INVESTIGATING MECHANISMS DRIVING FIBROSIS IN PATHOLOGICAL ROTATOR CUFF TENDONS

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Abstract

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Shoulder pain is common, often caused by rotator cuff inflammation and usually heals by fibrotic repair. The fibrotic signature associated with the resolution of inflammation is well documented in a number of human tissue pathologies, but not tendon. TGFβ and its associated superfamily are implicated as key drivers of fibrosis, and modulate extracellular matrix homeostasis. The aim of this thesis was principally to investigate the TGFβ axis in healthy and pathological tendons, and to study changes related to the epithelial to mesenchymal transition. We hypothesised that TGFβ family mediators would be dysregulated in pathological compared to healthy tendons. We also hypothesised that there would be evidence of epithelial to mesenchymal transition with tendon disease.

Healthy and diseased rotator cuff tendons were collected from patients presenting to an orthopaedic referral centre. Diseased tendinopathic (intact) and healthy rotator cuff tendons were collected via ultrasound-guided biopsy under general anaesthetic prior to surgery and torn tendons were collected during routine surgical debridement. Immunohistochemistry and qRT-PCR were used to investigate the protein and gene expression profiles of TGFβ superfamily members in these healthy and diseased rotator cuff tendons. Epithelial to mesenchymal transition was characterised with immunostaining for αSMA, E-Cadherin, and Vimentin. TLR4 and Collagen III were used to identify myofibroblast activation and collagen composition in tendon.

TGFβ signalling was dysregulated in human supraspinatus tendon pathology. Specifically, TGFβ-1, TGFβ R1 and TGFβ R2 proteins were significantly reduced ($p<0.01$) in pathological compared to healthy supraspinatus tendons, suggesting these pathways become down regulated with established disease. At the mRNA level, TGFβ R1 was significantly decreased whereas TGFβ R2 was significantly increased ($p<0.01$). BMP-2, BMP-7 and CTGF mRNA were unchanged with pathology. Immunostaining showed Vimentin was significantly decreased in pathology ($p<0.01$), however αSMA, E-Cadherin and TLR4 remained unchanged.

Pathological tendons showed dysregulation of the TGFβ axis compared to healthy tissues. Evidence for EMT in pathological tendons was inconclusive. Whilst Vimentin expression significantly decreased with pathology, immunostaining suggested co-localisation of Vimentin and αSMA along the edge of a massive tendon tear. We propose the down-regulation of TGFβ pathways could be a protective response to limit hypertrophic fibrosis. The disruption of the TGFβ axis with tendon pathology compared to healthy tissues demonstrates the importance of these pathways in tendon homeostasis. In the presence of suppressed TGFβ levels, alternative pathways such as TNFα signalling may drive fibrosis. In Dupuytren's disease TNFα is implicated as an important driver of myofibroblast activity, and could be the prevailing signal in chronic tendon pathology.

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Abbreviations

ACL	Anterior cruciate ligament
ADLs	Activities of daily living
aSMA	Alpha smooth muscle actin
BMP	Bone morphogenic protein
CD	Cluster of differentiation
COX	Cyclooxygenase
CSA	Cross sectional area
CTGF	Connective tissue growth factor
DAB	3,3'-diaminobenzidine
DAMPs	Damage associated molecular patterns
DNA	Deoxyribonucleic acid
E-Cadherin	Epithelial cadherin
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
EndMT	Endothelial mesenchymal transition
GC	Glucocorticoid
GP	General practitioner
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IRF	Interferon regulatory factor
JAK	Janus kinases
LAP	Latency associated peptide
NSAIDs	Non-steroidal anti-inflammatory drugs
OSS	Oxford shoulder score
OXPAT	Oxford Partial Thickness Rotator Cuff Tear and Tendinopathy Cohort
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear
POPO-1	propanediylbis[(dimethyliminio)-3,1-propanediyl-1(4H)-pyridinyl-4-ylidenemethylidyne]]bis[3-methyl]-, tetraiodide 169454-15-3
PROMs	Patient reported outcome measures
PRP	Platelet rich plasma
QALY	Quality adjusted life year
RNA	Ribonucleic acid
RT-qPCR	Real Time quantitative polymerase chain reaction
SAD	Subacromial decompression
SDFT	Superficial digital flexor tendon
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TGF R	Transforming growth factor receptor
Th	T helper type cells
TLR	Toll like receptor
TNF	Tumour necrosis factor
UKUFF	United Kingdom Rotator Cuff Trial
VEGF	Vascular Endothelial Growth Factor

Table of Contents

	Abstract	2
	Acknowledgements	3
	List of Abbreviations	4
	List of Figures	7
	List of Tables	9
Chapter 1	Introduction	10
	1.1 Overview of Tendon	10
	1.1.1 Function	10
	1.1.2 Structure	10
	1.1.3 Blood supply	12
	1.1.4 Innervation	12
	1.1.5 Composition	13
	1.1.6 Mechanical properties	14
	1.2 The Rotator Cuff	15
	1.2.1 Overview	15
	1.2.2 Rotator cuff injury	16
	1.2.3 Epidemiology of rotator cuff injury	17
	1.2.4 Tendinopathy	18
	1.2.5 Rotator cuff tear	19
	1.3 Clinical Management of Tendon Pathology	20
	1.3.1 Conservative management	20
	1.3.2 Interventional management	23
	1.3.3 Impact of age on management	24
	1.4 Fibrosis	25
	1.4.1 Overview	25
	1.4.2 Mechanisms of fibrosis	27
	1.4.3 Myofibroblasts	28
	1.4.4 Role of TGFβ in fibrosis	29
	1.4.5 TGFβ signalling	31
	1.4.6 Role of TGFβ in Epithelial Mesenchymal Transition	32
	1.4.7 Role of interleukin-13/-4 pathway in fibrosis	32
	1.4.8 Macrophages	33
	1.4.9 Bone Morphogenic Proteins	35
	1.5 Models of Fibrosis	35
	1.5.1 Scarless healing	35
	1.5.2 Animal models of fibrosis	36
	1.5.3 Manipulating tissue healing	37
	1.5.4 Fibrosis in musculoskeletal pathology	38
	1.6 Objectives and Hypothesis	39
Chapter 2	Methods	41
	2.1 Tissue sample phenotype	41
	2.2 Study approval	41
	2.3 Patient cohorts	42
	2.3.1 *Patient cohort for immunohistochemistry and immunofluorescence*	42

	2.3.2 *Patient cohort for gene expression analysis*	45
	2.4 Tissue processing	46
	2.4.1 *Immunohistochemistry protocol*	46
	2.4.2 *Image analysis for quantitative immunohistochemistry*	48
	2.5 Gene Expression	48
	2.5.1 *RNA extraction and cDNA synthesis*	48
	2.5.2 *Gene expression by RT-qPCR*	49
	2.6 Immunofluorescence	50
	2.6.1 *Immunofluorescence cell type and TGFβ co-localisation*	50
	2.6.2 *Protocol – Antigen retrieval*	50
	2.6.3 Staining for immunofluorescence	51
	2.7 *Confocal imaging for immunofluorescence*	52
	2.8 Statistical analysis	52
Chapter 3	Results	53
	3.1 Histological assessment of diseased rotator cuff tendons	53
	3.2 Pathological tendons show dysregulation of TGFβ family signalling proteins	53
	3.3 Pathological tendons show dysregulation of TGFβ family signalling at the mRNA level	60
	3.4 Epithelial to mesenchymal transition proteins and fibroblast activation	62
	3.5 Collagen III	63
	3.6 Immunofluorescence	64
Chapter 4	Discussion	67
	4.1 Limitations	73
	4.2 Future work	77
	4.3 Conclusions	78
	4.4 Summary	78
Appendix	A1 Related publications	80
	A2 Related presentations and scientific meetings	80
	Oral presentations	80
	Poster presentations	80
	A3 Courses attended	80
	A4 Prizes	
References		81

List of Figures

1. The hierarchical organisation of tendon structure (From Kannus 2000, based on the original drawing by Kastelic 1978) from individual collagen fibrils to tendon 10

2. Stress: Strain curve for human Achilles tendon by (Abrahams 1967) 14

3. Demonstrates the basic anatomy of the rotator cuff tendons. X demonstrates the most common location for a rotator cuff tear (within the supraspinatus, tears propagate from near the junction of the infraspinatus and supraspinatus at the posterior border (Kim *et al*. 2010) 16

4. PubMed search hits for tissue type, fibrosis and mediators of fibrosis demonstrating the paucity of research available on tendon fibrosis 26

5. TGFβ requires two heterodimeric receptors to initiate downstream Smad3 signalling. Adapted from (Huang *et al*. 2011). 1)➔2) TGFβ receptors 1 and 2 form heterodimers. 3) Ligand binds a homodimer of these two heterodimers to then activate downstream Smad signalling 30

6. Panel demonstrating the minimally invasive biopsy technique used for collection of healthy and tendinopathic supraspinatus tendon, as originally described by Murphy et al (2013) 44

7. Distributions of how many different samples were used for each experiment. 46

8. Photomicrographs for IHC of healthy, tendinopathic and torn supraspinatus, stained with antibodies to TGFβ1, TGFβ receptor 1, TGFβ receptor 2. DAB staining is brown. Nuclear counterstain is haematoxylin (blue). Scale bar = 20μm 55

9. Expanded photomicrograph of healthy TGFβ1 staining. DAB (brown) staining appears to be localised around the nuclei suggesting it is most highly expressed in either cytoplasm or on the cell membrane, rather than in the tendon ECM. Scale bar = 20μm 56

10. Expanded photomicrograph of tendinopathic TGFβ1 R1 staining. DAB (brown) immunopositive staining appears to be localised around the nuclei, and along the likely cell body shape. This would suggest increased expression on the cell membrane as would be expected. Scale bar = 20μm 57

11. Expanded photomicrograph of torn TGFβ1 R2 staining. DAB (brown) staining appears to be localised around the nuclei, and in a 58

few cases along the likely cell body shape. This would suggest increased expression on the cell membrane as would be expected. A – marks an area of DAB staining, B – marks a nucleus. Scale bar = 20μm

12. Quantitative analysis of immunopositive staining showing dysregulation of TGFβ family proteins in pathological compared to healthy rotator cuff tendons. (A) TGFβ1, healthy (n=11), tendinopathic (n=10), torn (n=10) (B) TGFβR1, healthy (n=11), tendinopathic (n=10), torn (n=9) and (C) TGFβR2, healthy (n=10), tendinopathic (n=10), torn (n=9). Bar represents the median value. Data were analysed using the Kruskal-Wallis test * $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$ 59

13. Expression of pro-fibrotic genes in healthy and diseased tendons. TGFβ family mediators (A-C), BMP's 2 & 7 (D, E) and CTGF (F) mRNA expression are shown in healthy hamstring (n=7) compared to pathological rotator cuff tendons (n=7). Gene expression is normalized to β-actin, bar shows median value. * = $p<0.05$ 61

14. Quantification of immunopositive staining for markers of EMT and fibroblast activation in pathological compared to healthy rotator cuff tendons. Healthy (n=10), tendinopathic (n=10), torn (n=10). Bar represents the median value. Data were analysed using the Kruskal-Wallis test * $p<0.05$ 63

15. Representative confocal image shows markers for alternative and classically activated macrophages on a massive supraspinatus tear. Markers include CD206 (green) for alternative macrophage activation, and TLR4 (red) and IRF5 (purple), which are both associated with classically activated macrophages. Nuclear counterstain was POPO-1, and scale bar represents 20μm 65

16. Representative confocal image shows markers for EMT on a massive supraspinatus tear. Markers for EMT include Vimentin (green), α-SMA (red), and CD163 (purple). CD163 is associated with, alternatively activated macrophages. Nuclear counterstain was POPO-1, and scale bar represents 20μm 65

17. Representative confocal image shows markers to co-localise TGFβ with alternatively activated macrophages on a massive supraspinatus tear. Markers include CD206 (green), CD163 (red), and TGFβ (purple). Nuclear counterstain was POPO-1, and scale bar represents 20μm 66

18. Shows a possible bimodal response of TGFβ in disease 68

19. Mechanisms of fibrosis in several organ tissues 76

List of Tables

1. Causes of supraspinatus tendinopathy and tears grouped as intrinsic and extrinsic factors 17

2. Patient cohort details for tissue samples 44

3. Antibody details 46

4. RT-qPCR information 49

Investigating mechanisms driving fibrosis in pathological rotator cuff tendons

1. Introduction:

1.1 Overview of Tendon

1.1.1 Function

Tendons connect muscle to bone, allowing force to be applied over one or more joints enabling effective limb movement. The myotendinous junction connects muscle to tendon, and the osteotendinous junction connects tendon to bone. Depending on the joint function, tendon can have different properties. Positional tendons undergo low levels of strain (±3%), and transmit muscle force directly to bone (Birch 2007). “Elastic” tendons store and release kinetic energy with strains reaching ±10% and help to improve the efficiency of locomotion (Alexander 2002).

1.1.2 Structure

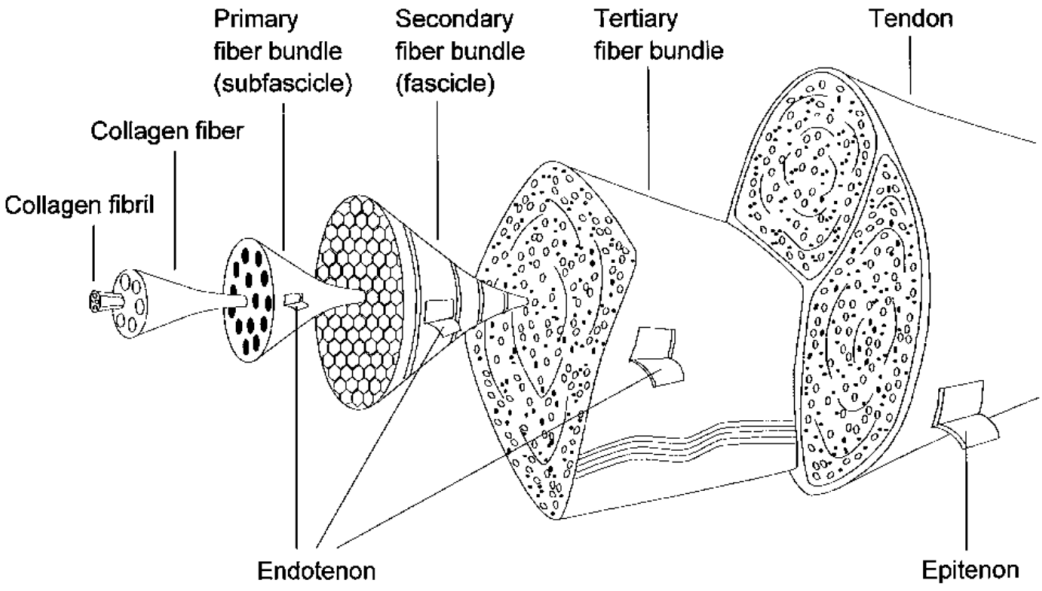


Figure 1: The hierarchical organisation of tendon structure (From Kannus 2000, based on the original drawing by Kastelic 1978) from individual collagen fibrils to tendon.

Tendons are oriented in the direction of stress, and are structured in a highly ordered hierarchical assembly (Figure 1) (Kastelic, Galeski and Baer 1978; Kannus 2000; Silver, Freeman and Seehra 2003). Within a tendon, multiple collagen fascicles are separated by a loose connective tissue layer called the endotenon, and surrounded by epitenon. Fascicles are formed of multiple bundles of collagen fibrils. The fibrils, which are themselves a bundle of collagen fibres, are mostly oriented longitudinally. A minority of fibrils form a multidirectional array, which helps to buffer the tendon against forces from directions other than that generated by its muscular attachment, and prevent disconnection of fibres (Józsa *et al.* 1991; Kannus 2000). The epi- and endotenon contain vascular, lymphatic and nerve supplies to the tendon. The epitenon is also surrounded by paratenon, a loose connective tissue layer that contains synovial cells on its inner lining. Synovial fluid acts as lubrication through tendon sheaths, as well as providing nutrition (Lundborg, Holm and Myrhage 1980).

Muscle and tendon merge at the myotendinous junction, which is characterised by finger-like projections formed of myocyte (muscle cell) recesses (Kvist *et al.* 1991). This allows for dissipation of force generated by myosin-complexes in the muscle over a greater surface area, reducing tensile stress. Bone and tendon merge at the osteotendinous junction or enthesis over four zones – tendon, fibrocartilage, mineralised cartilage, and bone, which broadly categorise the gradual change in composition and physical properties (Cooper and Misol 1970).

1.1.3 Blood supply

Tendons receive their blood supply from the musculo- and osteo-tendinous junctions (Kvist *et al.* 1995), surrounding paratenon and synovial sheath (Carr and Norris 1989). However, the oxygen consumption is 7.5 times less than that of skeletal muscle, which emphasises the relatively low metabolic activity of tendon compared to muscle (Vailas *et al.* 1978). This characteristic allows tendon to maintain tension for extended periods of time without becoming ischaemic. During acute injury and chronic tendinopathy, tendon vasculature increases its blood supply, thus aiding the supply of inflammatory and wound healing cells (Gelberman *et al.* 1981; Kvist *et al.* 1987; Aström and Rausing 1995). However, a region of hypovascularity has been described in the Achilles tendon, which corresponds with the area 2 to 6 cm from the insertion site that is most prone to rupture (Carr and Norris 1989). A similar region has been described in the supraspinatus, although it is dependent on position in the rotator cuff (Rathbun and Macnab 1970; Chansky and Iannotti 1991). Reduced tendon blood flow is associated with advancing age, mechanical loading, and being male (Aström 2000; Knobloch *et al.* 2008), factors that also predispose to tendon rupture (Hess 2010).

1.1.4 Innervation

Tendon innervation arises from associated muscle, cutaneous and peritendinous nerve trunks (Stilwell 1957). There is sparse innervation of the main tendon body, however peripheral nerve endings are present and act as

mechanoreceptors providing proprioception, nociception and autonomic responses (Ackermann *et al.* 2001). Both peripheral and central pathways are involved in pain processing and sensitisation, responding to mechanical changes in rotator cuff tendon, and consequently altering responses in central and peripheral nerves (Gangadharan *et al.* 2011; Gwilym *et al.* 2011). Homeostasis and tendon healing are also influenced by the peripheral nervous system. During the inflammatory phase of healing there is nerve in-growth. The subsequent proliferative and remodeling phases are in part regulated by the glutaminergic system and sensory nerves (Ackermann *et al.* 2003; Ackermann, Salo and Hart 2009). Neuropeptides such as Substance P are mechanoresponsive and are produced in response to loading, furthermore they may act as a regulatory component of tendon cell activity (Backman *et al.* 2011).

