope XT (Aperio Technologies, Vista, CA, USA). The samples were consistently evaluated by examiners who were blinded to the sample identity.

Tendon degeneration (tendinosis)

5 µm sections were stained with hematoxylin and eosin (H&E) for morphology and with Alcian Blue for sulphated glycosaminoglycans (GAG). Four diagnostic features of tendinosis – fibroblastic alterations (hyper-/hypo-cellularity), increased GAG, collagen disorganization or disarray, and hypervascularity or vascular remodeling were rated semiquantitatively. In papers I and II the extent of tendinosis was evaluated according to a modified Bonar scale (Cook et al. 2004). The validity of the Bonar scale in assessing tendon ruptures has been previously established (Maffulli et al. 2008). Using this scale, a completely normal tendon would score 0, and a maximally degenerated tendon would score 12.
All subscapularis tendons, both from patients and references, contained areas of chondrocyte-like cells, which may reflect zones of compression. Thus this was considered a normal phenomenon and accordingly the evaluation of Alcian blue staining was not included in the Bonar score of papers I & II.
In paper IV a revised version of the Bonar score was applied (Fearon et al. 2013). The revised Bonar score adds an evaluation of cell morphology, calcifications and intratendinous adipocytes to the original score. According to this revised score a completely normal tendon would score 0, and a maximally degenerated tendon would score 20.The evaluation was performed on supraspinatus tendon samples only, comparing smokers to non-smokers. Alcian blue staining was incorporated.

Tenocyte density

Papers I and II

The identity of H&E stained slides was masked with dark tape. Light microscopy was performed on a digital imaging workstation (Veritas, Molecular Devices), using a 20x objective lens (Zeiss). An automated scanning function was used to create a composite micrograph of the entire tissue section. Tenocyte density was determined by calculating the average for all individual fields which contained only tenocytes and which were free of significant artefact. Cell counting was done independently by 2 observers (A.S. and K.L.).

The procedure's reliability was evaluated by an interobserver test and was determined to be highly reproducible (r^2=0.97).

Paper IV
The stained samples were scanned and evaluated using an Aperio Scanscope XT (Aperio Technologies, Vista, CA, USA). Cell density (cell number per mm²) was calculated following cell counts on H&E-stains and area measurement using the tracing tool in Aperio Imagescope software.

Detection of cellular proliferation; tendon samples
Papers I, II, IV
Reagents and antibodies for Ki67 immunohistochemistry were obtained from a single supplier (Ventana Medical Systems Inc., Tuczon, AZ). Staining was carried out using an automated immunohistochemistry unit (Discovery XT) with an LSAB (labelled streptavidin-biotin) kit according to the manufacturer's instructions, using a universal secondary antibody with DAB as the substrate. Tissue sections were pre-treated with EDTA buffer (CC1). The primary antibody was incubated at 37°C for 60 minutes and the secondary at 37°C for 30 minutes. Human formalin-fixed paraffin embedded tonsil was used for optimization and positive control.

Paper I&II:
The identity of the slides was masked with dark tape. Light microscopy was performed on a digital imaging workstation (Veritas, Molecular Devices), using 400x total magnification. An automated scanning function was used to create a composite micrograph of the entire tissue section. Cells were counted independently by 2 observers. The ICC of the procedure was 0.96.

Paper I and IV
Proliferation index was calculated to estimate proliferative activity. It was defined as the percentage of Ki67+ cells within all fields of a given biopsy that demonstrated positive labelling with the assay.

Paper II
The amount of proliferation was expressed as the average number of Ki67+ cells per mm². In paper IV the stained samples were scanned and evaluated using an Aperio Scanscope XT (Aperio Technologies, Vista, CA, USA). The sample area (mm²) was calculated using the tracing tool in Aperio Imagescope software.

Detection of apoptosis; tendon samples

Paper I

ssDNA

A monoclonal antibody against single stranded DNA breaks (F7-26; Chemicon, Temecula, California, USA) was used to examine apoptotic cell death. Tissue sections were deparaffinised in xylene, then washed sequentially in 100%, 95%, and 70% ethanol and PBS. Slides were incubated in PBS containing 0.1% NP40 for 10 minutes followed by 20µg/ml proteinase K for 40 minutes. Slides were washed in distilled water and transferred to 60°C 50% formamide (v/v distilled water) for 20 minutes. Endogenous peroxidase activity was then blocked as above, followed by further blocking in goat serum for 20 minutes, and in avidin D and biotin solutions for 15 minutes each. The sections were incubated with F7-26 (1:10 dilution) for 30 minutes at room temperature, then with goat anti-mouse IgM (1:100; Dako) for 15 minutes. Sections were then processed with Vectastain ABC reagents according to the manufacturer's instructions. Formaldehyde-fixed mammary rat tissue with or without the primary antibody was used as positive and negative controls.

The apoptotic index (AI) was determined for the F7-26 staining. AI was defined as the percentage of apoptotic cells within all fields of a given biopsy that demonstrated positive labelling with the assay. The slide identity was masked with dark tape. Cell counting was done on a digital imaging workstation (Veritas, Molecular Devices), using 400 x total magnification. Composite digital micrographs of the entire tissue section were generated as described above. In all fields containing positive cells, the number of both positive and negative cells was counted. Cells were counted independently by 2 observers. The ICC of the procedure was 0.96.

Papers II and IV

Caspase-3

For assessment of apoptosis in papers II and IV, we identified cells that contained the active (cleaved) form of caspase-3, the common final executioner caspase for the apoptotic pathways (Blankenberg 2008). This was changed from paper I because the laboratory no longer could provide an analysis for single stranded DNA.

To identify activated caspase-3, we used a polyclonal antibody (Asp 175; Cell Signaling Technology, Beverly, CA, USA). Tissue sections were deparaffinised in xylene, then washed sequentially in 100, 95, and 70 % ethanol and PBS. Slides were incubated in phosphate-buffered saline (PBS) containing 0.1 % NP40 for 10 min followed by 20 lg/ml proteinase K for 40 min. Slides were washed in distilled water and transferred to 60°C 50 % formamide (v/v distilled water) for 20 min. Endogenous peroxidase activity was then blocked as above, followed by further blocking in goat serum for 20 min, and in avidin D and biotin solutions for 15 min each. The sections were permeabilised with methanol for 10 min. Endogenous peroxidases were quenched with 3 % hydrogen peroxide. The sections were incubated with the primary antibody (1:50) overnight at 4°C, then visualised using an avidin/biotin complex kit with diaminobenzidine as the substrate (Vector) and Harris' hematoxylin as the

counterstain. Formaldehyde-fixed mammary rat tissue with or without the primary antibody was used as positive and negative controls.

The average number of caspase-3+ cells per mm² was used to express the amount of apoptosis. Composite digital micrographs of the entire tissue section were generated in paper II as described above. In paper IV the sample area (mm²) was calculated using the tracing tool in Aperio Imagescope software.

p53

Papers I, II and IV

To further examine apoptotic cell death we assessed the presence of p53 protein. P53 is a powerful tumor suppressor and promotes programmed cell death via apoptotic pathways (ref Vogelstein B Nature 2000, Vousden KH 2002). A monoclonal antibody (M7001, Dako) was used in conjunction with automated immunohistochemistry (Ventana) as described above. Heat mediated antigen retrieval in EDTA buffer (CC1) was performed. The primary antibody was incubated at 37°C for 60 minutes at 1:100 dilution, followed by a secondary antibody at 37 °C for 30 minutes. Human formalin fixed breast cancer tissue was used for optimization and as a positive control.

Papers I and IV

The percentage of p53+ cells within all fields of a given biopsy that demonstrated positive labelling with the assay was calculated. The slide identity was masked with dark tape. Cell counting was done on a digital imaging workstation (Veritas, Molecular Devices), using 400 x total magnification. Composite digital micrographs of the entire tissue section were generated as described above. In all fields containing positive cells, the number of both positive and negative cells was counted.

Paper II

The number of positive cells per mm² was calculated as described above.

Paper I:

Gene expression analysis; p53

A subset of torn supraspinatus and reference subscapularis tendons were homogenate in a Mikrodismembrator (Sartorius, Germany) and the homeganate placed immediately in Trizol. RNA was extracted and purified using RNEasy columns. cDNA was generated via reverse transcription (all reagents from Applied Biosystems). Gene expression analysis was performed using RT Profiler PCR Arrays (SABiosciences, Frederick, MD) for p53-related genes. Data analysis was performed using SABiosciences Expression Analysis Software (v3.0). Genes for which both fold change >2.5 and $\alpha < 0.05$ were considered significant.

Paper III; muscle samples

Histologic evaluation of muscle degeneration

Five-µm tissue sections were stained with hematoxylin and eosin (H&E). Degeneration was evaluated by studying the density of centrally placed nuclei (nuclei /mm2), presence or absence of vacuoles within myofibers, and fatty infiltration (presence of lipid within myofibers, or of adipocytes surrounded by myofibers) (Scott et al. 2006).

Myofiber size analysis and myofiber grouping

Immunostaining for myosin heavy chain isoforms MHC1 and MHC2 was carried out on serial sections using an automated immunohistochemistry unit (Discovery XT Ultramap DAB, Ventana Medical Systems, Oro Valley, Arizona, USA) according to the manufacturer's instructions. It was recognized that muscle fiber type characteristics exist along a spectrum; therefore we selected two mouse monoclonal antibodies, MYSNO2 and NOQ7.5.4D, Abcam catalogue numbers ab75370 and ab11083 respectively) which stain largely non-overlapping myofiber populations. We defined the distinct and non-overlapping groups of muscle fibres stained by these two antibodies as MHC1+ and MHC2+, respectively. For ab75370, the primary antibody was incubated for 2 hours at room temperature, whereas for ab11083, antigen retrieval with protease1 for 8 minutes was used followed by 1 hour incubation at room temperature. Examination of stained muscle tissue sections confirmed that the two antibodies stained essentially mutually exclusive populations of muscle fibres. The smallest fiber diameter of MHC1+ and MHC2+ fibers in each sample were measured on a minimum of 100 fibers per sample. The interobserver reliability of fiber size measurements was tested; r2=0.90. Grouping of ≥15 fiber bundles of the same type, suggestive of denervation-reinnervation, was noted (Larsson et al. 1978, Lexell and Downham 1991).

MHC1 and MHC2 quantification

The amounts of MHC1 and 2 were expressed quantitatively using an automated method. Following a tuning protocol which was validated on a random selection of slides, positively stained pixels were digitally selected on all slides with the following parameters in ImageScope software (v 11.2, positive pixel count algorithm v 9.1): view width/height 1000, zoom 1, hue value 0.1, hue width 0.5, colour saturation threshold 0.04. The total positivity ratio of immunoreaction for the entire slide, i.e. the total number of positively stained pixels / total number of pixels, resulting in a theoretical range from 0 (no staining) to 1.0 (every pixel stained), although in no case was every pixel stained (only myofibers were positively stained, while other aspects of the tissue were negative). Different intensities of staining were not separately analysed.

Satellite cells

For CD56 (neural cell adhesion molecule; NCAM, used as a marker of satellite cells), a rabbit antibody (EPR2566) was used (Epitomics cat#2690-1) at 1:100 dilution, room temperature. Control tissue was human glioma. Satellite cells were defined as showing NCAM-positive

staining around the border of the cell, containing a nucleus, and being located at the periphery of a myofiber. We did not conduct special stains for the sarcolemma or the basal lamina. Positive cells were counted and the total number divided by the calculated muscle-sample area (mm^2) (calculated using the tracing tool in Aperio Imagescope software). The cells were counted and recounted after 2 days by the same blinded investigator. The intraobserver reliability coefficient was r2=0.76.

Proliferation

Proliferating nuclei were identified by the use of monoclonal mouse antibody against the Ki67 antigen (Lundgreen et al. 2011). Heat mediated antigen retrieval in EDTA buffer was performed. The primary antibody was incubated for 2 hours at 1:50 dilution. Tonsil tissue served as positive control. The number of positive cells located within or at the periphery of muscle fibers (and not in the connective tissue) was calculated and divided by the sample area (mm^2) (calculated using the tracing tool in Aperio Imagescope software) to allow direct comparison between samples.