1.1.5 Composition

Water accounts for 70% of human tendon mass, with the remaining 30% made up of dry mass, which is mainly extracellular matrix (ECM). The ECM consists of 65-80% type I collagen, <5% collagen III and 2% elastin (Birch *et al.* 1999; Kannus 2000; O'Brien 2007). Fibroblasts and tenocytes make up the majority of the cellular content of tendon (Kannus 2000) and are responsible for laying down ECM components.

The organisation and composition of different tendons at the intra-fascicular, inter-fascicular, and tendon level defines their various physical properties. Higher energy storing tendons such as the Achilles or equine

superficial digital flexor tendon (SDFT) have smaller diameter fibrils, higher levels of fibre organisation and greater inter-fascicular sliding (Birch 2007; Batson *et al.* 2010; Thorpe *et al.* 2012; Screen, Toorani and Shelton 2013). The physical properties of fascicles had previously been thought to define tendon mechanics, as they are the building blocks of tendon, however a number of groups have shown that the whole tendon is stiffer than the sum of the fascicles that compose it (Wren *et al.* 2001; Hirokawa and Hasezaki 2010; Thorpe *et al.* 2012). It is now believed that a combination of fascicles and properties of the inter-fascicular matrix help to define the overall properties of tendon.

1.1.6 Mechanical properties

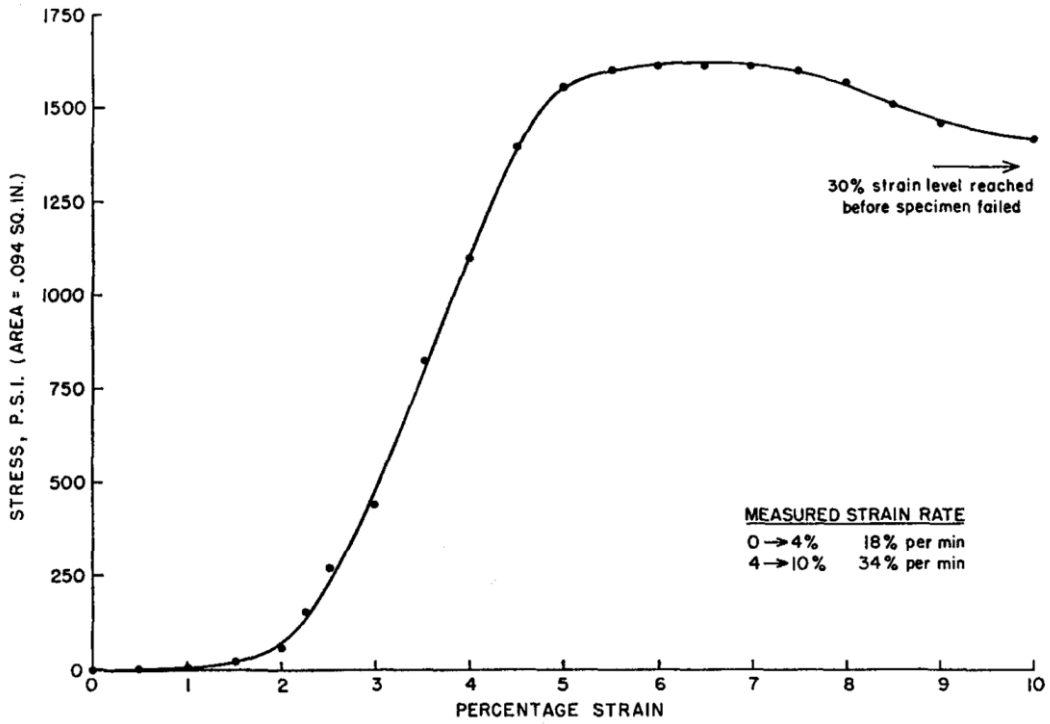


Figure 2: Stress:Strain curve for human Achilles tendon by (Abrahams 1967).

Tendons are viscoelastic tissues that display stress relaxation and creep. At rest, tendon fibrils have a crimped pattern, which straightens under a strain of up to 2% (Viidik 1973; Butler *et al.* 1978). Strains between 2-4% result in elastic deformation of the tendon, with inter-fascicular sliding, and intra-molecular sliding in collagen fibres (Mosler *et al.* 1985; Thorpe *et al.* 2012). Figure 2 demonstrates the initial low stress required to straighten the crimped tendon. The linear part of the curve represents the portion of elastic deformation of the tendon, before strain becomes too great. It is suggested that inter-fascicular sliding is particularly important in energy storing tendons, which are often present in limbs used for locomotion. A lower stiffness, permitting a greater amount of fascicle sliding allows more energy to be stored in the tendon without damaging fascicles themselves (Thorpe *et al.* 2012). Beyond 4% strain, plastic deformation of the tendon occurs, until there is complete failure at approximately 10% strain (Rigby 1959).

1.2 The Rotator Cuff

1.2.1 Overview

The rotator cuff is formed of four muscles: the subscapularis, infraspinatus, teres minor and supraspinatus, which fuse to form a complex of muscles and tendons that cover the front, back and top of the humeral head (Figure 3). As a complex, the rotator cuff provides stability to the glenohumeral joint in both normal and abnormal movement. Teres minor and infraspinatus tendons cover the back of the humeral head and the muscle group acts as an external rotator, subscapularis reaches forward to function

as an internal rotator and reinforce the anterior capsule of the glenohumeral joint. The supraspinatus tendon travels over the top of the humeral head and is a shoulder abductor.

1.2.2 Rotator cuff injury

Rotator cuff pathology can be classified as either tendinopathy, whereby the tendon is diseased but remains structurally intact, or as a tear in advanced disease, compromising the structural integrity of the tendon. The supraspinatus is the rotator cuff tendon most predisposed to injury and degenerative changes (Fu, Harner and Klein 1991).

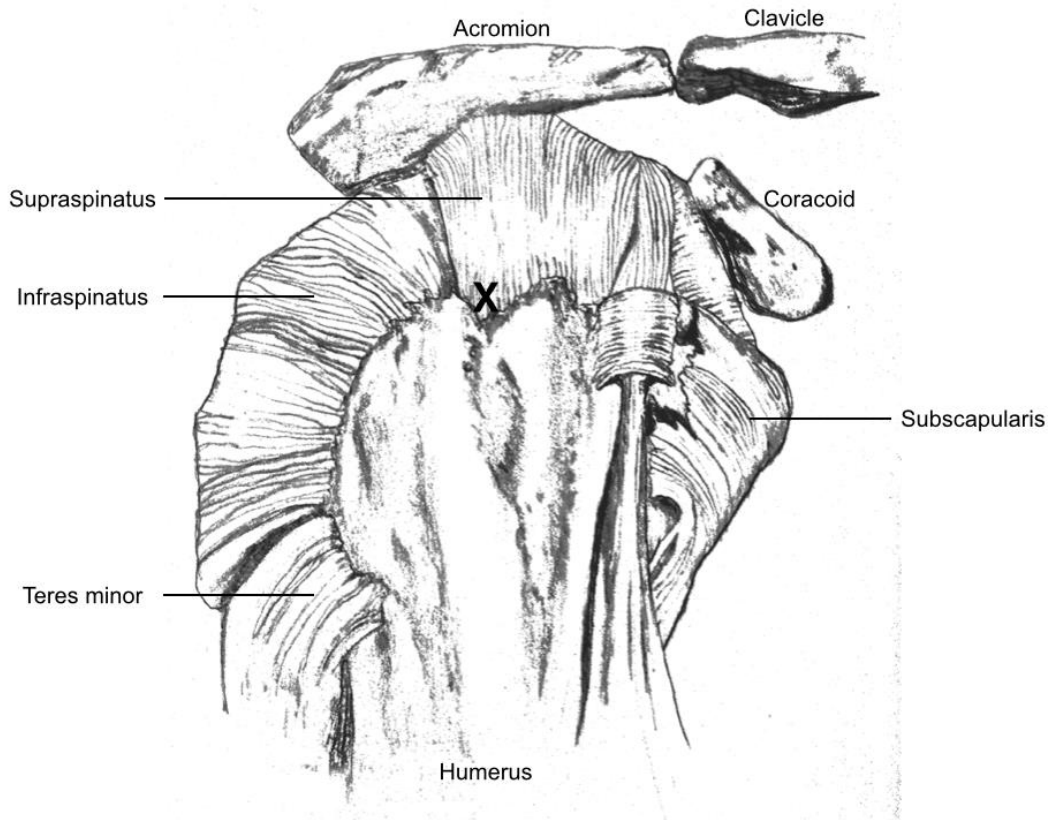


Figure 3. Demonstrates the basic anatomy of the rotator cuff tendons. X Demonstrates the most common location for a rotator cuff tear (within the supraspinatus, tears propagate from near the junction of the infraspinatus and supraspinatus at the posterior border (Kim *et al.* 2010))

1.2.3 Epidemiology of rotator cuff injury

Musculoskeletal diseases are common cause of pain and disability in well-resourced health systems (Vos, T. *et al* 2015). Shoulder pain is the third most common cause of musculoskeletal pain, accounting for 2.4% of all general practitioner consultations annually in the UK (Urwin *et al.* 1998; Murphy and Carr 2009). Rotator cuff pathology is reported as the most common cause, accounting for 70% of shoulder pain, other causes include trauma, frozen shoulder, calcific tendonitis and osteoarthritis (Chard *et al.* 1991; Mitchell *et al.* 2005). Estimates of the prevalence of rotator cuff tears vary between 10-30%; the mass screening of an entire Japanese village demonstrated 20% prevalence, and a study of 4629 cadaveric shoulders estimated prevalence at 23% (Sher *et al.* 1995; Tempelhof, Rupp and Seil 1999; Schibany *et al.* 2004; Reilly *et al.* 2006; Yamamoto *et al.* 2010; Minagawa *et al.* 2013).

Risk factors for rotator cuff pathology are classified as either intrinsic or extrinsic (Table 1). Extrinsic causes include external forces such as ‘impingement syndrome’ where the supraspinatus tendon is compressed between the humeral head and acromion during shoulder movement; this was originally argued by Neer (1972) to be a primary cause of pathology. However, intrinsic elements such as age, degeneration and dominant arm use are now known to be very important risk factors (Sano *et al.* 1997, 1999; Longo *et al.* 2008). A history of trauma, older age and dominant arm were found to be the most significant risk factors in a general population (Yamamoto *et al.* 2010).

Furthermore the prevalence of tears rises linearly with age after the third decade, from 13% in the 50s to 50% in the 80s (Milgrom *et al.* 1995; Yamamoto *et al.* 2010). Tendinopathy and tears can cause pain and disability, especially with overhead activities, and sleeping on the affected side.

Intrinsic Factors	Extrinsic Factors
Age (Milgrom *et al.* 1995; Sher *et al.* 1995; Tempelhof, Rupp and Seil 1999; Reilly *et al.* 2006; Yamamoto *et al.* 2010; Minagawa *et al.* 2013) **Degeneration** (Kannus and Józsa 1991; Sano *et al.* 1997, 1999; Longo *et al.* 2008) **Systemic disease** (obesity, steroid use)(Holmes and Lin 2006) **Dominant arm** (Yamamoto *et al.* 2010) **Genetics** (Gwilym *et al.* 2009)	**Impingement** (Neer 1972) **Overuse** (Sein *et al.* 2010) **Occupation** (Urwin *et al.* 1998; Yamamoto *et al.* 2010) **Trauma**

Table 1: Causes of supraspinatus tendinopathy and tears grouped as intrinsic and extrinsic factors.

1.2.4 Tendinopathy

Tendinopathy is a complex, multifactorial pathology encompassing the effects of ageing, exercise, genetic factors and inflammatory processes driving repair and fibrosis. Tendinopathy can be caused by brief or persistent pathological stimuli; regardless tendons demonstrate an inability to heal by generation of neo-tendon, leading to a fibrotic scar repair. Chronic overuse, overhead sports, and impingement syndrome are often cited as specific causes (Van der Windt *et al.* 1996; Selvanetti, Cipolla and Puddu 1997; Mitchell *et al.* 2005; Hess 2010). It is difficult to estimate the burden of

tendinopathy, although subacromial decompression surgery (SAD) which is performed in patients who do not respond to conservative management (rest, physiotherapy or glucocorticoid injections) for impingement syndrome was performed 21,355 times over 2009 / 2010 within NHS England (Judge *et al.* 2014). Disability is a major concern with supraspinatus tendinopathy due to pain and limited range of motion which can deny patients of their ability to work full time, perform activities of daily living (ADLs) or to lift any weight above their head (Chipchase *et al.* 2000; Cook *et al.* 2002; MacDermid *et al.* 2004). Furthermore, any rotator cuff injury or previous degeneration has been shown to predispose the tendon to complete tear which can be even more debilitating and may lead to secondary shoulder osteoarthritis (Kannus and Józsa 1991; Chaudhury *et al.* 2010). Moreover, tendon damage can occur within physiological limits and cumulative damage may not allow sufficient time for repair, resulting in a weakened tendon that is prone to rupture (Selvanetti, Cipolla and Puddu 1997; Ker 2002).

1.2.5 Rotator cuff tear

Torn tendons can be classified according to the size of the tear – small <1cm, medium 1-3cm, large 3-5cm, massive >5cm, or complete rupture (Post, Silver and Singh 1983). Smaller tears show increased fibroblast cellularity, blood vessel proliferation and a greater inflammatory response (Matthews *et al.* 2006). These changes may confer a superior propensity to heal for smaller tears after surgical debridement, and an increased risk of re-rupture after repair of larger more inert tears. Supraspinatus muscle tissue associated with

massive tears show down-regulation of all adipogenic and myogenic genes suggesting a lack of regenerative capacity (Choo *et al.* 2014).

As for tendinopathy where the tendon is diseased but remains intact, pain is the primary reason for consulting a General Practitioner (GP) for patients with a tear (Urwin *et al*. 1998). Symptomatic patients suffer from a reduced quality of life due to pain affecting activities of daily living, including sleeping on the affected side, raising their arm above shoulder height, and weakness lifting (Nakajima *et al.* 2012; Minns Lowe, Moser and Barker 2014). However, there is also a significant cohort of asymptomatic patients with rotator cuff tears (Yamamoto *et al.* 2010). These patients suffer no pain and often have limited functional deficit, but they are at an increased risk of developing symptoms. The natural history of asymptomatic tears leads to anatomic deterioration, increased tear size and reduced muscle quality, which then correlates with the onset and progression of symptoms (Milgrom *et al.* 1995; Yamaguchi *et al.* 2001; Moosmayer *et al.* 2013). Supraspinatus tears most commonly occur at an area of tendon approximately 15mm posterior to the biceps tendon, near the junction of the infraspinatus and supraspinatus tendon (Kim *et al.* 2010). However, it is likely that some tears have a multifocal origin.

1.3 Clinical Management of Tendon Pathology:

1.3.1 Conservative management

Management of rotator cuff pathology involves conservative, and interventional treatment. Conservative management can encompass rest, pain rehabilitation, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), physiotherapy, and glucocorticoid injections.

The success rate of conservative management varies considerably, with good outcomes between 33-82% (Wolfgang 1974; Itoi and Tabata 1992; Tanaka *et al.* 2010). Predictors of a good to excellent outcome include: integrity of the intramuscular tendon, supraspinatus muscle atrophy, negative impingement sign, and a greater ability for external rotation (Tanaka *et al.* 2010). Furthermore, patients with well preserved supraspinatus and infraspinatus function are deemed better candidates for conservative management (Itoi 2013). Physiotherapists can help to educate patients about appropriate rest, and unloaded or lightly loaded exercises to stimulate tendon, and prevent joint stiffness (Kuhn 2009). Rest must be appropriate, as tendon requires mechanical stimulus for optimal health and function, however there is currently no evidence base for how to optimally structure this management. In the sedentary population fully unloaded tendons can become weak, and it has been suggested that this can lead to asymptomatic degenerative tears (Kannus *et al.* 1997; Lewis 2010). In a prospective trial to assess the effectiveness of physical therapy for treatment of atraumatic full thickness rotator cuff tears, 25% of patients at their 2 year follow up had insufficient symptom control that they opted for surgery. The conservatively managed group reported mixed outcomes although some improvement was revealed in patient reported outcome measures (PROMs) (Kuhn *et al.* 2013).

NSAIDs can improve short term pain management, but there is evidence to suggest they have negative effects on tendon turnover, and tendon to bone healing (Cohen *et al.* 2006; Andres and Murrell 2008; Christensen *et al.* 2011; Boudreault *et al.* 2014). NSAIDs inhibit the cyclooxygenase enzymes (COX) -1 and -2, which catalyse the conversion of arachidonic acid to prostaglandin and thromboxane. Whilst COX-1 is ubiquitously expressed, COX-2 is an inducible enzyme primarily expressed by inflammatory cells (Seibert *et al.* 1994). Newer NSAIDs selectively inhibit COX-2 thereby protecting cellular homeostasis mechanisms that COX-1 is vital for, especially in the gastric mucosa (Crofford 1997). NSAIDs have a well documented inhibitory effect on bone healing, they lead to weaker ligament healing after surgical transection and may compromise the bone ingrowth and mineralisation necessary for effective tendon-bone healing (Elder, Dahners and Weinhold 2001; Krischak *et al.* 2007; Chen and Dragoo 2013; Su and O'Connor 2013). Moreover, they are suggested to cause less stable callus formation in fracture healing with decreased bone, and increased cartilage formation (Krischak *et al.* 2007), and in a rat model reduced tendon tensile strength after 5 days of healing (Virchenko 2004).

Glucocorticoid (GC) steroid injections are administered into the space below the subacromial bursa under ultrasound guidance, and aim to provide pain relief (Green *et al.* 2000; Wolf and Altchek 2003). However, there are clinical reports associating the use of GC injections with tendon rupture (Kleinman and Gross 1983; Clark *et al.* 1995; Smith *et al.* 1999). A meta-

analysis of treatment for Achilles tendinopathy showed no evidence for any benefit associated with GC injections (Shrier, Matheson and Kohl 1996). Animal studies also provide potentially concerning evidence of harm caused by corticosteroids on tendons, including reduced tensile load to failure, reduced tendon mass, and in healthy tendon a short term response similar to structural injury (Kapetanos 1982; Haraldsson *et al.* 2006; Wei *et al.* 2006).

1.3.2 Interventional management

Despite the success of conservative management in some scenarios, a primary care study by Van der Windt *et al* (1996) showed that after 1 year 40% of patients treated conservatively have persistent pain. If conservative management fails, there is significant pathology, or loss of function at presentation and imaging to support clinical findings, then surgery can be offered (Chaudhury *et al.* 2010).

Surgical management can include SAD in suspected impingement, and / or rotator cuff repair for torn tendons. In the USA approximately 300,000 rotator cuff repairs are performed at the cost of ~US$3billion per year (Aurora *et al.* 2007). In England ~20,000 SAD operations and 10,000 rotator cuff repairs occur per year (Judge *et al.* 2014) representing a significant socio-economic burden. There is unfortunately a dearth of evidence to support the outcomes of surgery compared to controls due to the inherent difficulties of such studies (Andres and Murrell 2008). Brox *et al* (1993) showed that surgery and exercise are better than placebo, but no significant difference

between surgery and exercise existed. It is reported that some patient cohorts benefit significantly from surgery, and being able to more successfully select these patients would be a valuable tool (Brox *et al.* 1993, 1999). The outcomes of surgical repair are variable, dependent on the extent of pathology, with re-tears of rotator cuff repair ranging from 5-40% after 14 months (Sugaya *et al.* 2007; Kim *et al.* 2012). Within the context of societal costs, surgery appears to provide a benefit over non-operative management (Mather *et al.* 2013). Vitale *et al* (2007) demonstrated a utility cost of $3,000-$13,000 for rotator cuff repair per quality adjusted life year (QALY), which is under the suggested $50,000 per QALY that is deemed an acceptable cost (Vitale *et al.* 2007).

1.3.3 Impact of age on management

Age is associated with tendon degeneration, fragmentation, loss of cellularity, and reduced vascular supply (Brewer 1979). Aged tendons are also more susceptible to a loss of tensile strength from cyclical loading, due to load induced matrix metalloproteinase activity (Dudhia *et al.* 2007). However, rotator cuff repair is supposedly just as beneficial to the elderly as it is in younger populations (Rhee, Cho and Yoo 2014). The outcome of SAD has also been compared to rotator cuff repair in the elderly, to investigate whether the reported benefit was related to debridement, decompression and placebo. There is still a significant clinical benefit to rotator cuff repair in older populations compared to SAD, although tendon healing was nevertheless the main determinant of outcomes in rotator cuff repair (Flurin *et al.* 2013; Jacquot

et al. 2014). However, conservative management has comparatively better outcomes in the older population, although this could be related to reduced activity and expectations of what ADLs can be accomplished in the elderly (Kijima *et al.* 2012).

1.4 Fibrosis:

1.4.1 Overview

Fibrosis is the complex formation of fibrous scar tissue that results from excess deposition of ECM proteins and pathological activation of myofibroblasts (Verrecchia and Mauviel 2007). Injured tendons heal by fibrotic repair and do not form neo-tendon. The development of a repair scar leads to structurally and functionally inferior tendon with altered proteomic composition, that increases the risk of re-injury (Józsa *et al.* 1990; Kannus and Józsa 1991; Dakin *et al.* 2014). If it were possible to moderate fibrosis, tendon healing and disease outcomes could be significantly improved.

Inflammation and the wound-healing cascade are the primary drivers of fibrosis, with recruited cells producing excess ECM. Inflammation mediated fibrosis is well documented in skin, liver, renal and pulmonary fibrotic diseases (Border and Noble 1994; Wynn 2007). A considerable amount of investigation has been completed with regards to these organs; however, far less is known about the mechanisms by which fibrosis occurs in pathological rotator cuff tendons as demonstrated by a PubMed search in Figure 4 (below). Inflammation has been identified in sub-acute and chronic equine tendon

injury (Dakin *et al.* 2012). Animal models of surgically induced tendon injury show the presence of inflammatory cells by immunohistochemistry (Marsolais, Côté and Frenette 2001; Wong *et al.* 2009).

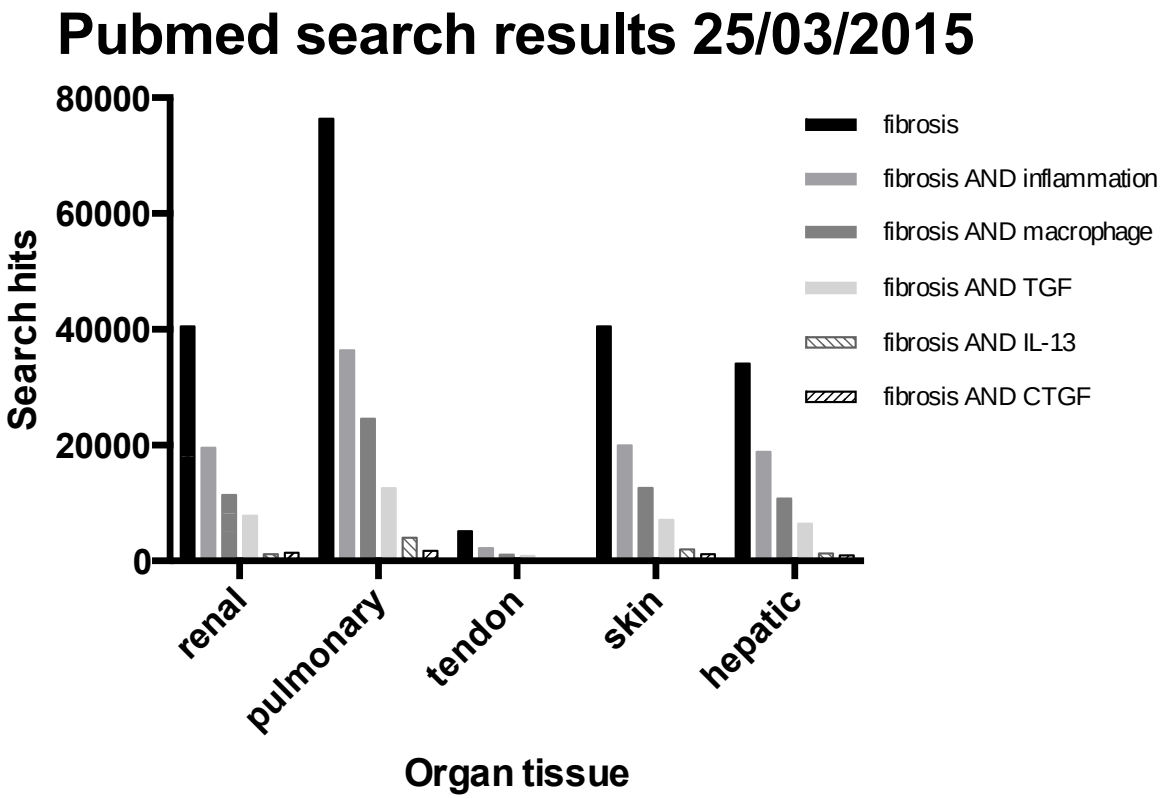


Figure 4: Pubmed search hits for a tissue type, fibrosis and mediators of fibrosis demonstrating the paucity of research available on tendon fibrosis.

Fibrosis can also be subdivided by its cause: Reparative wound healing or 'scarring' is a normal wound healing response and returns tissue to good function. Reactive fibrosis is a pathological response to a stimulus (e.g. Hepatitis C virus – liver, or chronic overuse – tendon) and encompasses what is traditionally known as fibrosis. Aberrant fibrosis can occur when a particular pathway is abnormally triggered, as has been suggested in some forms of scleroderma and pulmonary fibrosis (Chilosi *et al.* 2003; Sato *et al.* 2004; Abraham and Varga 2005).

1.4.2 Mechanisms of fibrosis

The key effector cells of fibrosis include macrophages, fibroblasts, lymphocytes, and eosinophils. When an injury occurs endothelial and epithelial cells release inflammatory mediators, which initiate the clotting cascade. Platelet activation causes vasodilation, increased vessel permeability, aggregation, clot formation and haemostasis. The release of growth factors and chemokines stimulates the recruitment of leucocytes, polymorphonuclear cells (PMNs), and myofibroblasts. These cells clear cellular debris and signal lymphocyte recruitment. Lymphocytes then release further cytokines, including TGFβ, and IL-13, which cause activation of fibroblasts that consequently express α-SMA (becoming myofibroblasts). Myofibroblasts are a population of fibroblasts usually activated during wound healing and are responsible for laying down the majority of ECM and driving wound contraction (Krieg, Abraham and Lafyatis 2007). A single or sustained tissue insult can lead to pro-fibrotic mediators (e.g. IL-13, TGFβ) being elevated chronically, causing tissue fibrosis (Yamamoto *et al.* 1994; Sime *et al.* 1997; Branton and Kopp 1999).

Both the innate and adaptive immune system are involved in the propagation of fibrotic disease with IL-13 one of the dominant adaptive pro-fibrotic cytokines; increasing collagen synthesis from fibroblasts and regulating alternatively activated macrophages (Chiaramonte *et al.* 1999; Aliprantis *et al.* 2007). TGFβ is the most frequently investigated (innate)

effector of fibrosis and regulator of extracellular matrix (ECM) turnover. However, there is considerable overlap and feedback loops between the two systems, IL-13 is shown in some settings to achieve its pro-fibrotic signalling via TGFβ (Lee *et al.* 2001; Fichtner-Feigl *et al.* 2006).

1.4.3 Myofibroblasts

Kvist *et al* (1985) initially found myofibroblasts in injured Achilles, and suggested their role in wound contraction. More recently myofibroblasts were implicated in collagen turnover and homeostasis of tendons in a murine treadmill running model (Szczodry *et al.* 2009). In this study, myofibroblast levels increased after exercise in healthy mice, and produced higher total collagen levels. Myofibroblasts are derived from multiple sources including local tissue, circulating fibroblast-like bone marrow derived stem cells, epithelial and endothelial-mesenchymal transition (EMT, EndMT) (Bucala *et al.* 1994; Kalluri and Neilson 2003; Forbes *et al.* 2004; Ebihara *et al.* 2006; Quan, Cowper and Bucala 2006). There are several mechanisms proposed in the regulation of myofibroblasts across both the innate and adaptive ends of the immune system. Fibroblasts can express pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs), which recognise pathogens and damage associated molecular patterns (PAMPs, DAMPs) causing them to activate and differentiate into myofibroblasts (Wang *et al.* 2011). Expression of connective tissue growth factor (CTGF) and TGFβ can also differentiate mesenchymal cells into myofibroblasts as well as stimulate fibroblasts to proliferate (Battegay *et al.* 1990; Lee *et al.* 2010).

1.4.4 Role of TGFβ in fibrosis

The TGFβ superfamily modulates a number of vital cellular processes in development, differentiation and homeostasis; importantly for tendon it is a key regulator of ECM (Wynn 2008; Wu and Hill 2009). In particular it drives collagen production through fibroblast activation (Roberts *et al.* 1986; Lee *et al.* 2010). TGFβ can also be expressed via non-Smad mechanisms associated with the adaptive immunity end of the wound healing spectrum (Moustakas and Heldin 2005). TGFβ receptors interact with non-Smad proteins, initiating signalling that is complimentary to Smad pathways. There are three mammalian isotypes of TGFβ – 1, -2, -3, exhibiting similar activity, although scarring models report profibrotic effects of TGFβ -1 and -2, and anti-scarring effects from TGFβ -3 (Shah, Foreman and Ferguson 1992, 1995; Gorelik and Flavell 2002). Fibroblasts stimulated by TGFβ demonstrate a similar fibrotic phenotype to those involved in systemic sclerosis and TGFβ is also linked to stimulating EMT in pulmonary fibrosis (Abraham and Varga 2005; Kasai *et al.* 2005; Zhou *et al.* 2005; Willis and Borok 2007).

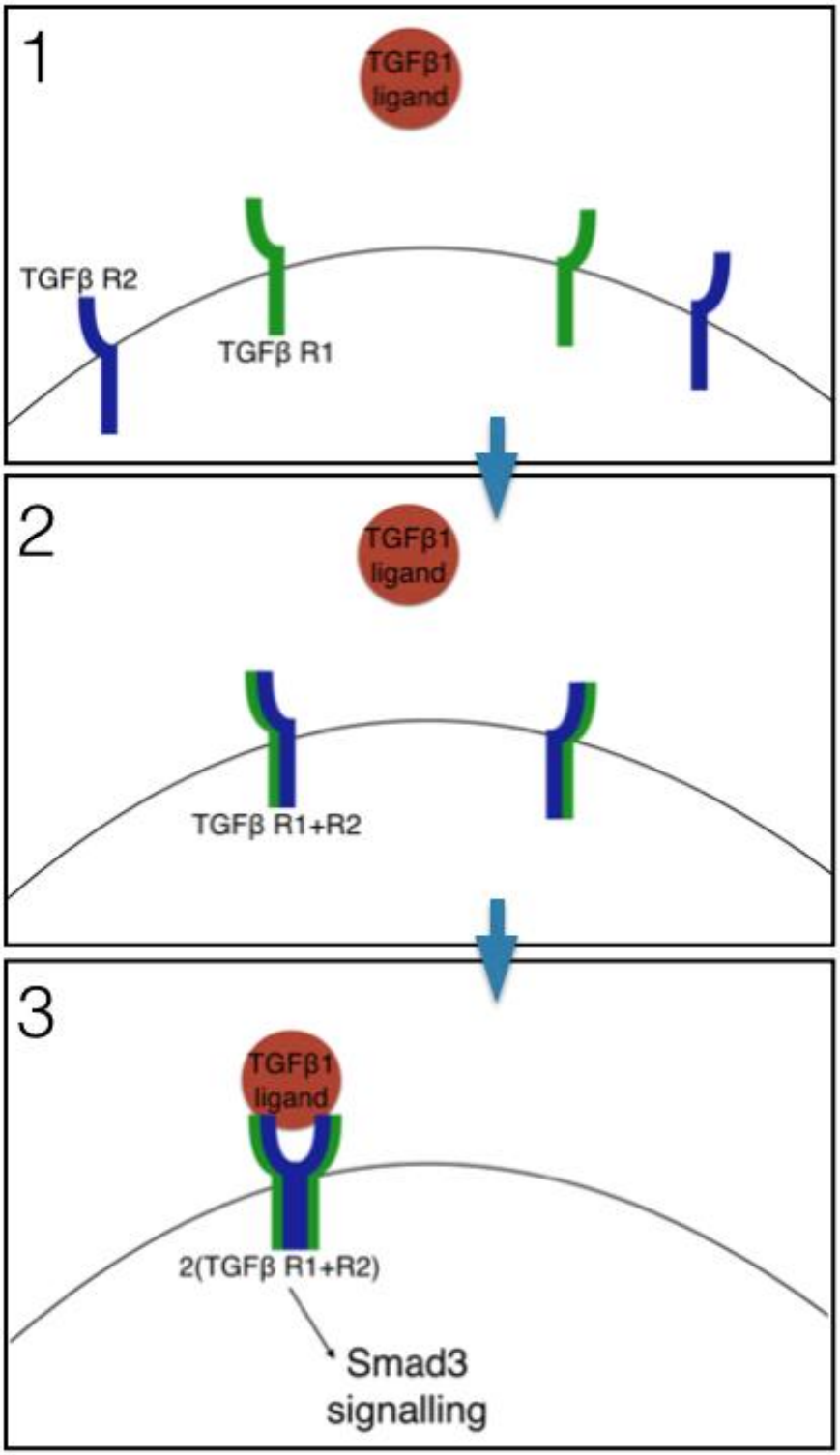


Figure 5: TGFβ requires two heterodimeric receptors to initiate downstream Smad3 signalling. Adapted from (Huang *et al.* 2011).
1)➔2) TGFβ receptors 1 and 2 form heterodimers. 3) Ligand binds a homodimer of these paired heterodimers to then activate downstream Smad signalling.

1.4.5 TGFβ signalling

TGFβ signalling is mediated through a complex of two heterodimers of TGFβ receptor 1 and 2 (Figure 5). In response, intracellular Smad3 is phosphorylated and forms complexes with Smads 2, 4 and other proteins, which then modulate transcription of target genes in the nucleus (Wu and Hill 2009). Betaglycan or TGFβ receptor 3 (TβRIII) is a co-receptor for the TGFβ superfamily. TβRIII acts by presenting ligands to other receptors and regulating their trafficking and localisation (López-Casillas, Wrana and Massagué 1993; López-Casillas *et al.* 1994; Chen *et al.* 2003; Kirkbride *et al.* 2008). TGFβ is synthesised as a pro-peptide and stored in a latent form bound to latency-associated protein (LAP). This pro-peptide is abundant within ECM, platelets and macrophages, but TGFβ must dissociate from LAP before it can bind to receptors.

TGFβ-1 is of particular importance during wound healing and homeostasis, but is also implicated in multiple disease states associated with excess collagen deposition (Sporn *et al.* 1983; Roberts *et al.* 1986; Li *et al.* 2005; Choo *et al.* 2014). TGFβ signalling increases collagen I expression via Smad3 and fibroblast activation (Chen *et al.* 1999). Fibroblasts are induced to express CTGF, which stimulates further fibroblast differentiation and proliferation, and collagen synthesis (Duncan *et al.* 1999). CTGF has been shown to directly bind to TGFβ in the extracellular space, and increase activity by enhancing its receptor-binding ability (Abreu *et al.* 2002).

1.4.6 Role of TGFβ in Epithelial Mesenchymal Transition

The most studied roles of TGFβ centre on its recruitment of pro-fibrotic cells, and initiating downstream signalling via Smads, but it also appears to be responsible for the phenotypic conversion of epithelial and endothelial cells into mesenchymal cells (Borthwick *et al.* 2012). This process changes the expression of certain proteins that are associated with the cell phenotype, such as elevating α-smooth muscle actin (αSMA), vimentin, and reducing E-Cadherin (Zhang *et al.* 1996; Hills *et al.* 2012). EMT has been extensively investigated in renal and pulmonary fibrosis (Zeisberg and Kalluri 2004; Willis and Borok 2007). The process involves the dedifferentiation of epithelial cells into mesenchymal, fibroblast-like cells, which then produce Collagen type I and proliferate in fibrotic conditions (Iwano *et al.* 2002). However, it has more recently come into question whether EMT does occur or if some areas of fibrosis research have been misguided (Kriz, Kaissling and Le Hir 2011; Quaggin and Kapus 2011). Cell fate mapping studies have shown that epithelial cells do not begin to express fibroblast markers, and are not found in regions of fibrosis such as the renal interstitium (Humphreys *et al.* 2010; Koesters *et al.* 2010; Li *et al.* 2010).

1.4.7 Role of interleukin-13/-4 pathway in fibrosis

IL-13 has been shown in hepatic and pulmonary fibrosis models to develop a profibrotic phenotype via both TGFβ dependent and independent mechanisms (Lee *et al.* 2001; Kaviratne *et al.* 2004; Fichtner-Feigl *et al.* 2006).