Apoptosis

For assessment of apoptosis we identified the activation of caspase-3. Sections were pre-treated with EDTA buffer, incubated with primary rabbit monoclonal cleaved caspase 3 antibody (Asp 175; Cell Signaling, Danvers, MA, USA) at 1:50 for 2 hours. Human tonsil tissue served as control. We counted positively stained cells within or at the periphery of muscle fibers, and calculated the density of positive cells per mm2 of muscle sample to allow a direct comparison between samples.

Statistical analyses

Paper I:

To compare the histological and immunohistochemical data between normal and tendinopathic tendons the Mann-Whitney U test was conducted with P-values less than 0.05 considered significant. Data were analyzed using SPSS. Reliability testing and correlation of variables within patients was conducted using Pearson's correlation coefficient.

Paper II:

To compare the histological and immunohistochemical data of normal and tendinopathic tendons, the Mann-Whitney U test and Wilcoxon signed-rank test were used with p values <0.05 considered significant. Data were analysed using SPSS. Reliability testing and correlation of variables within patients was conducted using Pearson's correlation coefficient.

Paper III:

To compare the histological and immunohistochemical data of muscle samples from PTRCT and FTRCT (fibre size, fibre type area ratio, CD56 and Ki67 density), student's t-test or Mann Whitney U test were applied, depending on whether the samples satisfied the condition of

equal variances; p-value < 0.05 was considered significant. To compare the presence of fatty infiltration in PTRCT vs FTRCT, a Fisher exact test was used. Data were analysed using online statistical software available through Vassar stats, Vassar College, New York, USA. Reliability testing and correlation of variables within patients were conducted using Pearson's correlation coefficient. Data were expressed as means +/- standard deviation.
Paper IV:
For all the antibodies used only non-vascular cells of fibroblastic appearance, embedded within the tendon extracellular matrix, were counted and analysed. Mann-Whitney U test αwas applied to compare the histological and immunohistochemical data of smokers and non-smokers' tendons; p-values less than 0.05 were considered significant. T- Test for independent variables was used to compare age between the 2 groups. Data were analysed using online statistical software available through Vassar stats, Vassar College, New York, USA. Reliability testing and correlation of variables within patients was conducted using Pearson's correlation coefficient.

Results

Paper I

Tenocyte apoptosis in the torn rotator cuff: a primary or secondary pathological event?
Lundgreen K, Lian ØB, Engebretsen L, Scott A.
Br J Sports Med. 2011 Oct;45(13):1035-9. doi: 10.1136/bjsm.2010.083188. Epub 2011 Apr 10.

The apoptotic index was significantly increased in torn supraspinatus tendon and matched subscapularis tendon. Cell density and proliferation rate were also elevated in torn supraspinatus compared to reference subscapularis tendons. A significant increase in p53 occurred specifically in torn supraspinatus tendon, and several genes encoding p53-inhibiting proteins were down-regulated in association, including HDAC1, MDM4 and PPM1D.

The extent of tenocyte apoptosis in our study is relatively small, but the magnitude of increase in the injured rotator cuff compared to healthy reference tendon (8.2% in torn supraspinatus tendon versus 2.8% in reference subscapularis tendon) is in keeping with the 2-3-fold elevation reported in previously published studies (Yuan et al. 2002, Yuan et al. 2003, Tuoheti et al. 2005, Benson et al. 2010). A possible explanation for the discrepancy between our numbers and the previous publications may be the exclusion of smokers/nicotine-users, diabetics, and patients with systemic inflammatory disorders. Another explanation may be the choice of diagnostic tool to identify apoptosis. We identified apoptosis by studying the presence of ssDNA, a hallmark of apoptotic cell death(Frankfurt et al. 1996). Previous publications applied TUNEL which does not discriminate between necrotic, apoptotic and autolytic cell death (Grasl-Kraupp et al. 1995, Van and Van Den Broeck 2002, Unglaub et al. 2010). This was illustrated in the study by

Tuoheti et al; comparing ssDNA- analysis and TUNEL they found a discrepancy of approximately +6% when TUNEL was applied on their material (Tuoheti et al. 2005).

Apoptosis was increased in the torn supraspinatus and the matched subscapularis tendon compared to the reference subscapularis tendon. Intriguingly we found that a significant increase of p53-activity only occurred in the torn supraspinatus tendon and not in the matched subscapularis tendon. This may imply a different, p53-independent pathway of apoptosis to occur in the matched subscapularis. Yuan et al were the first to report increased apoptosis in rotator cuff tears (Yuan et al. 2002). They also described a general increase of apoptosis in a subgroup of patients with supraspinatus tears and matched subscapularis tendon samples. This generalized increase of apoptosis may be due to aging (reference patients are younger), to changed loading profile of the rotator cuff following a tear, and to systemic effects of inflammatory mediators or other signal substances (Osawa et al. 2005, Shindle et al. 2011).

We confirm an association between increased tenocyte density and proliferation rate in rotator cuff tendinopathy (Matthews et al. 2006), but extend this knowledge by demonstrating an association between increased proliferation and increased apoptosis. This indicates that apoptosis is a feature of rotator cuff tendinopathy, however its contribution to tendon degeneration requires further research.

Paper II

Increased levels of apoptosis and p53 in partial-thickness supraspinatus tendon tears.
Lundgreen K, Lian Ø, Scott A, Engebretsen L.
Knee Surg Sports Traumatol Arthrosc,DOI10.1007/s00167-012-2226-9;Epub 2012 Oct 6.

We found an increase of apoptotic, p53+tendon cells in supraspinatus tendons demonstrating partial-thickness tears compared to reference subscapularis tendons. This phenomenon also extended into the matched subscapularis tendon confirming previous reports (Yuan et al. 2002, Lundgreen et al. 2011). Previous publications on apoptosis in rotator cuff tendinopathy without an established tear were contradictory. Benson et al stated apoptosis to be a feature of full-thickness tears, whereas Tuoheti et al documented a significant increase of apoptosis in supraspinatus tendinopathy without tear (Tuoheti et al. 2005, Benson et al. 2010). Recently Murphy et al demonstrated a significant increase of apoptosis and inflammatory cells in early rotator cuff tendinopathy (Murphy et al. 2013).