IL-13 is secreted by a number of cell types, but most significantly by T helper type 2 cells (Th2). The Th2 response has a central role in precipitating fibrosis. IL-13 is closely related to IL-4 and has pleiotropic effects including tissue remodelling and fibrosis (Wynn 2003). Both IL-4 and -13 use receptor IL-4Rα as a signalling component, which via Janus Kinases (JAKs) initiates signal transducer and activator of transcription6 (STAT6) (Jiang, Harris and Rothman 2000). IL-13 levels are significantly reduced in the absence of IL-4, although even low levels are sufficient to implement its *in vivo* functions (Chiaramonte *et al.* 1999).

IL-13 has been shown to exert fibrotic effects by up-regulating TGFβ, especially in macrophages (Lee *et al.* 2001; Fichtner-Feigl *et al.* 2006). Furthermore, it has been reported that IL-13 can induce its pro-fibrotic effects in a TGFβ-independent manner (Kaviratne *et al.* 2004). IL-13 is able to stimulate a time and dosage-dependent increase of CTGF via Smad1 and 2, independent of the TGFβ-STAT6 pathway (Liu *et al.* 2011).

1.4.8 Macrophages

Macrophages are a diverse population of cells with phenotypes that engage in a spectrum of roles, from early responders of wound healing to orchestrators of tissue remodelling in later phases. The early responders react to chemokines, and begin the process of clearing tissue debris and pathogens. They are further activated by cytokines including TGFβ, IL-13 and Platelet-derived Growth Factor (PDGF) (Hesse *et al.* 2001; Li *et al.* 2006; Parsons,

Takashima and Rippe 2007). Rodent models of surgically induced tendon injury have shown that macrophages accumulate after a peak of neutrophils between 5 and 21 days post-injury (Marsolais, Côté and Frenette 2001; Wong *et al.* 2009). Macrophages are a major, sustained source of TGFβ in murine wound models (Ashcroft *et al.* 1999; Martin *et al.* 2003). The different subsets of macrophages present during different stages of healing have commonly been classified as M1 and M2 macrophages, although it is now thought that there is a spectrum of phenotypes rather than separate classes (Martinez and Gordon 2014). In acute equine SDFT injury $CD14^+$ $CD206^-$ (M1-type) macrophages predominate, and have a pro-inflammatory role, whereas a potential phenotypic switch to $CD206^+$ (M2-type) anti-inflammatory macrophages is seen in chronic injury (Dakin *et al.* 2012). This study showed there is also a reduction in the expression of pro-resolving proteins in chronic equine tendon disease. In human rotator cuff tissue a similar picture has been found with expression of pro-resolving proteins increased in early-stage disease, which counteract inflammation. In chronic tendon disease macrophages with an M2 like signature predominated with reduced expression of pro-resolving proteins, and subsequent disorganisation of the ECM (Dakin *et al.* 2015).

The Th2 response is stimulated by increased expression of IL-4 and IL-13 by macrophages, which subsequently leads to a rise in alternatively activated macrophages (Hesse *et al.* 2001). It is suggested that this happens in chronic disease because there is dysregulated resolution to counter the

pro-inflammatory cascade that follows from injury, allowing the development of chronic inflammation and fibrotic repair to ensue (Dakin *et al*. 2014).

1.4.9 Bone Morphogenic Proteins

Bone morphemic proteins (BMPs) are a sub-group of the TGFβ superfamily, and are known to have developmental and homeostatic functions in most tissues (Wang *et al.* 2014). They have been investigated for their role in tendon-bone healing, stimulation of rotator cuff cell activity and collagen production (Nishimura *et al.* 1998; Martinek *et al.* 2002; Ma *et al.* 2007). BMPs-2 and -7 can increase collagen production in rotator cuff cells, and so could also play a role in driving fibrosis(Mihelic 2004; Pauly *et al.* 2012). Furthermore BMP-2 may act as a mechano-transducer in response to strain and help regulate collagen expression in tendon homeostasis (Rui *et al.* 2011).

1.5 Models of Fibrosis:

1.5.1 Scarless healing

During the first two trimesters of embryonic development, wounds heal with minimal inflammatory response, and without generating a scar (Adzick and Longaker 1992; Hopkinson-Woolley *et al.* 1994). These findings have led research into defining the differences between adult and embryonic healing. Some of the key differences include; lower levels of pro-fibrotic cytokines (e.g. TGF-β1, 2) and increased anti-fibrotic cytokines (e.g. TGF-β3); reduced degranulation of platelets; reduced macrophage recruitment with fewer

activated in embryos; and a predominance of Collagen III, which benefits cell migration and proliferation in foetal wounding (Nath *et al.* 1994; Cowin *et al.* 1998; Hsu *et al.* 2001; Beanes *et al.* 2002; Larson, Longaker and Lorenz 2010). If it was possible to replicate the conditions necessary for foetal healing a number of fibrotic disease processes could be prevented or treated. It was believed that the foetal environment might be beneficial to wound healing. However, marsupials leave the sterile amniotic sac before they are immunologically mature, but they do not scar like adults until day 9 in the pouch (Armstrong and Ferguson 1995). Moreover, when transplanted onto the dorsa of lambs, full thickness adult sheep dermis still heals with a scar after incisional wounding, (Longaker *et al.* 1994). It would then follow that there is an intrinsic ability of foetal tissue to heal via regeneration rather than repair (Lorenz *et al.* 1992; Lo *et al.* 2012). Since these discoveries a number of investigators have tried altering components of wound healing to decipher which elements influence fibrosis.

1.5.2 Animal models of fibrosis

Several rodent models have been developed to mimic rotator cuff and upper limb injury, through repetitive and high strain actions with the aim of better understanding the tissue injury response (Barbe *et al.* 2003; Abdelmagid *et al.* 2012; Gao *et al.* 2013; Sato *et al.* 2014). In these studies, rodents were made to perform repetitive and / or high strain tasks, and as exposure increased, grip strength and the ability to tolerate mechanical load decreased. Further analysis showed increased inflammation, markers of

collagen synthesis, and fibrotic signalling (TGFβ-1, CTGF), potential culprits for the development of fibrosis.

1.5.3 Manipulating tissue healing

Suppression of TGF-β1 and 2, immediately after wounding of adult rodents resulted in reduced scarring and more foetal-like healing (Shah, Foreman and Ferguson 1992, 1994). Addition of exogenous TGF-β3 to a similar model also acted to reduce scarring (Shah, Foreman and Ferguson 1995). Chang *et al* (1997) created a flexor tendon wound healing model that showed an increase in TGF-β1 expression during healing compared to healthy tendon. Addition of anti-TGF-β1 to this model resulted in a greater range of motion after healing (Chang *et al.* 2000). Furthermore the fibrotic phenotype of tendon cells has been suppressed by using the same neutralising antibody for TGF-β1 (Zhang *et al.* 2004). Moreover, downstream disruption of the TGFβ signalling after cutaneous irradiation using Smad3 knockout mice reduces myofibroblast recruitment and fibrosis (Flanders *et al.* 2002). A number of studies manipulating TGFβ signalling in an array of tissues (tendon, liver, kidney, skin) show a reduction in fibrosis (Akagi *et al.* 1996; Ashcroft *et al.* 1999; Martin, Lefaix and Delanian 2000; Okuno *et al.* 2001; Li 2002; Lu *et al.* 2005; Bates *et al.* 2006; Gressner *et al.* 2008).

However, disrupting TGFβ signalling can lead to a reduction of tendon strength, which needs to be better understood before practical application is

realistic (Jørgensen *et al.* 2005; Katzel *et al.* 2011). TGFβ is a well know regulator of ECM and likely to be very important in tendon homeostasis. TGFβ is released from ECM in response to exercise and strain, thus acting as a mechanical transducer of collagen synthesis, allowing the tendon to respond and adapt to mechanical demands (Heinemeier *et al.* 2003; Maeda *et al.* 2011).

Several macrophage depletion studies have shown that by reducing early macrophage infiltration fibrosis diminishes, but later macrophage depletion prevents successful wound healing (Duffield *et al.* 2005; Imamura *et al.* 2005; Hays *et al.* 2008). M1 pro-inflammatory macrophages release cytokines including TGFβ and may be responsible for driving an initial stimulus for fibrosis. As alternatively activated macrophages start to predominate they become important in wound healing.

1.5.4 Fibrosis in musculoskeletal pathology

There is little literature available on the development of inflammation-mediated fibrosis in tendons. However, some other connective tissues have been investigated to a greater depth such as the liver, lungs, skin and kidneys. Knowledge from these organ systems may be translatable to allow a better understanding of fibrosis in tendon.

Dupuytren's contracture is an inherited proliferative connective tissue disorder characterised by fibrosis and fibrotic nodules of the palmar fascia.

The palmar fascia has a similar composition to tendon, it is formed mostly of ECM, in particular collagen I. Collagen III levels are usually very low, but in Dupuytren's Collagen III has been shown to accumulate much like tendon disease (Brickley-Parsons *et al.* 1981; Melling *et al.* 1999). Myofibroblasts are implicated in driving the fibrotic phenotype, and increase Collagen III with persistent expression of α-SMA (Murrell, Francis and Bromley 1991; Dolmans *et al.* 2011; Verjee *et al.* 2013). Increased TGFβ expression is shown to drive myofibroblasts in Dupuytren's (Kloen *et al.* 1995; Krause, Kloen and Ten Dijke 2011). Furthermore inflammation plays a crucial role in the fibrosis of Dupuytren's, with the presence of immune cells, in particular macrophages, shown to correlate with the number of myofibroblasts (Andrew *et al.* 1991).

Hypertrophic burn scars involve the excessive production of ECM by wound healing fibroblasts, under the influence of persistently high fibrotic cytokines such as TGFβ (Ghahary *et al.* 1993; Scott *et al.* 1995; Wang *et al.* 2000). Similarly Cui *et al* (1995) demonstrated the homeostatic role of TGF-β1 and TGFβ R2 in epidermal hyperplasia, and its role in growth sensitivity through up regulation of TGFβ R2 increasing sensitivity to TGF-β1. Contrary to other studies Fenwick *et al* (2001) did not find evidence of TGFβ-1 expression, or TGFβ R1 in healthy or pathological tendons. They used tissue from ruptured Achilles and healthy cadaveric tendon to evaluate TGFβ, and receptor expression.

1.6 Objectives and Hypothesis

The aim of this study is to investigate the fibrotic signature of healthy, tendinopathic, and torn human supraspinatus tendons. Expression of TGFβ family signalling members was investigated at the mRNA and protein levels. Genes investigated include TGF-β1, TGFβ R1, R2, and BMP2 & 7. Expression of myofibroblast-related proteins α-SMA, Vimentin, E-Cadherin, and TLR4 were also studied.

It is hypothesised that there would be dysregulation of TGFβ family genes and proteins and increased α-SMA in diseased compared to healthy tendons. No study to date has investigated the fibrotic signature in detail in human tendinopathic and torn tendon in comparison to healthy tendons. This study will help to define the potential mediators of fibrosis in rotator cuff tendon disease.

2. Methods

2.1 Tissue sample phenotypes

Healthy and diseased human rotator cuff (supraspinatus) tendons were collected to investigate protein expression of potential key mediators of fibrosis. Healthy hamstring tendon and diseased supraspinatus tendon were used for gene analysis due to sample size constraints

Exclusion criteria for all patients in this study included previous shoulder surgery, dual shoulder pathological lesions, significant problems in the other shoulder, rheumatoid arthritis or systemic inflammatory disease, osteoarthritis or significant neck problems.

2.2 Study Approval

Torn supraspinatus tissue was used from the United Kingdom Rotator Cuff Trial (UKUFF), funded by the National Institute for Health Research Health Technology Assessment Programme in the United Kingdom (project no. 05/47/02). Multicentre research committee and local ethics committee approved the study (REC reference 10/H0402/24).

Tendinopathic supraspinatus tissue was from the OXPAT study (Oxford Partial Thickness Rotator Cuff Tear and Tendinopathy Cohort) investigating post-operative patients with rotator cuff tendinopathy (REC reference 10/H0605/35).

Healthy supraspinatus tissue was also from the UKUFF trial. The ethical approval for this study was granted by the Oxfordshire ethics committee (REC reference 09/H0605/11). All tissue samples were collected at the same orthopaedic referral centre. For gene expression analysis, torn supraspinatus tendons and healthy hamstring tissue samples were collected via the Oxford Musculoskeletal Biobank in compliance with national and institutional ethical compliance (REC reference 09/H0606/11). Full informed consent according to the Declaration of Helsinki was obtained from all patients.

2.3 Patient cohorts

2.3.1 Patient cohort for immunohistochemistry and immunofluorescence

Torn human supraspinatus tendon samples were collected from the edges of torn tendons during surgical debridement for rotator cuff repair from symptomatic male and female patients with full thickness tears aged between 50 and 78 years. Tears were grouped according to their size using the accepted classification by Post *et al*. (1983) into small <1cm, medium 1-3cm, large 3-5cm, massive >5cm (Table 2).

Samples of tendinopathic supraspinatus were collected from male and female patients at the time of sub-acromial decompression surgery. Tendon specimens were collected from patients presenting to a referral shoulder clinic. Patients had failed non-operative treatment, including a course of physical

therapy, and / or glucocorticoid injections into the sub-acromial space, but had not received glucocorticoid treatment or PRP for 12 weeks before surgery and had experienced pain for a minimum of 6 months. A shoulder specialist diagnosed sub-acromial impingement syndrome. Painful arc tests and external rotation resistance tests were positive in all patients. High definition ultrasound examination (GE Medical Systems, UK, Voluson-i B02117) was performed in all patients to determine if there was evidence of a rotator cuff tear and identify abnormal echogenic changes at the supraspinatus footprint. All patients had loss of shoulder function and / or shoulder pain as reflected in a median Oxford Shoulder Score (OSS) of 29 (Dawson, Fitzpatrick and Carr 1996). Tendinopathic tissue was collected from the patient via biopsy under general anaesthesia prior to sub-acromial decompression (Table 2).

Healthy supraspinatus tendon samples were obtained from patients who underwent shoulder surgery for instability, and who had an intact non-degenerative supraspinatus tendon on ultrasound and confirmed at surgery. Healthy patients were aged between 18 and 29 years. Tissue collection for tendinopathic and healthy samples was performed as described by Murphy *et al*. (2013) (Figure 6). A percutaneous ultrasound guided biopsy technique was performed to acquire healthy tendon tissue 5 to 10mm posterior to the anterior edge of the tendon while the patient was under general anaesthesia prior to shoulder instability surgery (Movin *et al.* 1997) (Table 2).

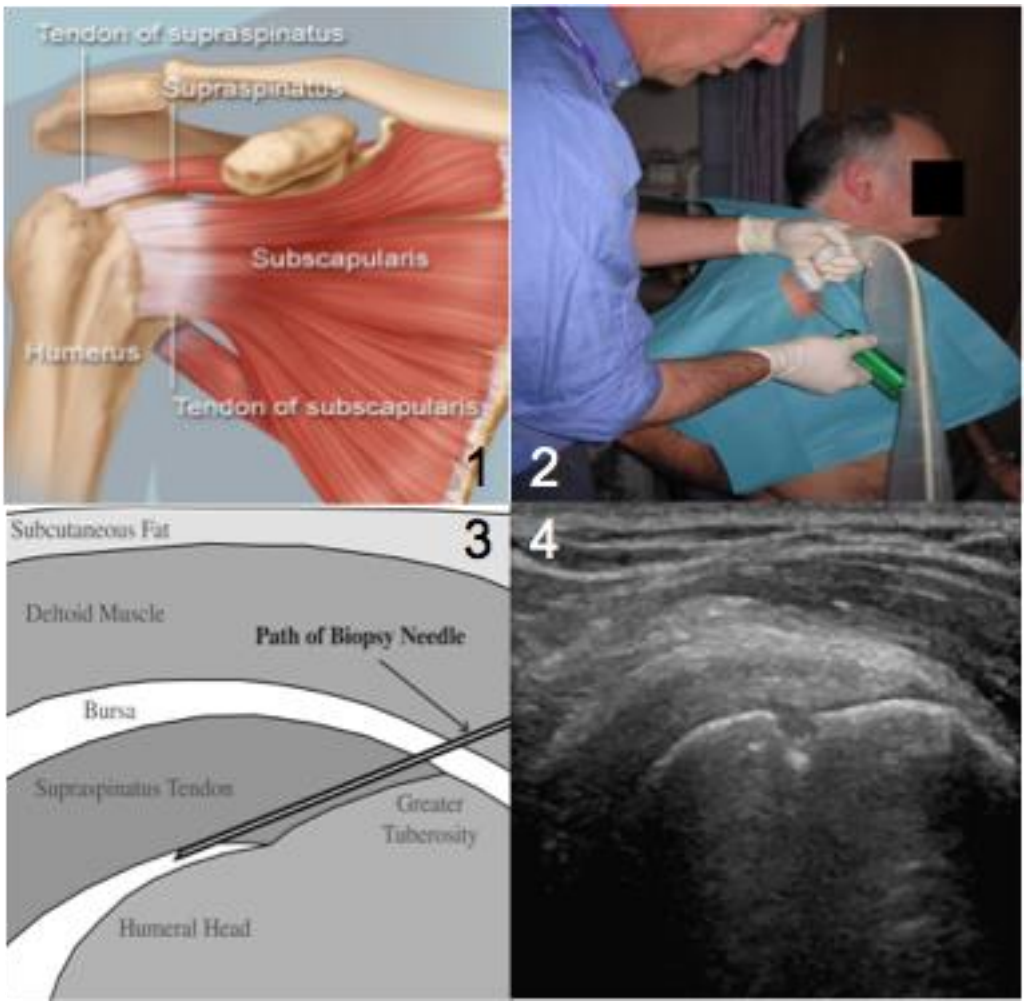


Figure 6: Panel demonstrating the minimally invasive biopsy technique used for collection of healthy and tendinopathic supraspinatus tendon, as originally described by Murphy et al (2013). 1) Anatomical image demonstrating rotator cuff anatomy. 2) Professor Carr performing biopsy of the supraspinatus tendon. 3) Illustration of the intended path of the biopsy needle. 4) Ultrasound image demonstrating the visualisation of the supraspinatus tendon to guiding the surgeon.

2.3.2 Patient cohort for gene expression analysis

Healthy and diseased human tendons were collected to investigate gene expression of potential key mediators in fibrosis of rotator cuff tendons. Torn supraspinatus tendons (n=7) and healthy hamstring tendon (n=7) samples were collected to investigate the fibrotic mRNA signatures in these samples. Healthy hamstring tendons were collected during Anterior Cruciate Ligament (ACL) reconstruction surgery, from male and female patients aged between 22 and 49 (mean 31 ± 9.5 years). Torn supraspinatus tissues were collected from the same study as the IHC cohort, and included male and female patients aged 40 to 67 (mean 52.5 ± 9 years) (Table 2). The fresh tissue samples were immediately snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

Cohort	**n**	**Median age**	**Sex M:F**	**History**	**Treatment**	**Median OSS**
Healthy Supraspinatus	11	24 (18-29)	9:1	Shoulder instability	Shoulder stabilisation	N/A
Healthy Hamstring (mRNA)	7	31 (22-49)	3:4	ACL rupture	ACL repair	N/A
Tendinopathic Supraspinatus	23	54 (36-73)	13:10	Shoulder Sx 1-10 years (mean 2.5 years)	1-2 steroid injections, SAD ± PRP	29 (11-38)
Torn Supraspinatus	30	69.5 (50-78)	18:12	5 Small 9 Medium 9 Large 7 Massive	Rotator cuff repair	20.5 (7-35)
Torn Supraspinatus (mRNA)	7	52.5 (40-67)	5:2	3 Medium 2 Large 2 Massive	Rotator cuff repair	26 (9-31)

Table 2: Patient cohort details for tissue samples.

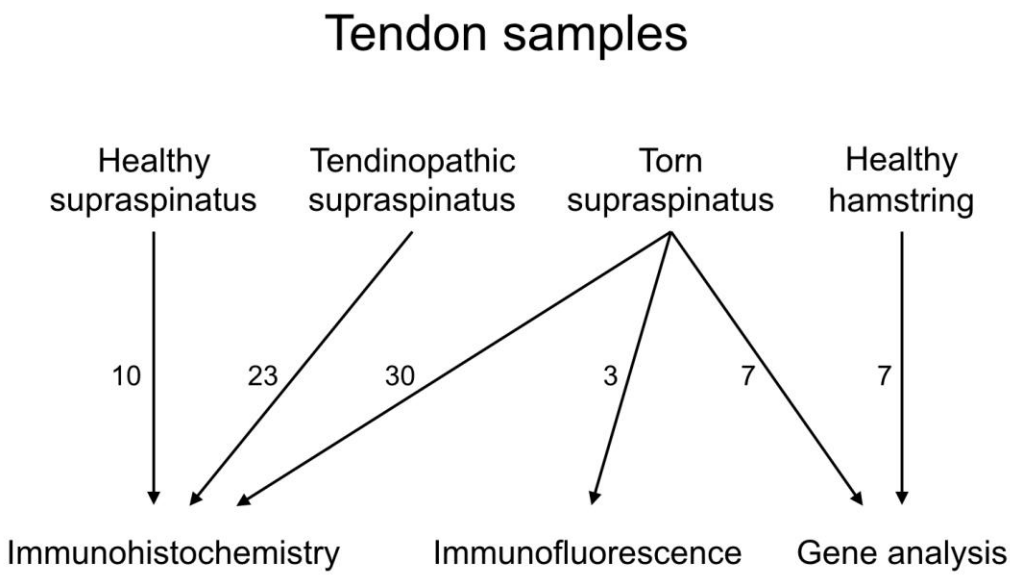


Figure 7: Distributions of how many different samples were used for each experiment.

2.4 Tissue processing

Samples were immersed in 10% buffered formalin and left for approximately 0.5mm/hr, and then embedded in paraffin wax for storage. The wax blocks were sectioned as 4µm segments using a rotary microtome (Leica Microsystems Ltd, UK). Sectioned samples were collected on Leica X-tra adhesive glass slides (Leica Biosystems, UK) and baked at 60°C for 30 minutes and 37°C for 60 minutes.

2.4.1 Immunohistochemistry protocol

Before antigen retrieval, slides were baked at 60°C for 60 minutes. Combined deparrafinisation and antigen retrieval was performed by submerging slides in FLEX TRS antigen retrieval fluid using a PT Link machine (Dako, Glostrup, Denmark). Immunostaining was performed using an

Autostainer Link 48 machine using the EnVision FLEX visualisation system (Dako). Primary antibodies against TGFβ signalling pathways included TGFβ-1, TGFβ R1 and TGFβ R2. Antibodies used to investigate EMT and fibroblast activation included Anti-TLR4, Anti-α smooth muscle Actin antibody, Anti-E Cadherin, and Anti-Vimentin (Table 3). Finally, to investigate changes in tendon composition consistent with fibrosis Anti-Collagen III antibody was used (Abcam Cambridge, UK). Antibody binding was visualized using FLEX 3,3'-diaminobenzidine (DAB) substrate working solution and haematoxylin counterstain (Dako) as per protocols provided by the manufacturer. Isotype control antibodies included mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM, (Dako). All antibodies were validated in house to ensure the recommended concentration produced positive immunostaining with minimal artefact on supraspinatus tendon sections. After staining slides were rehydrated in industrial methylated spirit (alcohols) and xylene and mounted using Pertex mounting medium (Histolab, Gothenburg, Sweden).

Antibody to	**Abcam number**	**Dilution used**	**Isotype**
TGF-β1	ab64715	1:50	IgG1
TGFβ R1	ab31013	1:50	IgG polyclonal
TGFβ R2	ab78419	1:50	IgG1
TLR4	ab22048	1:100	IgG2b
α-SMA	ab7817	1:100	IgG2a
Vimentin	ab8978	1:200	IgG1
E-Cadherin	ab76055	1:200	IgG1
Collagen III	ab7778	1:5000	IgG polyclonal
CD163 (purple) (red)	ab189915 LifeSpan BioSciences	1:200 1:200	IgG rabbit IgG2a mouse
CD206	ab117644	1:200	IgG1
Pan-TGF-β	ab66043	1:100	IgG rabbit
IRF 5	ab175317	1:200	IgG rabbit
POPO-1	Life Technologies		

Table 3: Antibody details

2.4.2 Image analysis for quantitative immunohistochemistry

Twenty images of each immunostained section (or until exhausted) were acquired on a Zeiss inverted microscope (Zeiss, Cambridge, UK) using Axiovision software (Zeiss) at x100 magnification with oil immersion. Images were collected systematically to ensure no overlap and to avoid areas of artefact or fold in the tissue sample.

ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to analyse the acquired images, employing a previously validated algorithm that quantifies DAB staining by a colour deconvolution method (Ruifrok and Johnston 2001; Rizzardi *et al.* 2012). For each antibody stain the threshold level was manually adjusted to best represent the staining seen with visual comparison in full colour. The quantification system used normalised the number of nuclei to the amount of immunopositive staining, thus accounting for tissue cellularity.

2.5 Gene Expression

2.5.1 RNA extraction and cDNA synthesis

Frozen tissue samples (~100mg) stored at -80°C were defrosted and homogenised in 1mL of RNABee (AMS Biotechnology, UK), using an IKA Ultra Turrax T8 Homogeniser (Fischer Scientific, UK). After homogenisation in RNABee, chloroform was added (180 µL), and the sample left on ice for 5 minutes. RNA was then phase separated via centrifugation and precipitated

with isopropanol to isolate a pellet. After drying, the pellet was reconstituted in 30µL RNAse free water and vortexed. RNA was then cleaned as per manufacturer's protocol using an RNeasy mini kit (Qiagen, Limburg, Netherlands). RNA concentration and quality were determined by measuring the ratio of absorbance at 260:280nm using a NanoDrop 100 spectrophotometer. (Thermo Scientific, MA, USA) with all samples achieving a minimum 1.80. RNA (250ng) was reverse transcribed into cDNA using High Capacity Reverse Transcription Kit (4368813, Applied Biosystems). cDNA synthesis was performed using a Veriti Thermal cycler (Applied Biosystems, CA, USA). Cycle conditions consisted of three stages, 10 minutes 25°C, 2 hours 37°C, 5 minutes 85°C, then cooled to 4°C as advised by Applied Biosystems manufacture instructions.

2.5.2 Gene expression by RT-qPCR

cDNA was diluted to 2.5ng/µL with RNase free water and 5µL was used in a 20µL reaction with Fast SYBR Green Master Mix (4385612, Applied Biosystems). Validated human primers from Qiagen included *TGFB1* (QT00000728), *TGFBR1* (QT00083412), *TGFBR2* (QT00014350), *BMP2* (QT00012544), *BMP7* (QT00068936), *CTGF* (QT00052899), *ACTB* (QT00095431), and *GAPDH* (QT00079247). Duplicate reactions for each gene were run on a ViiA7 qPCR machine (Applied Biosystems, CA, USA). Three stages were run on the qPCR machine: a holding stage at 95°C for 20 minutes, the PCR stage with 40 cycles, from 95-60°C with rapid heating (2.63°C/s) and cooling (2.42°C/s) followed by 20 minutes at 60°C and then a

melt curve stage which has 15 minutes at 95°C, 1 hour at 60°C and then finally 15 minutes at 95°C reached by slow heating (0.05°C/s) (Table 4). Results were calculated using the ddCt method and normalised against β-actin and GAPDH reference genes. Results were consistent with both reference genes and data shown are normalised to β-actin.

Heating cycles and speeds for RT-qPCR				
RT-qPCR protocol	°C	Minutes	Cycles	ΔTemperature/s
Holding	95	20	1	2.63
PCR	60-95	75	40	2.42-2.63
Melt curve	60-95	90	1	0.05-2.63

Table 4: RT-qPCR information

2.6 Immunofluorescence

2.6.1 Immunofluorescence cell type and TGFβ co-localisation

Triple antibody labelling (Table 3) was performed on formalin fixed paraffin embedded sections of torn supraspinatus tissue to identify whether macrophages and / or non-macrophages were expressing TGFβ. Vimentin and α-SMA were used as markers of fibroblast-like cells. CD206 and CD163 were used as markers of alternatively activated macrophages, and IRF5 and TLR4 for classically activated macrophages (Martinez and Gordon 2014).

2.6.2 Protocol - Antigen retrieval

Slides were baked at 60°C for 60 minutes in an oven. Prior to antibody staining, tissue sections were taken through deparaffinisation and target retrieval steps (high pH heat mediated antigen retrieval) using an automated

PT Link FLEX TRS system (Dako, Denmark). Samples were then incubated in PBS tween (PBST) containing 998 mL of PBS and 2mL Tween 20 (Sigma P1379) to prevent drying prior to staining.

2.6.3 Staining for immunofluorescence

The protocol was modified from Dakin *et al* (2015). An ImmEdge pen (Vectashield) was used to create a hydrophobic barrier around the tissue section. Slides were placed in a humid chamber. To prevent non-specific antibody binding, tissues were blocked in 5% normal goat serum (Sigma G9023) in PBS for 45-60 minutes at room temperature. Slides were then incubated with the primary antibody combination (Table 3) diluted in 5% normal goat serum in PBS at room temperature for 2 hours. For negative controls the primary antibody was substituted for universal isotype control antibodies: cocktail of mouse IgG_1, IgG_{2a}, IgG_2b, IgG_3 and IgM (Dako IR750) and rabbit immunoglobulin fraction of serum from non-immunised rabbits, solid phase absorbed (Dako IR600). After washing 3 times in PBST for 5 minutes each time, slides were incubated with the secondary antibody combination. Secondary antibodies were goat anti-mouse IgG1 (Southern Biotech), Alexa Fluor 568 goat anti-mouse IgG2a and Alexa Fluor 633 anti-rabbit IgG (Life Technologies). Antibodies were incubated at 1 in 200 dilution in PBS containing 5% normal equine serum (Sigma) for 2 hours at room temperature and shielded from light. After washing in PBST, nuclei were counter stained with 2μM POPO-1 (Life Technologies) diluted in PBS containing 0.05 Saponin (Sigma) for 20 minutes at room temperature. After

washing with PBST the slides were then incubated in a solution of 0.1% Sudan Black B (Applichem A1407) in 70% ethanol for 5 minutes to quench tissue autofluorescence. Slides were mounted in VectaShield fluorescent mounting medium (H1000) and coverslip secured with nail varnish. Slides were stored in a humid box at 4°C until imaging.

2.7 Confocal imaging for immunofluorescence

Immunofluorescence images were acquired on a Zeiss LSM 710 confocal microscope using a ×40 oil immersion objective following a protocol from Dakin *et al* (2015). The fluorophores of POPO-1, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 633 were excited using the 405nm, 488nm, 561nm, and 633nm laser lines respectively. To reduce bleed-through, all channels were acquired sequentially. Averaging was set to 2 and the pinhole was set to approximately 1 Airy unit. Two- and three-dimensional image reconstructions were created using ZEN 2009 (Zeiss).

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, CA, USA). Normality was tested for using a Shapiro-Wilk test. The Kruskal-Wallis test was used to compare expression of TGFβ superfamily and related fibrotic mediators at the mRNA and protein levels in samples of healthy, tendinopathic and torn tendons. $p<0.05$ was considered statistically significant.

3. Results

3.1 Histological assessment of diseased rotator cuff tendons

Tendon tissues from patients with supraspinatus tendinopathy or tears showed marked disorganization of the tendon extracellular matrix and collagen fibrils and increased vascularity. Histological assessment of tendons collected from this study cohort was performed on Haematoxylin and Eosin stained sections using the Bonar scoring system (0-12) evaluating tissue structure and is reported in a previous published study (Dakin *et al.* 2015). Median and interquartile ranges of healthy supraspinatus tendons exhibited more normal tissue architecture (Bonar score of 2, range 1-2) compared to tendinopathic (7, range 6-8) and torn supraspinatus tendons (10, range 8.25-10).

3.2 Pathological tendons show down regulation of TGFβ family signalling proteins

Expression of TGFβ-1, TGFβ R1, and TGFβ R2 proteins were investigated in healthy, tendinopathic and torn rotator cuff tendons (Figure 12). Quantitative analyses of immunopositive staining showed TGFβ-1, TGFβ R1, and TGFβ R2 proteins were significantly reduced in pathological tendons compared to healthy samples. Specifically, TGFβ-1 protein was reduced in torn ($p=0.0123$, 2.6 fold reduction) and tendinopathic tendons ($p=0.0064$, 3 fold reduction) compared to healthy tendons. TGFβ R1 protein was reduced

between healthy and torn tendon (p=0.0002, 30 fold reduction) but showed no significant difference between healthy and tendinopathic tendons. TGFβ R1 expression was reduced in torn tendons compared to tendinopathic tissues (p=0.0018, 21 fold reduction). TGFβ R2 was reduced in torn tendons compared to healthy tendon (p=0.0087, 7 fold reduction); tendinopathic tissues showed almost no expression of TGFβ R2 compared to healthy tendon (p<0.00001, 183 fold reduction).

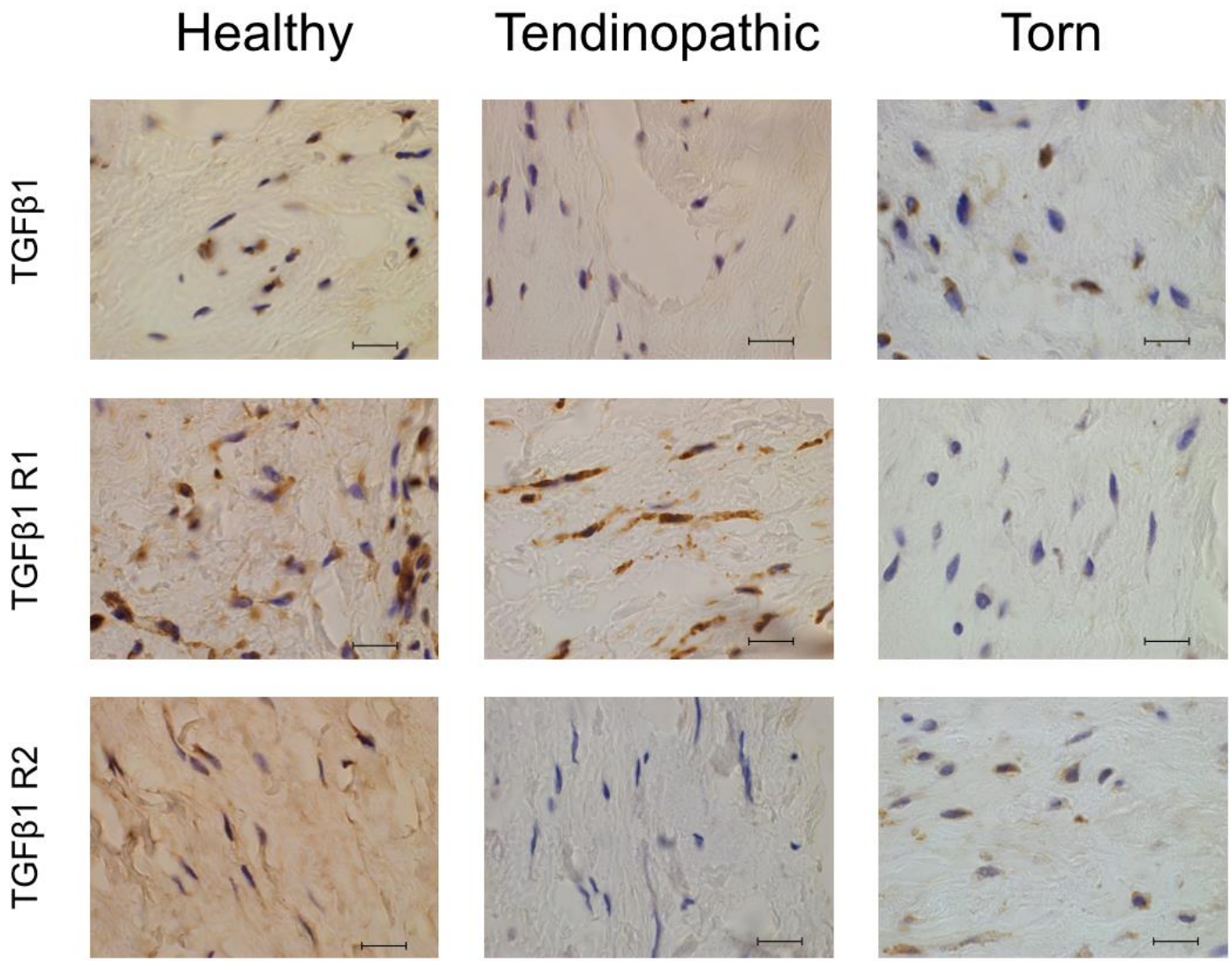


Figure 8: Photomicrographs for IHC of healthy, tendinopathic and torn supraspinatus, stained with antibodies to TGFβ1, TGFβ receptor 1, TGFβ receptor 2. DAB staining is brown. Nuclear counterstain is haematoxylin (blue). Scale bar = 20μm