There was an association of increased apoptosis and cellular proliferation in partial-thickness tears, indicating apoptosis potentially to be part of a failed adaptation or reparative response. Increased apoptosis has been described at both early and late stages of tendon repair and during altered stimulation of tenocytes in vitro (Barkhausen et al. 2003, Chuen et al. 2004, Lui et al. 2007, Egerbacher et al. 2008, Wu et al. 2010, Wu et al. 2012). Taken together the existing information implies that apoptosis could be triggered at different steps

in the development of tendinopathy by more than one mechanism. This renders increased apoptosis a potentially early feature of tendinopathy and a possible target for therapeutic intervention.

Paper III

Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears

Lundgreen K, Lian ØB, Engebretsen L, Scott A.

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The establishment of a full-thickness tear was associated with reduced muscle proliferative capacity, myofiber atrophy and loss of MHC1-content compared to partial-thickness tears. These changes and a tendency to fatty infiltration may illustrate a transition evolving by a pathomechanical pathway; tendon continuity is lost and the muscle adapts to reduced/lacking force transmission (Meyer et al. 2004). The reduced MHC1-content involved a loss of endurance-type fibers which also has been described in reports on muscle disuse atrophy (Jozsa et al. 1990, Grossman et al. 1998, Pette and Staron 2001). This phenomenon differs from the muscle aging processes which commonly encompass a transition towards type1-fiber phenotype (Klitgaard et al. 1990, Short et al. 2005, Korhonen et al. 2006, Lee et al. 2006). Interestingly our results are in contrast to a previous study documenting MHC2- fibers to be more prone to atrophy whereas we find both fiber types to display similar degreees of atrophy (Irlenbusch and Gansen 2003).

There were no signs of capsase-3 dependent apoptosis in our muscle samples. This may support the hypothesis that fiber atrophy is not caused by apoptosis or other caspase-dependent processes (Steinbacher et al. 2010). However, alternate explanations which must be acknowledged are (1) that apoptosis occurred, but followed a caspase-independent pathway, or (2) that the cross-sectional nature of our study missed a crucial time point when apoptosis of satellite cells or myofibers occurred (e.g directly following the loss of muscle tension after tendon rupture). Information regarding the most important pathway in atrophy-induced apoptosis is lacking; there appears to be a selective activation of specific apoptosis pathways depending on age, muscle type and the nature of atrophying condition (Dupont-Versteegden 2005, Dirks et al. 2006, Nagano et al. 2008, Marzetti et al. 2010).

Despite the general absence of fatty infiltration on MRI, fat infiltration was in fact histologically confirmed in 5 of 15 patients presenting FTRCTs, and in 1 of 9 patients with PTRCTs. If fat accumulation occurs before it can be detected on MRI, this may represent evidence in favour of early operation i.e. to prevent establishment of significant irreversible fat infiltration which is associated with reduced healing potential (Goutallier et al. 1994, Goutallier et al. 2003, Gladstone et al. 2007, Cho and Rhee 2009). Our findings highlight the need for a validation of MRI classification of fatty infiltration.

Paper IV

Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis.

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This is according to our knowledge the first study to assess the effect of smoking on microscopic tendon degeneration including cellular alterations, proliferation and apoptosis. Comparing non-smoking to smoking rotator cuff tear patients we found smokers to be younger, demonstrate more pronounced tendinopathic changes with reduced cellularity, increased proliferation index and increased apoptosis. Our findings are in line with previous clinical reports of tear onset at younger age, larger tears, poorer functional outcome and reduced healing ability after repair in smokers (Mallon et al. 2004, Kane et al. 2006, Baumgarten et al. 2010, Kukkonen et al. 2012, Carbone et al. 2012a). Whether the negative effects of smoking are due to nicotine or other substances contained in cigarettes is not clear. There are studies indicating that nicotine as such is not responsible for the negative effects (Akhter et al. 2003, Yang and Liu 2004, Benatti et al. 2005, Skott et al. 2006, Dahl and Toksvig-Larsen 2007). Nicotine seems to present a dose-dependent effect on cellular metabolism, but the dose and time limits are unknown.

Interestingly tendon samples from smokers demonstrated an increase of tenocyte proliferative activity accompanying reduced cell density. This may illustrate a compensatory alteration of cell metabolism in an attempt to restore tissue balance.

Our findings indicate that smoking needs to be controlled for in studies on tendinopathy; the inconsistency regarding information of smoking status in previous reports may explain the reported discrepancies and variation in the estimated extent of apoptosis (Yuan et al. 2002, Tuoheti et al. 2005, Benson et al. 2010, Lundgreen et al. 2011). Our findings substantiate the harmful effects of smoking on the musculoskeletal system, and reinforce the need for further research on tobacco products. Knowledge of the role of nicotine and non-nicotine substrates is mandatory to improve smoking cessation strategies and modify guidance of smokers. This may aid in the prevention of rotator cuff tendinopathy and guide the choice of rotator cuff repair strategies to improve healing and reduce the socioeconomic burden of this patient group.

General conclusions

Paper I

Our results suggest that tenocyte apoptosis results from more than one mechanism in the injured rotator cuff, including both intrinsic factors related specifically to the torn suprapsinatus tendon, as well as a more generalized effect which also affects the matched subscapularis tendon.

Paper II

Patients with partial thickness tears of the supraspinatus tendon demonstrated an increased density of apoptotic, p53+ tendon cells. The fact that apoptosis was accompanied by increased tendon cell proliferation suggests that apoptosis may be related to an ongoing injury-repair process. Increased tenocyte apoptosis may be a relatively early feature in rotator cuff tendinopathy and could represent a possible target for therapeutic intervention.

Paper III

Full-thickness tears demonstrate significantly reduced muscle proliferative capacity, myofiber atrophy and loss of MHC1 content compared to partial-thickness supraspinatus tendon tears.