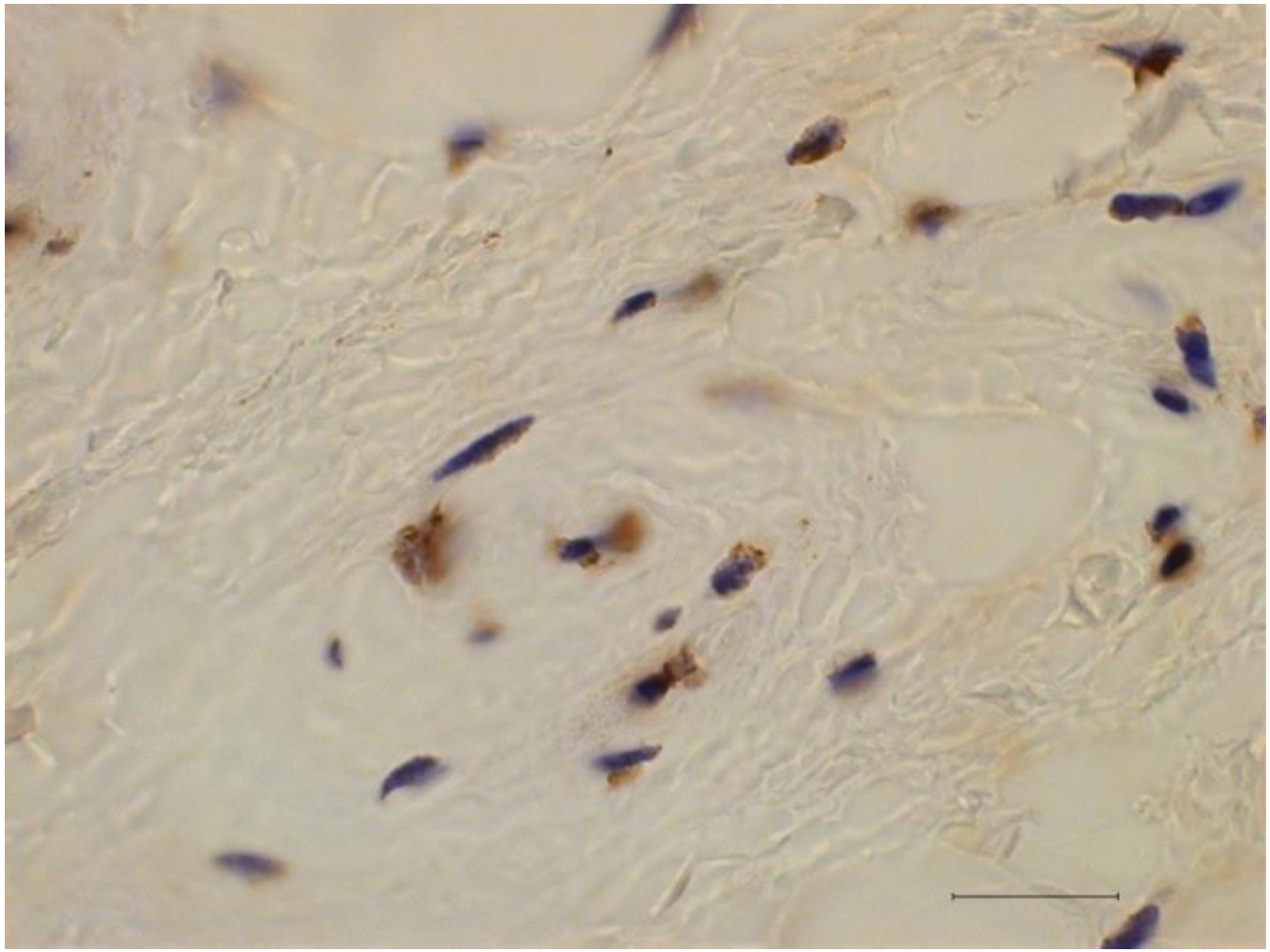


Figure 9: Expanded photomicrograph of healthy TGFβ1 staining. DAB (brown) staining appears to be localised around the nuclei suggesting it is most highly expressed in either cytoplasm or on the cell membrane, rather than in the ECM. Scale bar = 20μm

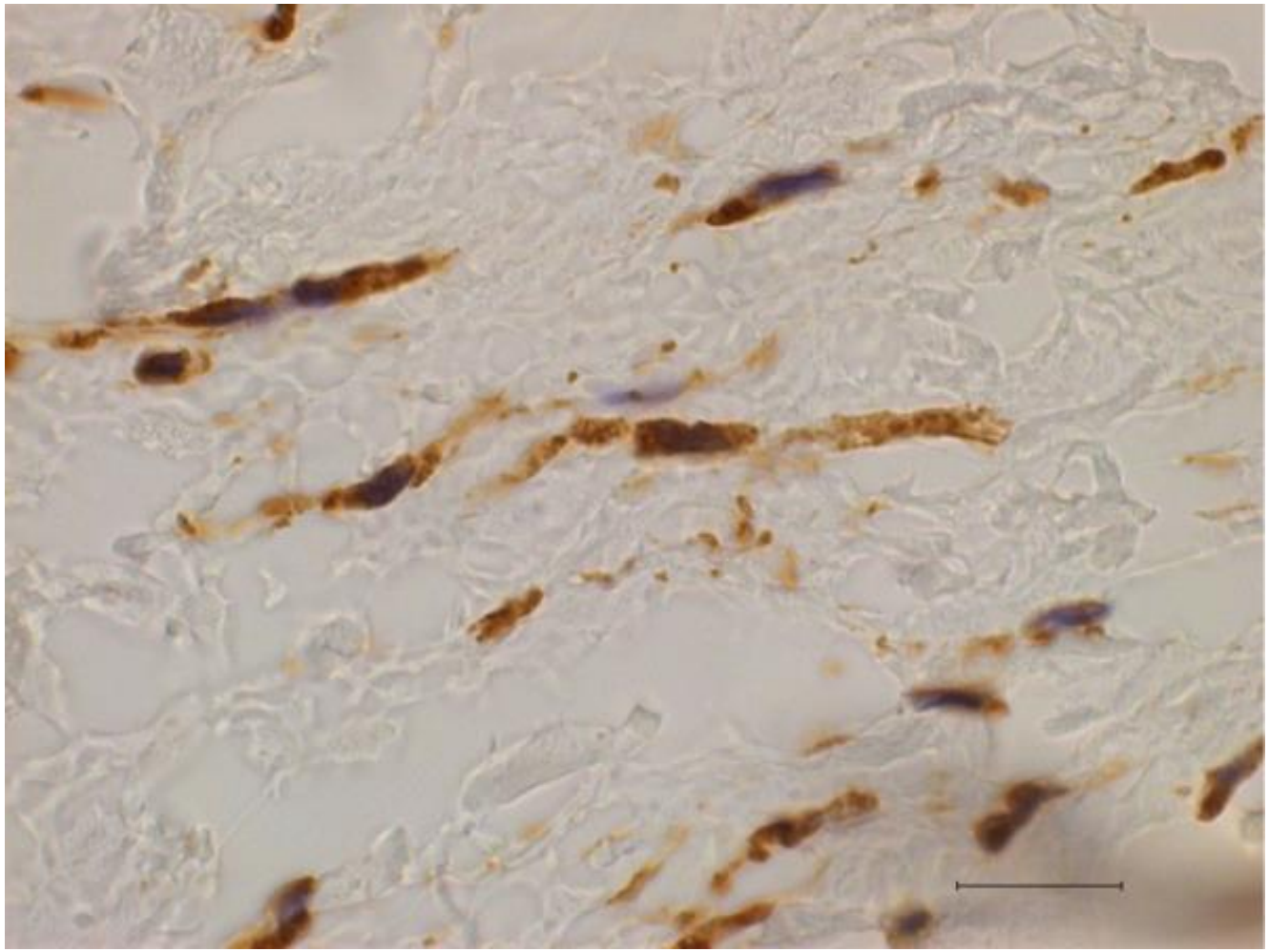


Figure 10: Expanded photomicrograph of tendinopathic TGFβ1 R1 staining. DAB (brown) staining appears to be localised around the nuclei, and along the likely cell body shape. This would suggest increased expression on the cell membrane as would be expected. Scale bar = 20μm

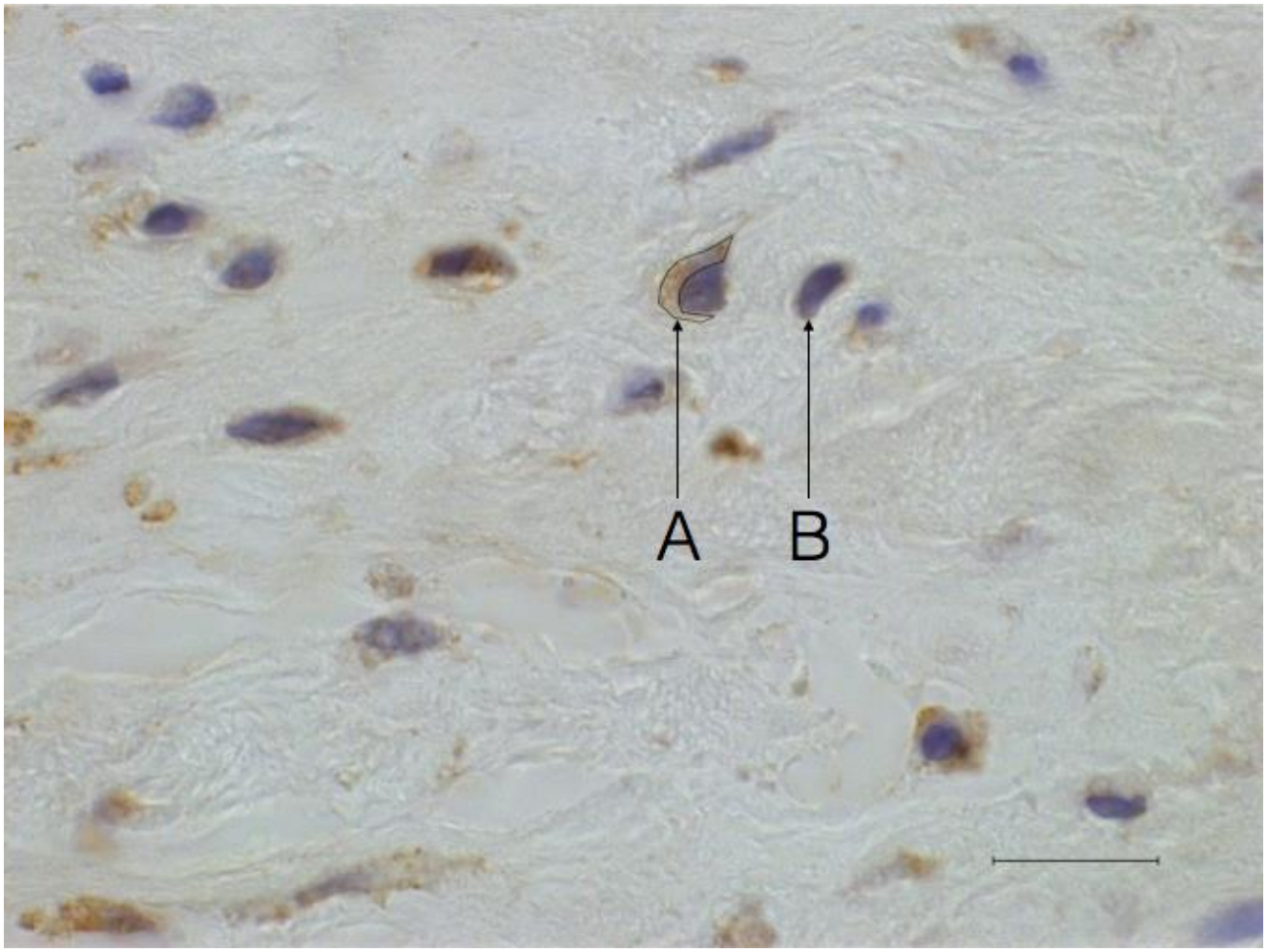


Figure 11: Expanded photomicrograph of torn TGFβ1 R2 staining. DAB (brown) staining appears to be localised around the nuclei, and in a few cases along the likely cell body shape. This would suggest increased expression on the cell membrane as would be expected. Scale bar = 20μm
A – marks an area of DAB staining, B – marks a nucleus.

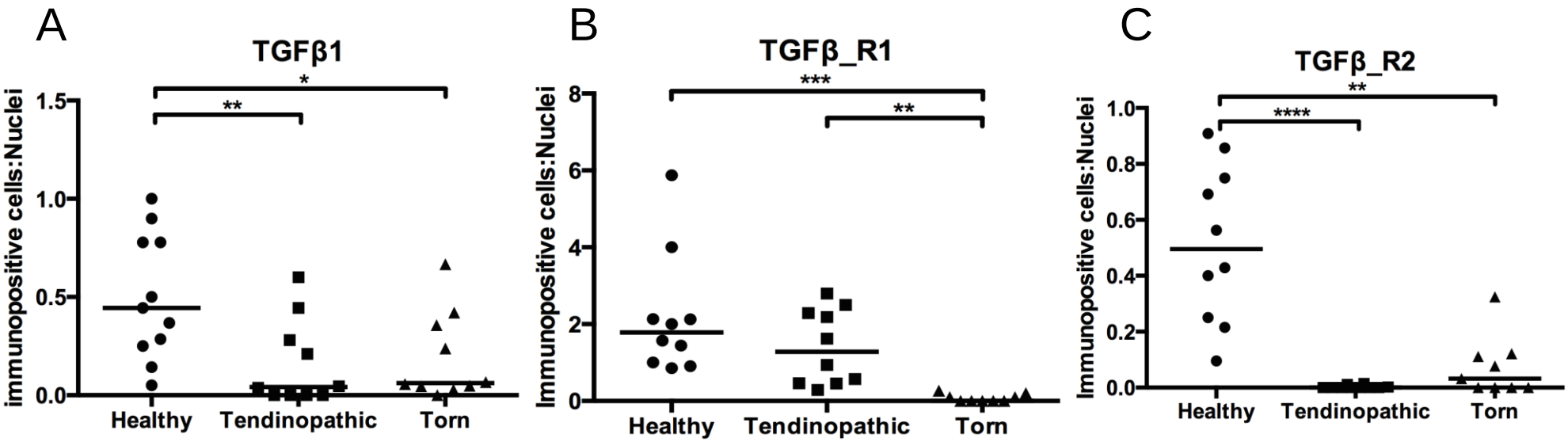


Figure 12: Quantitative analysis of immunopositive staining showing dysregulation of TGFβ family proteins in pathological compared to healthy rotator cuff tendons. (A) TGFβ1, healthy (n=11), tendinopathic (n=10), torn (n=10) (B) TGFβR1, healthy (n=10), tendinopathic (n=10), torn (n=9) and (C) TGFβR2, healthy (n=10), tendinopathic (n=10), torn (n=9). Bar represents the median value. Data were analysed using the Kruskal-Wallis test * $p<0.05$ ** $p<0.01$ *** $p<0.001$ **** $p<0.0001$

33 Pathological tendons show dysregulation of TGFβ family signalling at the mRNA level

Expression of *TGFB1, TGFBR1*, and *TGFBR2* were investigated in torn rotator cuff tendons and healthy hamstring tendon at the mRNA level (Figure 13). *TGFBR1* was decreased in torn rotator cuff compared to healthy tissue (p=0.048, 2 fold reduction). In contrast *TGFBR2* increased (P=0.048, 3 fold increase). There was a trend for reduced *TGFB1* in pathological tendons compared to healthy hamstring.

BMPs have been shown to signal via homodimeric receptors from the TGFβ superfamily, and are also implicated in fibrotic pathways, hence were investigated in the current study. There was no significant difference in *BMP2* between healthy hamstring and torn rotator cuff (Figure 13). *BMP7* was not detected in torn rotator cuff tendons, however there was no significant difference compared with healthy hamstring. CTGF was also investigated as a pro-fibrotic cytokine and downstream effector of TGFβ. There was a trend for reduced *CTGF* in torn rotator cuff tendons compared to healthy hamstring.

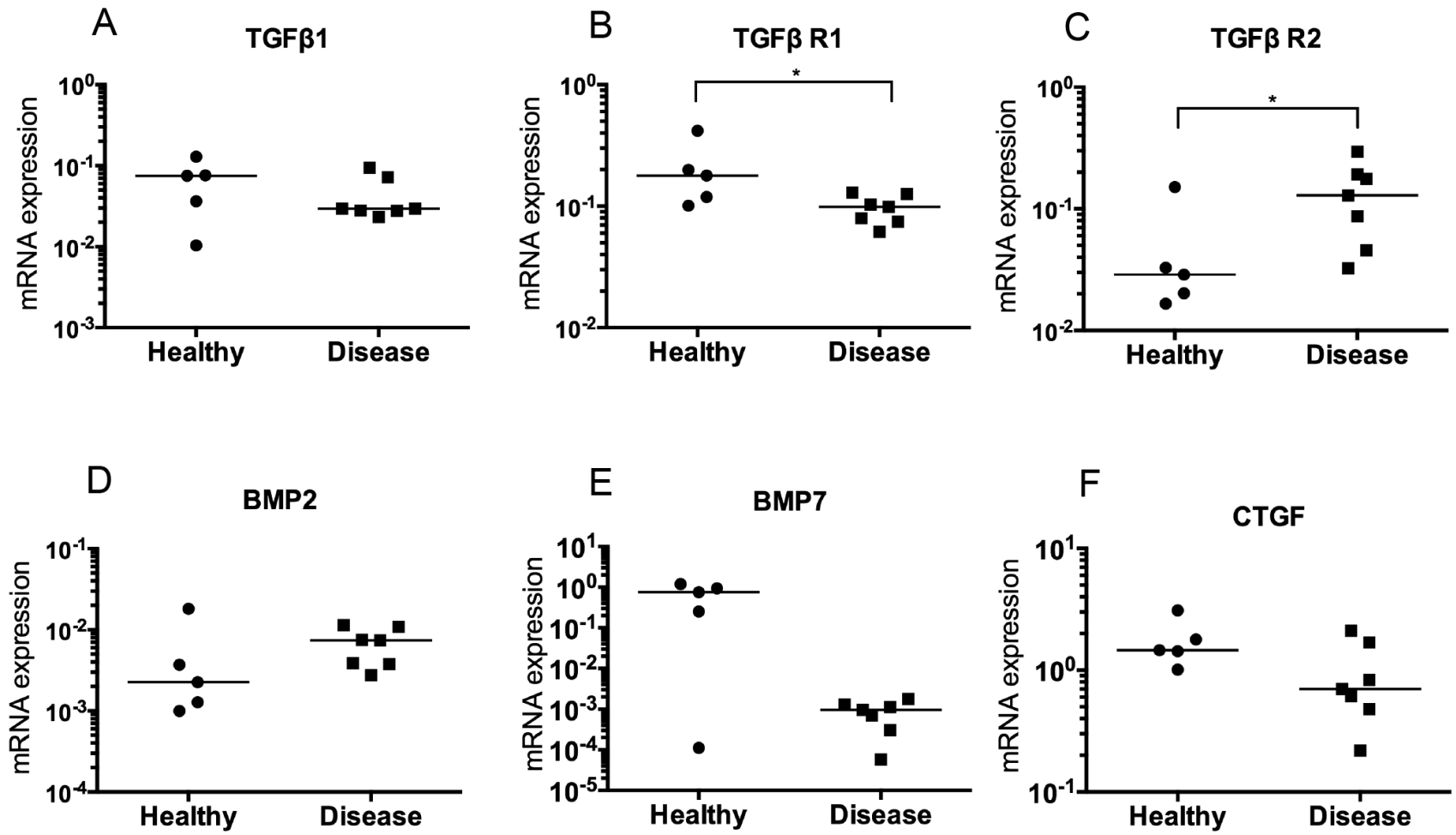


Figure 13: Expression of pro-fibrotic genes in healthy and diseased tendons. TGFβ family mediators (A-C), BMP's 2 & 7 (D, E) and CTGF (F) mRNA expression are shown in healthy hamstring (n=5) compared to pathological rotator cuff tendons (n=7). Gene expression is normalized to β-actin, bar shows median value. * = $p<0.05$.

3.4 Epithelial to mesenchymal transition proteins, and fibroblast activation

In order to determine if EMT was occurring in tendon pathology, the presence of Vimentin, α-SMA, and E-Cadherin proteins were investigated in healthy, tendinopathic and torn rotator cuff tendons (Figure 14). Vimentin was decreased in torn tendons compared to healthy ($p=0.016$, 4 fold decrease) and tendinopathic ($p=0.0039$, 7 fold decrease). There was no significant difference detected between any tendon conditions for α-SMA and E-Cadherin.

TLR4 was investigated to identify the level of myofibroblast activation. There was no significant difference between tendon conditions for TLR4.

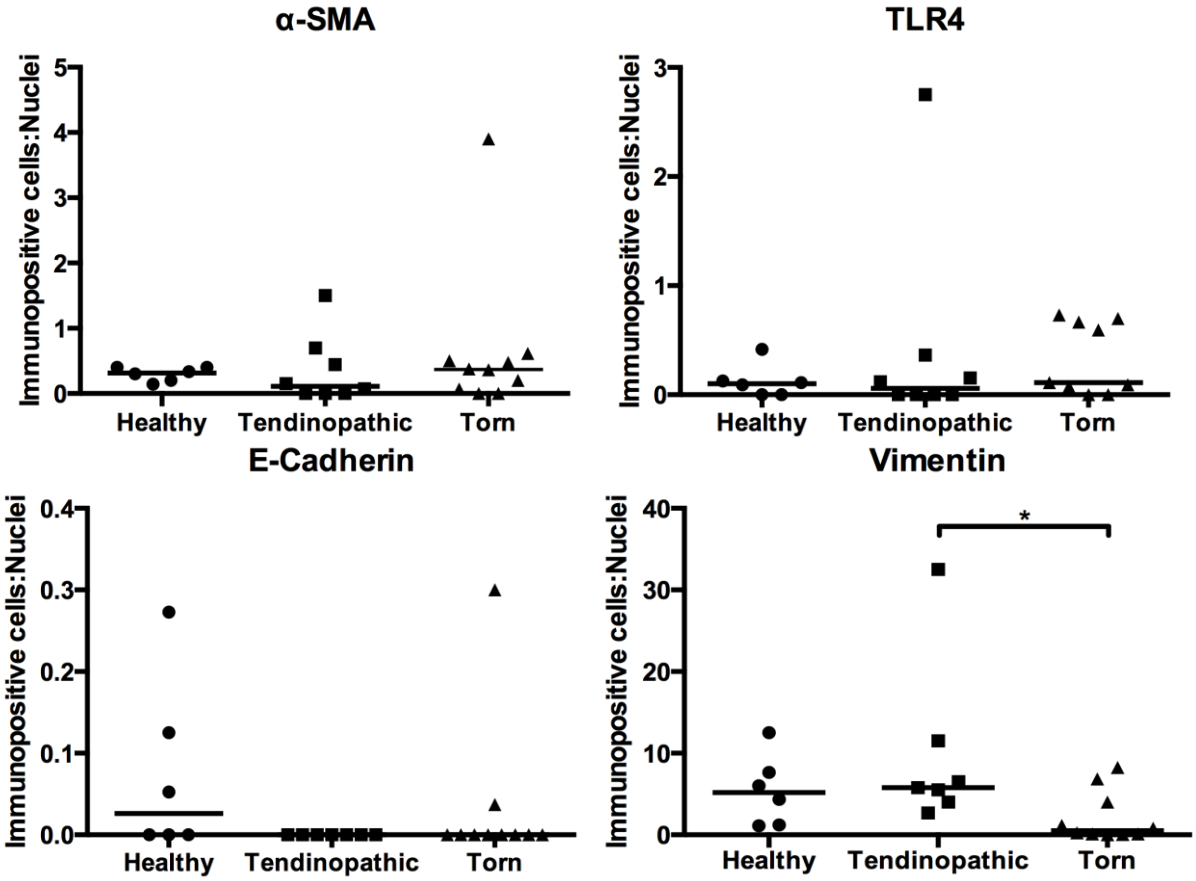


Figure 14: Quantification of immunopositive staining for markers of EMT and fibroblast activation in pathological compared to healthy rotator cuff tendons. (A) TGFβ1, healthy (n=6), tendinopathic (n=8), torn (n=10). Bar represents the median value. Data were analysed using the Kruskal-Wallis test * p<0.05

3.5 Collagen III

It was not possible to titre the antibody for Collagen III to a meaningful level using immunohistochemistry, as the antigen was too ubiquitous to be able to compare levels of DAB staining.

3.6 Immunofluorescence

Confocal images illustrating the edge of a massive supraspinatus tendon tear are shown in Figures 15, 16, and 17. The sample showed co-localisation of Vimentin and alpha SMA, which were predominantly expressed on $CD163^{negative}$ cells. This would suggest that torn tendons contain both tendon fibroblasts and macrophage cell populations (Figure 15).

Macrophages are known to be important immune cell populations in diseased rotator cuff tendons and their activation is known to change with disease stage (Dakin *et al.* 2015). TLR4 and IRF5 stain for classically activated macrophages and were present around the edge of the tear in Figure 16. Macrophages expressing CD206 were found to reside away from the edge of the section, possibly suggesting a subset of alternatively activated macrophages were also present in the sample.

We investigated if myeloid cells in samples of chronic fibrosed tendon tears expressed pan-TGFβ. Antibody labelling with macrophage markers CD206 and CD163 (representing STAT-6 and glucocorticoid receptor macrophage activation pathways respectively) and pan-TGFβ showed co-localisation of these 3 markers in sections of a massive supraspinatus tendon tear.

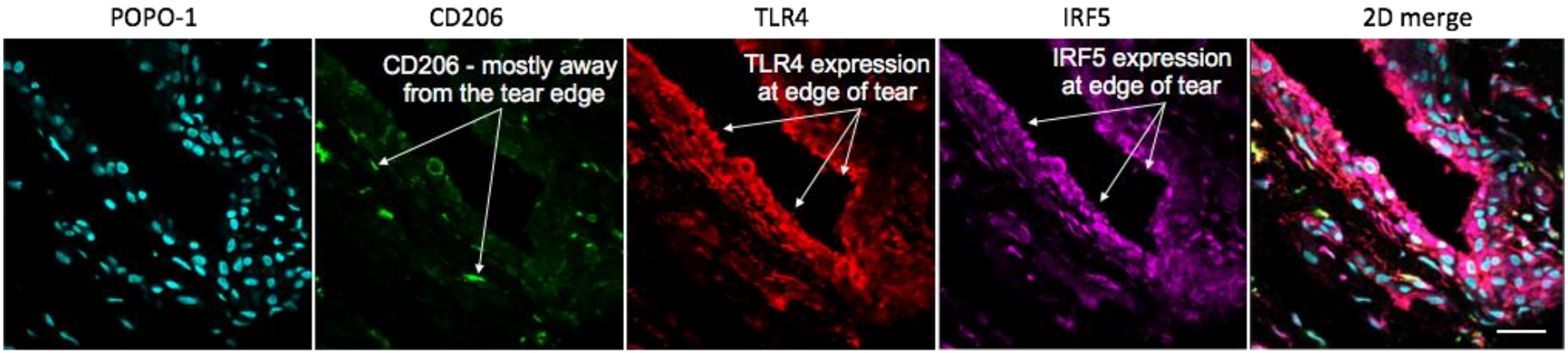


Figure 15: Representative confocal image shows markers for alternative and classically activated macrophages on a massive supraspinatus tear. Markers include CD206 (green) for alternative macrophage activation, and TLR4 (red), and IRF5 (purple), which are both associated with classically activated macrophages. Nuclear counterstain was POPO-1, and scale bar represents 20μm

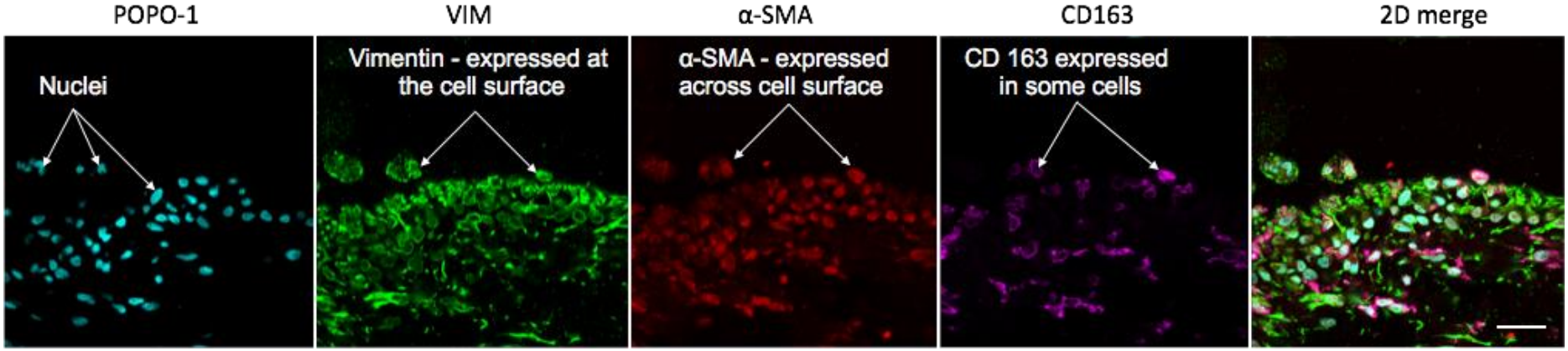


Figure 16: Representative confocal image shows markers for EMT on a massive supraspinatus tear. Markers for EMT include Vimentin (green), and α-SMA (red), and CD163 (purple), which is associated with, alternatively activated macrophages. Nuclear counterstain was POPO-1, and scale bar = 20μm

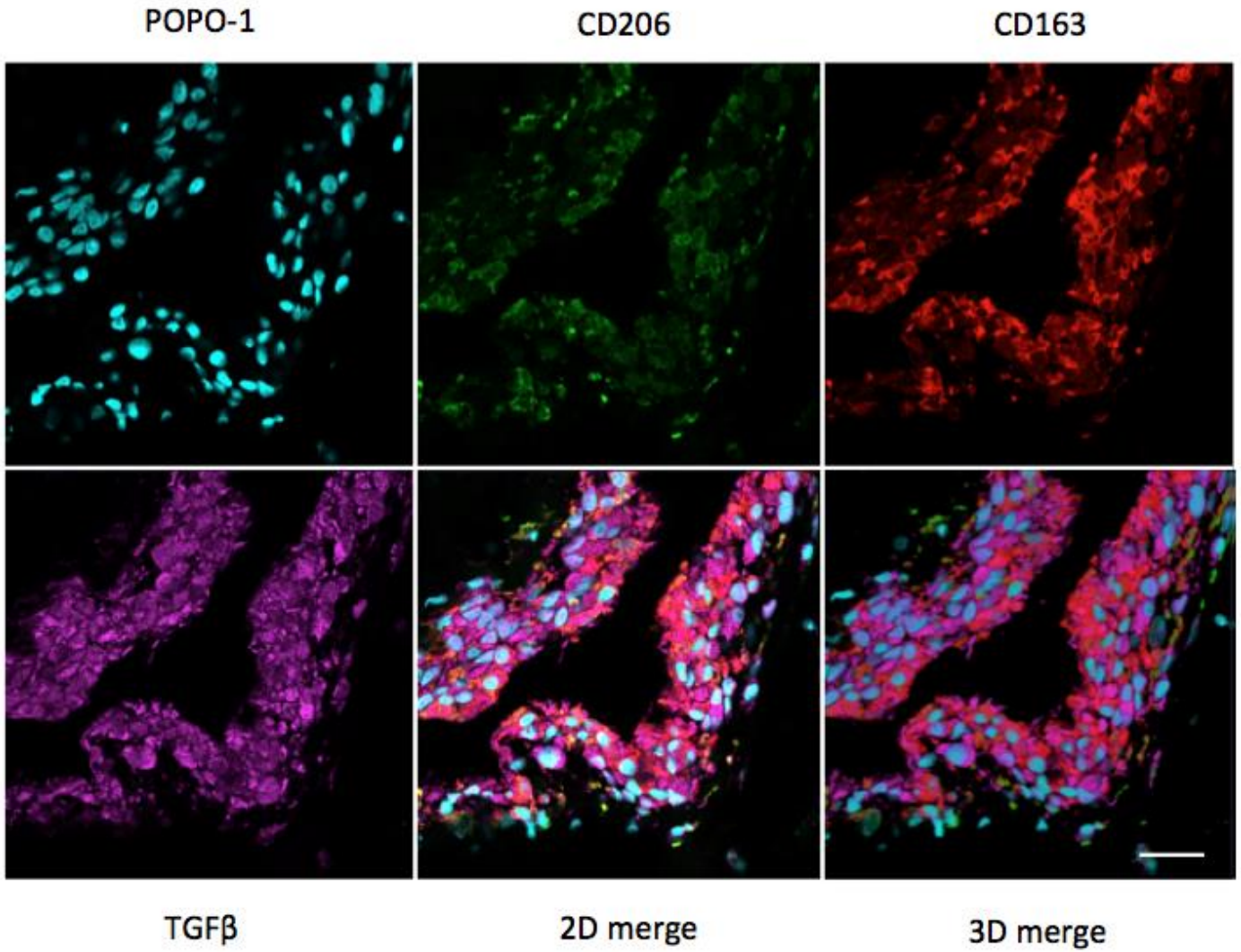


Figure 17: Representative confocal image shows markers to co-localise TGFβ with alternatively activated macrophages on a massive supraspinatus tear. Markers include CD206 (green), CD163 (red), and TGFβ (purple). Nuclear counterstain was POPO-1, and scale bar represents 20μm

4. Discussion

The purpose of this study was to investigate the mechanisms driving fibrosis in pathological rotator cuff tendons. Tendon tissues were collected from well phenotyped patient cohorts, including healthy, tendinopathic, and torn supraspinatus, to identify changes in the fibrotic signature of pathological tendons. In addition hamstring tendon tissues were collected for gene analysis, for use as a surrogate as it was not possible to ethically collect a significant enough quantity of healthy supraspinatus tissue.

Pathological tendons showed dysregulation of TGFβ family members compared to healthy tendons. Specifically, TGF-β1, TGFβ R1, and TGFβ R2 protein levels and TGF-β1 and TGFβ R1 mRNA expression, were suppressed in chronic pathology. Conversely, TGFβ R2 mRNA was elevated in torn rotator cuff compared to healthy tissue. Evidence for EMT in pathological tendons was inconclusive. Whilst vimentin expression significantly decreased with pathology, immunostaining suggested co-localisation of vimentin with αSMA along the edge of a massive tendon tear, and there was also no change found in e-cadherin or αSMA expression.

Previous work investigating pro-fibrotic mediators in tendon pathology is limited. A study of human cadaveric Achilles tendon showed no expression of TGF-β1 in pathology, but also failed to find it in cadaveric controls (Fenwick *et al.* 2001). However a number of studies have suggested roles for TGFβ in tendon homeostasis (Heinemeier *et al.* 2003; Maeda *et al.* 2011). It is evident

from this work that TGFβ family mediators are dysregulated with established tendon disease. Other studies of tendon fibrosis and TGFβ expression have utilised surgical or collagenase based injury models and so investigate the more acute wound healing response of tendon. Their findings suggest that TGFβ genes and proteins can be elevated in response to injury, and that this drives fibrosis, and recruitment of a number of cells. The results of the current study provide an insight into the signalling of chronic pathology in human patients. Taken together, it is possible that a bimodal response (Figure 18) of TGFβ expression occurs throughout the early and late phases of tendon pathology, although further investigation of this concept is required in both animal and human models of tendon disease.

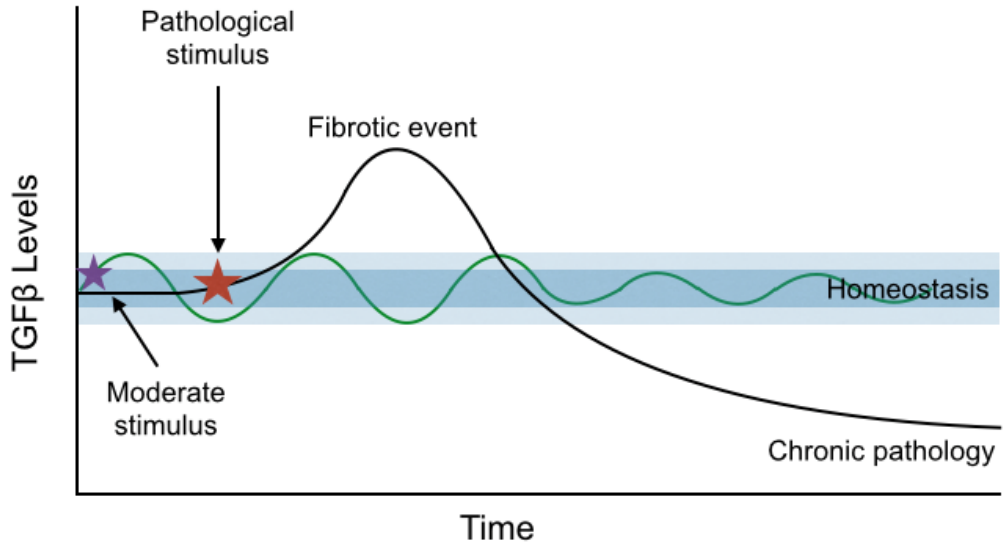


Figure 18 showing a proposed bimodal response of TGFβ in tendon

This study shows that TGFβ protein levels are suppressed in chronic fibrotic tendon pathology, which could be due to a number of mechanisms. It is known that TGF-β1 can modulate the inflammatory response to tendon injury, including fibroblast and macrophage recruitment, stimulation of

collagen production, down-regulation of proteinase activity, and regulation of EMT (Yamamoto *et al.* 1994; Klein *et al.* 2002; Willis and Borok 2007).