Paper IV

Smoking is associated with worsened supraspinatus tendon histopathology and increased apoptosis.

Contributions to existing knowledge

Paper I

Information regarding the role for apoptosis in tendinopathy is scarse. Our findings indicate increased apoptosis to be a general feature of the torn rotator cuff possibly evolving along different pathways.

Paper II

Controversy exists regarding the onset of increased apoptosis in rotator cuff tendinopathy. We document increased apoptosis to be a feature of partial-thickness rotator cuff tears, and to be accompanied by increased proliferation possibly participating in an ongoing repair process.

Paper III

Information regarding early histological and immunohistochemical muscular changes accompanying rotator cuff tendinopathy in humans is scarce. We report a loss of muscle regenerative potential in full-thickness tears compared to partial-thickness tears. The muscular changes are not associated with an increase of caspase-3 dependent apoptosis.

Paper IV

Clinical reports have indicated smoking to deteriorate the results of rotator cuff repair. The knowledge of histopathologic tendon changes in the rotator cuff associated with smoking in humans is poor. Comparing smoking to non-smoking rotator cuff tear patients we demonstrate a significant deterioration of tendon quality and increased apoptosis in smokers. This may indicate reduced tendon healing capacity in smokers.

Implications for future research

Increased apoptosis is clearly a feature of tendinopathy. But whether it is a cause or a consequence of tendinopathy remains unclear. We need to identify pro- and anti-apoptotic factors to modulate the pathway of tendinosis aiming to prevent progression and improve healing at the time of repair.
Our finding of fatty infiltration despite negative MRI indicates a need for a validation of MRI grading systems regarding this phenomenon.
Our study adds to previous reports on the harming effects of smoking on the musculoskeletal system and reinforces the need for further research on tobacco products. We need to clarify which substances cause pathology. To give proper guidance for treatment strategies we need to establish dose and time dependency rates and improve knowledge on reversibility of tobacco tendon effects. Improved knowledge may improve prevention and repair strategies.

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II

III

Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears

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Background and purpose **Rotator cuff tears are associated with secondary rotator cuff muscle pathology, which is definitive for the prognosis of rotator cuff repair. There is little information regarding the early histological and immunohistochemical nature of these muscle changes in humans. We analyzed muscle biopsies from patients with supraspinatus tendon tears.**

Methods **Supraspinatus muscle biopsies were obtained from 24 patients undergoing arthroscopic repair of partial- or full-thickness supraspinatus tendon tears. Tissue was formalin-fixed and processed for histology (for assessment of fatty infiltration and other degenerative changes) or immunohistochemistry (to identify satellite cells (CD56+), proliferating cells (Ki67+), and myofibers containing predominantly type 1 or 2 myosin heavy chain (MHC)). Myofiber diameters and the relative content of MHC1 and MHC2 were determined morphometrically.**

Results **Degenerative changes were present in both patient groups (partial and full-thickness tears). Patients with full-thickness tears had a reduced density of satellite cells, fewer proliferating cells, atrophy of MHC1+ and MHC2+ myofibers, and reduced MHC1 content.**

Interpretation **Full-thickness tears show significantly reduced muscle proliferative capacity, myofiber atrophy, and loss of MHC1 content compared to partial-thickness supraspinatus tendon tears.**

Rotator cuff tendon tears are accompanied by secondary changes in the rotator cuff muscles, including muscle atrophy and fatty infiltration. These muscular changes are especially pronounced in patients with large and massive tears, and are associated with reduced healing potential following rotator cuff repair and poor functional outcome. Whether rotator cuff muscle atrophy may partially or completely reverse after tendon repair is a matter of controversy, but fatty infiltration is undisputedly regarded as a later, irreversible change (Thomazeau et al. 1997, Goutallier et al. 2003, Gladstone et al. 2007).

Atrophy and fatty infiltration (also termed fatty degeneration) can be graded on CT-scans and MRI (Goutallier et al. 1994, Thomazeau et al. 1997, Fuchs et al. 1999). Whether this grading accurately reflects the true muscular changes is not clear. There is little information regarding early histological and immunohistochemical muscular changes accompanying rotator cuff tears in humans. The data that are available are mainly from animal studies (Gerber et al. 2004, Rubino et al. 2007, Liu et al. 2011, Kim et al. 2012) and a few cadaveric and biopsy studies (Nakagaki et al. 1996, Steinbacher et al. 2010).

Satellite cells are known to play a key role in the adaptive response of muscle to exercise, and in the maintenance of the regenerative capacity of muscle. Several studies have shown that a loss of mechanical stimulus (i.e. unloading) reduces the number of satellite cells in muscle; this reduction is thought to result from an impairment of proliferation and/or an increase in the level of satellite cell apoptosis, leading to reduced muscle mass and protein content (Darr and Schultz 1989, Matsuba et al. 2009) The possible association of satellite cells with rotator cuff muscle atrophy has not been studied.

The main objective of the present study was to compare the following histological and immunohistochemical features in the supraspinatus muscle of patients with partial-thickness rotator cuff tears (P) to those from patients with full-thickness tears (F): myofiber diameter, muscle protein content including myosin heavy chains 1 and 2 (MHC1/MHC2), degenerative changes including fatty infiltration, satellite cell number, proliferative activity, and apoptosis. We hypothesized that the group with full-thickness rotator cuff tears would have a reduced satellite cell number and a greater degree of myofiber atrophy.

Patients and methods

24 patients undergoing arthroscopic repair of partial-thick-

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ness (P) or full-thickness (F) supraspinatus tendon tears were included. The tears were classified by the operating surgeon intraoperatively. P tears were defined as articular- or bursal-side tears depending on the location of the tendon defect. F tears were classified according to Post et al. (1983).

9 patients presented with P tears: mean age was 54 (45–60) years and 5 were men. There were 5 bursal- and 4 articular-side tears. The F group consisted of 15 patients: mean age was 58 (49–69) years and 10 were men. 13 tears were medium-sized and 2 were small. Median duration of symptoms in the P and F groups was similar (13 (6–24) months and 11 (1–72) months). In both groups, most of the tears were degenerative. In the F group, 4 of 9 patients reported a traumatic onset and 2 of 9 reported an "acute on chronic" onset. In the P group, 2 out of 7 patients reported a traumatic onset.

Preoperative MRIs were performed at different institutions. In the F group, 8 of 15 patients had atrophy of grade 1 according to Thomazeau et al. (1997) and 1 of these 8 showed concomitant fatty infiltration of grade 1 according to a 5-stage grading system originally introduced by Goutallier et al. (1994) for CT. Fuchs et al. (1999) showed that this grading system was also reproducible on MRI. 2 patients in the P group had atrophy of grade 1; none showed fatty infiltration on MRI.

Patients suffering from systemic inflammatory disorders or diabetes and patients using nicotine were excluded. The study was approved by the regional committee for research ethics (1.2007.728), and informed, written consent was obtained from all participants.

Muscle biopsies of the supraspinatus were harvested arthroscopically from the fascial side of the supraspinatus muscle belly with a 3-mm biopsy punch passing through the subacromial space after completion of the rotator cuff repair (single-row technique). The samples were fixed in fresh 10% buffered formalin for 16–24 h at 4ºC and then dehydrated, mounted with fibers oriented transversely, and paraffin-embedded. The stained samples were scanned and evaluated using an Aperio Scancope XT (Aperio Technologies, Vista, CA). The examiners were blind as to the identity of samples.

Histological evaluation of muscle degeneration

5-µm tissue sections were stained with hematoxylin and eosin. Degeneration was evaluated as previously described by studying the density of centrally placed nuclei (nuclei/mm^2), presence or absence of vacuoles within myofibers, and fatty infiltration (the presence of lipid within myofibers, or of adipocytes surrounded by myofibers) (Scott et al. 2006).

Myofiber size analysis and myofiber grouping

Immunostaining for myosin heavy chain isoforms MHC1 and MHC2 was carried out on serial sections using an automated immunohistochemistry unit (Discovery XT Ultramap DAB; Ventana Medical Systems, Oro Valley, AZ) according to the manufacturer's instructions. We recognized that there is a range of muscle fiber type characteristics; we therefore selected 2 mouse monoclonal antibodies, MYSNO2 and NOQ7.5.4D (Abcam catalogue numbers ab75370 and ab11083 respectively), which stain largely non-overlapping myofiber populations (Figure 1). We defined the distinct and non-overlapping groups of muscle fibers detected by these 2 antibodies as MHC1+ and MHC2+, respectively. For ab75370, the primary antibody was incubated for 2 h at room temperature, whereas for ab11083, antigen retrieval with protease 1 for 8 min was used followed by 1 h of incubation at room temperature. Examination of stained muscle tissue sections confirmed that the 2 antibodies stained essentially mutu-

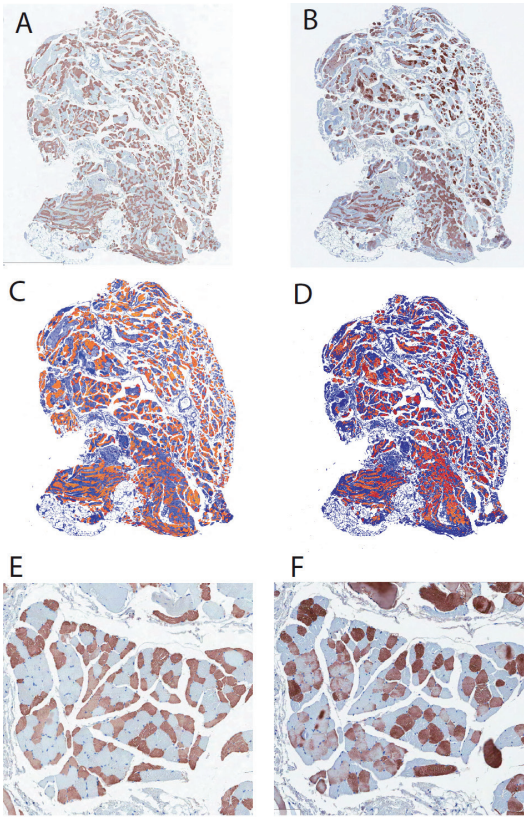

Figure 1. Immunohistochemistry of supraspinatus muscle, showing MHC1+ (left panels) and MHC2+ fibers (right panels). A and B. Serial sections were stained with the 2 relevant antibodies (see Methods for details). C and D. The same pixel selection algorithm was used to select positive staining (orange/red) and negative staining (blue). E and F. Higher-magnification view of area shown in A and B. Note the generally non-overlapping pattern of MHC1 and MHC2 immunostaining. The vast majority of myofibers have been stained with one of the antibodies, but not both, while a very small minority have not been stained with either.

ally exclusive populations of muscle fibers. The smallest fiber diameter of MHC1+ and MHC2+ fibers in each sample were measured on a minimum of 100 fibers per sample. The inter-observer reliability of fiber size measurements was tested (r^2 = 0.90). Grouping of ≥ 15 fiber bundles of the same type, suggestive of denervation-reinnervation, was noted (Lexell and Downham 1991).

Quantification of MHC1 and MHC2

The amounts of MHC1 and 2 were expressed quantitatively using an automated method. Following a tuning protocol that was validated on a random selection of slides, positively stained pixels were digitally selected on all slides with the following parameters in ImageScope software (v 11.2, positive pixel count algorithm v 9.1): view width/height 1000, zoom 1, hue value 0.1, hue width 0.5, and color saturation threshold 0.04. The total positivity ratio of immunoreaction for the entire slide, i.e. the total number of positively stained pixels divided by the total number of pixels, resulted in a theoretical range from 0 (no staining) to 1.0 (every pixel stained), although in no case was every pixel stained (only myofibers were positively stained, while other aspects of the tissue were negative). Different intensities of staining were not analyzed separately.

Satellite cells

For CD56 (neural cell adhesion molecule (NCAM), which is used as a marker of satellite cells), a rabbit antibody (EPR2566) was used (Epitomics cat. 2690-1) at a dilution of 1:100 and at room temperature. The control tissue was human glioma. Satellite cells were defined as showing NCAM-positive staining around the border of the cell, containing a nucleus, and being located at the periphery of a myofiber. We did not conduct special staining for the sarcolemma or the basal lamina. Positive cells were counted and the total number was divided by the muscle-sample area (mm^2) (as calculated using the tracing tool in Aperio Imagescope software). The cells were counted and recounted after 2 days by the same blinded investigator. The intra-observer reliability coefficient (r^2) was 0.76.

Proliferation

Proliferating nuclei were identified by the use of monoclonal mouse antibody to the Ki67 antigen. Heat-mediated antigen retrieval in EDTA buffer was performed. The primary antibody was incubated for 2 h at a dilution of 1:50. Tonsil tissue served as positive control. The number of positive cells located within or at the periphery of muscle fibers (and not in the connective tissue) was calculated and divided by the sample area (mm^2) (as calculated using the tracing tool in Aperio Imagescope software) to allow direct comparison between samples.

Apoptosis

For assessment of apoptosis, we identified the activation of caspase-3, which is the common final executioner caspase for the apoptotic pathways. Sections were pretreated with EDTA buffer and incubated with a primary rabbit monoclonal antibody targeting cleaved caspase 3, 1:50 dilution (Asp 175; Cell Signaling, Danvers, MA) for 2 h. Human tonsil tissue served as control. We counted positively stained cells within or at the periphery of muscle fibers and calculated the density of positive cells per mm^2 of muscle sample to allow direct comparison between samples.

Statistics

To compare the histological and immunohistochemical data of muscle samples from the P and F groups (fiber size, fiber type area ratio, CD56 and Ki67 density), Student's t-test or Mann-Whitney U test were used, depending on whether the samples satisfied the condition of equal variances. Any p-value < 0.05 was considered significant. To compare the presence of fatty infiltration in the P and F groups, Fisher's exact test was used. Data were analyzed using online statistical software available through Vassar Stats (Vassar College, New York, NY). Reliability testing and correlation of variables in individual patients were conducted using Pearson's correlation coefficient. Data are expressed as mean (SD).

Results

General histological observations

There were no statistically significant differences in the general degenerative histological features of muscle from P tears and F tears. Fatty infiltration was present between fibers both in F tears (5 of 15 samples) and in P tears (1 of 9 samples) (Fisher's exact test, p = 0.2) with a tendency to be more pronounced in the F group. The density of centralized nuclei, a secondary indicator of histological degeneration, was not significantly different between the 2 groups (3.1 (3.1) vs. 1.78 (1.2); p = 0.4). The presence of vacuoles within myofibers was similar in both groups (5 of 15 vs. 3 of 8).

Myofiber size and grouping

F tears showed a statistically significantly smaller average diameter of fibers that were immunopositive either for MHC1 or MHC2, compared to P tears (Figure 2). The mean fiber diameters of MHC1 and MHC2 myofibers in F tears were 39 (6.6) µm and 38 (8.8) µm, as compared to 48 (8.2) µm and 44 (12) µm in P tears (p < 0.01 for both MHC types). Fiber grouping was present in 4 patients with P tears (only affecting MHC1+ fibers) and in 9 patients with F tears (affecting both MHC1+ and MHC2+ fibers).

Muscle protein content

The MHC type 1 content was lower in F patients than in to P patients (0.40 (0.19) vs. 0.50 (10); p<0.05). Conversely, MHC type 2 content between the 2 groups was similar (F: 0.45 (0.13) vs. P: 0.47 (0.15)).

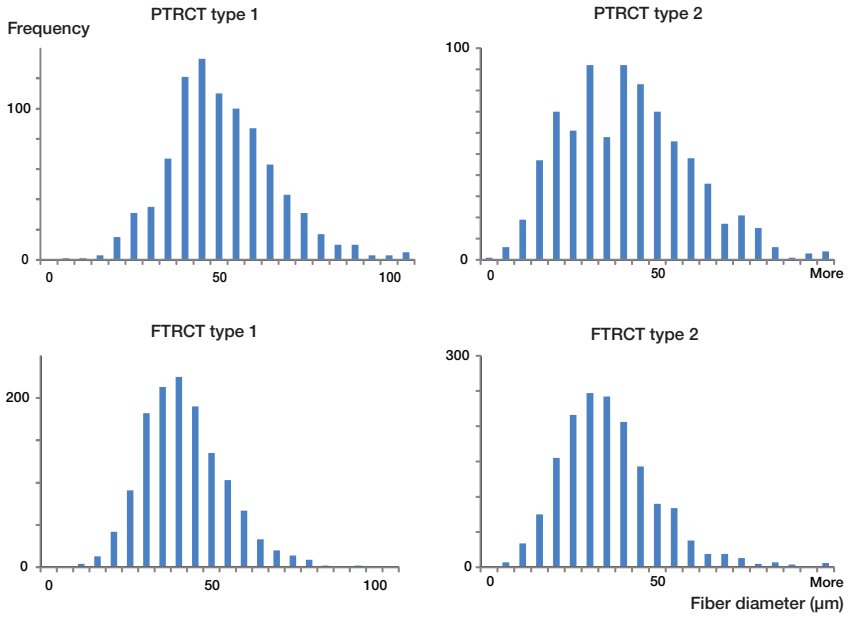

Figure 2. Sample fiber size distribution of type 1 and type 2 myofibers in the supraspinatus muscle of patients with partial-thickness rotator cuff tears (PTRCT, top panels) or full-thickness rotator cuff tears (FTRCT, bottom panels). Note the smaller size (a leftward shift in myofiber histogram) of both myofiber types in the FTRCT patients.

Satellite cells, proliferation (Figure 3)

The number of cells showing CD56 expression, indicating the presence of satellite cells, was significantly less in F patients (3.1 (2.9) cells/mm^2) than in P patients (8.1 (10.2) cells/mm^2; $p < 0.05$). The number of proliferating cells (defined as Ki67+ nuclei) was 0.35/mm^2 in F patients and 1.0/mm^2 in P patients ($p < 0.05$), indicating a reduced number of proliferating cells in F tears.

Apoptosis

There was no detectable apoptosis involving the activation of caspase-3 in either sample group. In contrast, there was substantial apoptosis involving the activation of caspase-3 in control (human tonsil) tissue.

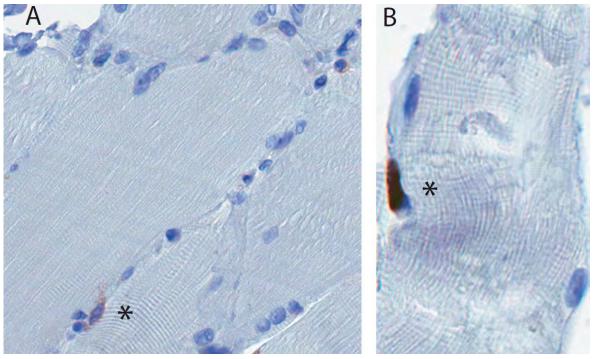

Figure 3. A. Satellite cells were defined as CD56+ cells at the periphery of the myofiber (marked with an asterisk). B. A Ki67+ cell (asterisk), which appears to lie outside the sarcolemma, in the expected location of a satellite cell.

Discussion

We observed a statistically significant reduction in satellite cell number and reduced proliferative activity in the supraspinatus muscle of patients with a full-thickness rotator cuff tear, in comparison to those with a partial-thickness tear. This was also associated with muscle atrophy, which affected both MHC1+ and MHC2+ fibers without gross signs of fiber degeneration, and a tendency to fatty infiltration. These changes may illustrate a transition evolving by a pathomechanical pathway when tendon continuity is lost, resulting in loss of force transmission through the muscle (Meyer et al. 2004).

The MHC1 content was reduced in patients with an F tear,

perhaps involving a shift in myofiber phenotype in which endurance-type fiber characteristics are lost. This finding is in line with previous reports describing a similar phenomenon in muscle-disuse atrophy (Jozsa et al. 1990, Grossman et al. 1998, Pette and Staron 2001), as opposed to the process of muscle ageing, which commonly involves a transition towards an increased type I fiber phenotype (Klitgaard et al. 1990, Korhonen et al. 2006, Lee et al. 2006). However, it must be acknowledged that we did not conduct a complete assessment of myofiber phenotypes but focused our attention on 2 of the predominant MHC types (1 and 2). Interestingly, our observations are in contrast to a study reporting that type 2 fibers are more prone to atrophy than type 1 fibers with progression of supraspinatus tendinopathy (Irlenbusch and Gansen 2003), whereas we report here that MHC1+ and MHC2+ fibers showed similar degrees of atrophy in F tears.

We found that muscle atrophy appears independently of degenerative muscle changes, which is in line with previous publications (Einarsson et al. 2011, Gumucio et al. 2012).

There were no signs of caspase-3-dependent apoptosis in the muscle samples examined, which may support the hypothesis that fiber atrophy is not caused by apoptosis or by other caspase-dependent processes (Steinbacher et al. 2010). However, alternative explanations are (1) that apoptosis occurred, but followed a caspase-independent pathway, or (2) that the cross-sectional nature of the present study missed a crucial time point when apoptosis of satellite cells or myofibers occurred (e.g directly following the loss of muscle tension after tendon rupture). There is very little information regarding the most important pathway in atrophy-induced apoptosis; there appears to be a selective activation of specific apoptosis pathways depending on age, muscle type, and the nature of the atrophying condition (Dupont-Versteegden 2005, Marzetti et al. 2010).

Despite the general absence of fatty infiltration on MRI, fat infiltration was in fact histologically confirmed in 5 of 15 patients with F tears, and in 1 of 9 patients with P tears. If fat accumulation occurs before it can be detected radiologically, this may favor early operation—i.e. to prevent establishment of significant irreversible fat infiltration which is associated with reduced healing potential.

Muscle biopsies from both P and F patients contained areas of fiber type grouping. Grouping of muscle fibers occurs in ageing human muscle and is thought to arise from a continuous, progressive process of denervation and partial reinnervation. This may be due to a slow, progressive neurogenic ageing process involving loss of motor neurons in the spinal cord and/or loss of functioning motor units in ageing human muscle (Tomonaga 1977, Lexell 1995). The age of patients with or without evidence of fiber type grouping was similar.

MRIs were performed at different institutions and this clearly is a limitation, allowing for variation in the quality of the examination and in the radiological evaluation of the extent of muscle changes. This precludes a conclusive evaluation of correlation between MRI findings and histological findings in our study. However, we found histological evidence of fatty infiltration despite the fact that this feature was not visible on the MRIs. This raises the question of whether there is a role for early fatty muscle infiltration in rotator cuff tendinopathy preceding more pronounced changes that are detectable on MRI. A comparative study between histology and MRI to evaluate the sensitivity and specificity of MRI and CT to early muscle changes is yet to be published.

Another limitation inherent in muscle biopsy studies is that changes may not be distributed uniformly in the muscle. According to a previous study, the main changes of atrophy occur primarily at the fascial side of the muscle, whereas fatty infiltration is more pronounced on the scapular side (Meyer et al. 2005). Our biopsies were harvested arthroscopically, reaching the muscle from the subacromial space presumably representing the fascial side of the muscle belly.

With regard to the presence of fiber type, grouping electromyography—which was not performed—might have added valuable information.

Our findings should be interpreted with caution, due to the small number of patients and the limited size of the biopsies. Muscle atrophy, fatty infiltration, and fiber grouping may also develop without a rotator cuff tear, as part of the ageing of muscle tissue—a possibility which could have been evaluated further with an age-matched reference group without any rotator cuff pathology (Lexell 1995).

In summary, full-thickness rotator cuff tears are associated with a substantial reduction in satellite cell number and muscle proliferative capacity, general myofiber atrophy, and loss of MHC1 content compared to partial-thickness tears.

KL, OL and LE designed the clinical study (patient recruitment and biopsy procedure). KL assessed the patients and obtained the biopsy tissue. KL and AS designed and tested the methods, and conducted data collection and statistical analysis. All authors contributed to the writing of the manuscript.

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