TGFβ is an important transducer of mechanical load, being released from ECM in response to strain and exercise (Heinemeier *et al.* 2003; Maeda *et al.* 2011). In the current study, TGFβ family members could be suppressed because patients with tendon pathology are unwilling or unable to load the tendon to its previous level, or in the presence of a tear, the tendon is unable to be loaded sufficiently to stimulate TGFβ signalling. This process would lead to further degradation of the ECM due to reduced collagen production and turnover, and increased likeliness in progression of pathology as is often seen with rotator cuff disease (Yamaguchi *et al.* 2001; Moosmayer *et al.* 2013).

Wound healing is a normal response for tissue repair but it can become dysregulated, resulting in hypertrophic fibrosis as seen in tendinopathy. A reduction in TGFβ may, however, be a protective response to limit this chronic uncontrolled fibrosis. Dermal wounding often leads to hypertrophic scarring (Ghahary *et al.* 1993; Scott *et al.* 1995; Wang *et al.* 2000) which although large and unsightly does not cause significant dysfunction. If the same were to occur in tendon it would likely cause disabling immobility due to the tight compartments within which they operate. Suppression of pro-fibrotic mediators could serve to limit a dysfunctional increase in cross-sectional area (CSA) of the tendon in response to injury. CSA increases with age alongside an associated decrease in tensile strength and increased stiffness which could be associated with accumulation of micro-scarring and products of

advanced glycation (Magnusson *et al.* 2003). CSA is also increased in regular runners, which may be a response to cope with continued loading, but could also be related to accumulation of fibrosis. The major fibrotic processes occur in early disease with increased TGFβ expression detected early in surgical tendon wound models and less expression detected in chronic rotator cuff pathology (Chang *et al.* 1997; Choo *et al.* 2014).

Conversely it may be possible that suppression of TGFβ expression encourages progression of pathology, but does not compensate sufficiently to drive wound resolution. Without TGFβ signalling, tendon would have a lower turnover of ECM, and would not produce the collagen required for repair and return of tensile strength. With suppression of TGFβ expression, alternative pathways such as TNFα could drive fibrosis. For instance in Dupuytren's disease TNFα signalling is implicated as an important driver of myofibroblast activity, and may be the prevailing signal in chronic pathology (Verjee *et al.* 2013).

TGFβR1 and TGFβ1 protein and RNA were consistently down regulated with established tendon disease. However, *TGFBR2* mRNA was up regulated in rotator cuff tears compared to healthy tissue. This did not translate into protein levels, where TGFβR2 was down regulated; demonstrating the dysregulation of the TGFβ axis is in disease. In skin TGFβR2 is an important sensitizer for TGF-β1 signalling and is increased by pro-fibrotic stimuli, it is also a regulator of homeostasis (Cui *et al.* 1995).The increase in *TGFBR2* mRNA may represent a potential aberration in the

translation of *TGFBR2* mRNA into protein. Up-regulation of *TGFBR2* could also be an attempt to support higher levels of alternatively activated macrophages which would help with wound resolution (Gong *et al.* 2012). It is possible the observed up regulation of *TGFβ R2* is a compensatory response to try and overcome the loss of TGFβR1 and reduction in TGFβ1. However, it is known that both TGFβ receptors 1 and 2 are required for TGFβ signalling (Huang *et al.* 2011).

BMPs are a sub-group of the TGFβ superfamily, and are known to play developmental and homeostatic functions in most tissues (Wang *et al.* 2014). BMP-2 and -7 have been shown to increase collagen production in rotator cuff cells, and could both be implicated in the development of a fibrotic phenotype (Pauly *et al.* 2012). Like TGFβ, BMP-2, is reported to increase in response to strain (Rui *et al.* 2011). In the current study, there was no significant difference in *BMP2* or *BMP7* mRNA in healthy and diseased tendons. However, there was a trend for *BMP7* to decrease in pathology, as there were almost undetectable levels in torn rotator cuff cells. This correlates with the pattern of reduced TGFβ signalling mediators in pathology.

CTGF is an effector cytokine of TGFβ that increases deposition of ECM and promotes myofibroblast differentiation. With significantly reduced TGFβ in tendon pathology it might be expected that CTGF would also be decreased. In the current study, no significant difference was seen in *CTGF* expression between healthy and pathological tendons. It is possible that other signalling pathways could influence CTGF mRNA expression overriding any

impact of reduced TGFβ. It has been suggested that CTGF can be independently expressed via the IL-13 pathway (Liu *et al.* 2011), which is implicated in alternative activation of macrophages. This is an interesting observation in light of the high levels of monocytes and macrophages observed in chronic tendinopathy (Dakin *et al.* 2012).

Confocal images of massive supraspinatus tendon tears showed tendon fibroblasts and classically activated macrophages around the edge of a tear, with alternatively activated macrophages residing further from the edge. Fibroblasts are vital to lay down new ECM, and classically activated macrophages would be expected to react with DAMPs during injury. Alternatively activated macrophages then assist resolution of wound healing, and reduce pro-inflammatory signalling. We identified $CD206^{high}$ and $CD163^{high}$ macrophages that co-expressed pan TGFβ in a massive tendon tear, suggesting M2 macrophages may be a source of TGFβ in advanced stage tendon disease. In active fibrosis, fibroblasts and myofibroblasts should be present in greater numbers than in healthy tendon. Vimentin, an intermediate filament protein present in mesenchymal cells, however, was decreased in pathology, which would suggest the presence of fewer fibroblasts. There was no significant difference in the levels of TLR4, which can activate fibroblasts, suggesting that there is no specific TLR4 mediated on-going DAMP-based stimulus driving the inflammatory process in the samples from this patient cohort. There was also no significant difference in levels of other markers of myofibroblasts and EMT such as alpha-SMA or E-cadherin. Models where myofibroblasts have been shown to be present in

fibrosis involve acute pathology, and so their activity may be relatively diminished in the cohort of patients being investigated given that the phenotype is known to be chronically fibrotic.

4.1 Limitations

In tendon ECM TGFβ is bound LAP as a pro-peptide and then dissociates as it is released in response to a stimulus (Rifkin 2005). The antibody used for this study binds to TGFβ whether it is bound to LAP or not. Therefore, we are not able to discern whether the suppression in TGFβ detected is definitively associated with a detectable change in signalling output. Further work could investigate downstream mediators such as phospho Smad2/3, which would be expressed with successful TGFβ signalling.

We also did not investigate expression of other isoforms of TGFβ, namely TGF-β2 and TGF-β3. These isoforms are indicated to be important in muscle wound healing (McLennan and Koishi 1997). Much like TGF-β1, they are involved in embryogenesis and mediate many cellular interactions (Pelton *et al.* 1991; Hsu *et al.* 2001). TGF-β2 has many similar actions to TGF-β1 during development and embryology, but it has also been shown to correlate with fibrosis of the eye during retinal detachment (Connor *et al.* 1989). Interestingly, TGF-β3 has been demonstrated to play different roles to TGF-β1 and 2 in a wound healing model where exogenous TGF-β3 reduced the fibrotic phenotype (Shah, Foreman and Ferguson 1995).

It was not possible to accurately quantify Collagen III using IHC due to ubiquitous staining in tendon tissues. The antibody worked effectively even at extremely low concentrations, thus the small percentage changes that might be expected in fibrotic tendon are unlikely to be detectable. ELISA combined with RT-qPCR or more complex proteomic analyses such as Mass Spectrometry would perhaps been better suited to measuring the percentage changes of Collagen III between healthy and pathological samples.

Due to the new and rare nature of the healthy samples there was a limited amount of tissue available for both IHC and gene analysis. With a greater number it may have been possible to detect significant differences such as for *BMP7* and *CTGF* mRNA expression, and TLR4 and alpha-SMA protein levels. Gene analysis required a significant proportion of tissue, so healthy hamstring tendon had to be used instead of healthy supraspinatus, which although similar is not a perfectly comparable tissue. However, hamstring was known to be healthy as it was evaluated at the time of surgery and also from live donors. We believe this is a more suitable comparator than cadaveric rotator cuff tendon tissues where little is known about whether the tendons were healthy or diseased and tendons were not affected by post mortem changes.

EMT is thought to be a significant source of myofibroblasts during the development of fibrosis in some tissues. As the name of the process would suggest, EMT is initiated in epithelial cells, which in tendon surround the

periphery. The majority of EMT research has focussed on the kidneys. Renal tubules are arranged throughout the kidney, and so there are epithelial cells well distributed within the parenchyma to provide a significant source for EMT and its detection. The IHC component of this study investigated core biopsies of tendon, and may not have included suitable material to truly investigate EMT. Furthermore, image processing aimed not to include bare space near the edges of the tendon samples, so as not to skew the quantification process, this will have also reduced the amount of imaging of any edges included in the tissue. Additionally, although there is evidence suggestive of myofibroblasts due to the levels of alpha-SMA and Vimentin present in tendon, there were no changes in E-cadherin between different pathologies, which would have suggested their presence is due to EMT. Moreover, alpha-SMA is not an entirely specific marker for myofibroblasts; there are currently no highly specific markers.

Wound healing and fibrosis are complex processes that involve a number of interactions between cells and activation of downstream signalling cascades. Figure 19 demonstrates some of the pathways investigated in the literature leading to fibrosis, focussing on TGFβ. The complexity of these pathways highlights the difficulty in selecting a therapeutic target to modulate the TGFβ axis in the prevention of fibrosis. The multitude of TGFβ effects in acute tendon disease and ECM regulation must be further understood before therapeutic targets to moderate fibrosis can be identified.

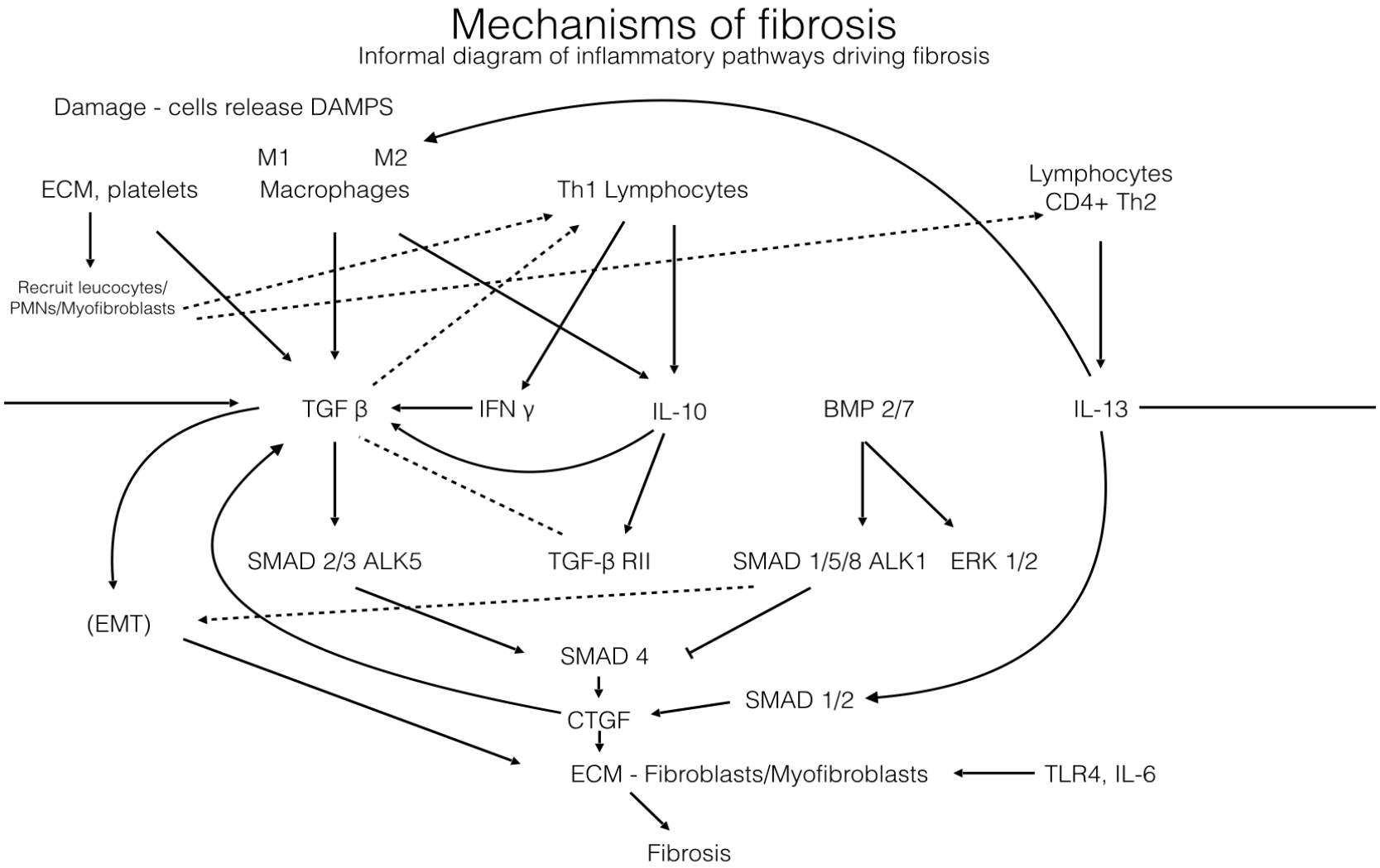


Figure 19 Mechanisms of fibrosis in several organ tissues. Diagram illustrates the complex interplay of cytokines and signalling pathways in fibrosis.

4.2 Future work

Future work should focus on quantification of the levels of active and latent TGFβ signalling. There are currently no antibodies that can distinguish TGFβ bound or unbound to LAP. To identify active signalling it would therefore be necessary to investigate downstream Smad signalling, in particular Smad 2/3 phosphorylation. The photomicrographs (figures 8-11) show that the DAB immunopositive staining for TGFβ1, R1 and R2 is minimal within the ECM and so it is possible that LAP inhibits binding of TGFβ1 or they are of insufficient density to be well represented by the stain. ELISA could be an alternative modality to differentiate active and latent TGFβ1, using extracts of healthy and diseased tendon tissue.

Identifying other pathways involved in driving fibrosis, such as TNFα, would help characterise other signalling pathways that may influence the fibrotic phenotype. TNF receptor 2 is inducible and up-regulated in chronically inflamed tissues and thus suggested to drive the fibrotic phenotype of Dupuytren's contracture (Verjee *et al.* 2013). Given the suppression of TGFβ expression, it is possible that other pathways are important in inducing fibrosis, and thus warrant further investigation.

The temporal nature of the development of fibrosis would also be interesting to investigate. Acute tendon injury and surgical incision animal models generate increased TGFβ and are associated with increased collagen production (Shah, Foreman and Ferguson 1995; Chang *et al.* 1997; Zhang *et*

al. 2004). The ability to map critical time points in the early development of fibrosis and tendinopathy may reveal potential treatment targets. However, this would present a number of technical challenges in human tendon tissue. We do not fully understand the timing of symptoms and development of tendinopathy, and furthermore presentation to clinic would need to be prospective or at least accelerated.

4.3 Conclusions

This study is the first to investigate mediators of fibrosis in non-cadaveric healthy and diseased human tendons. Furthermore, it has been able to use healthy tendon from a unique cohort of patients thanks to the advent of a novel, minimally invasive, biopsy technique used to biopsy supraspinatus tendons in patients undergoing shoulder stabilisation surgery under general anaesthetic.

The findings from this study suggest that patients with chronic diseased fibrotic tendon would be unlikely to benefit from therapeutic TGFβ blockade, which has been investigated as a treatment strategy in several animal models. Future work should focus on identification of fibrotic pathways activated in early stage human tendon disease.

4.4 Summary

This work enhances our understanding of the dysregulation of TGFβ family mediators in chronic tendon pathology. The suppression of TGFβ mediators suggests a reduction in TGFβ pathway signalling. In the presence of on-going inflammation other fibrotic pathways may therefore be involved in driving the chronic fibrotic phenotype associated with tendon pathology. Furthermore, there is likely to be a point at which TGFβ expression alters from being increased in acute injury models to being suppressed in chronic disease.

Appendix

Appendix A

A1 Related publications

Comparison of TGFβ expression in healthy and diseased human tendon Henry CJ Goodier; Andrew J Carr; Sarah JB Snelling; Lucy Roche; Kim Wheway; Bridget Watkins; Stephanie Georgina Dakin, Arthritis Research & Therapy (In Press)

A2 Related presentations in scientific meetings

Oral and poster presentations

Dysregulation of TGFβ family mediators in human supraspinatus tendon pathology. NDORMS postgraduate seminar, April 2015

Dysregulation of TGFβ family mediators in human supraspinatus tendon pathology. Dr Henry Goodier Dr Stephanie G Dakin, Dr Sarah Snelling, Professor Andrew Carr.

At: 1) British Society of Immunology, Oxford 2015

2) Advances in tendon research: from bench to bedside, QMUL London, 7-8 September 2015

A3 Courses and conferences Attended

2014 Good clinical Practice on-line training course

2014 International Scientific Tendinopathy Symposium Conference

2015 Statistics: Designing clinical research and biostatistics

A4 Prizes

James Turner Prize for work on TGF-β in joint inflammation

References

Abdelmagid SM, Barr AE, Rico M *et al.* Performance of repetitive tasks induces decreased grip strength and increased fibrogenic proteins in skeletal muscle: role of force and inflammation. *PLoS One* 2012;**7**:e38359.

Abraham DJ, Varga J. Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol* 2005;**26**:587–95.

Abrahams M. Mechanical behaviour of tendon in vitro. A preliminary report. *Med Biol Eng* 1967;**5**:433–43.

Abreu JG, Ketpura NI, Reversade B *et al.* Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. *Nat Cell Biol* 2002;**4**:599–604.

Ackermann PW, Li J, Finn A *et al.* Autonomic innervation of tendons, ligaments and joint capsules. A morphologic and quantitative study in the rat. *J Orthop Res* 2001;**19**:372–8.

Ackermann PW, Li J, Lundeberg T *et al.* Neuronal plasticity in relation to nociception and healing of rat achilles tendon. *J Orthop Res* 2003;**21**:432–41.

Ackermann PW, Salo PT, Hart DA. Neuronal pathways in tendon healing. *Front Biosci* 2009;**14**:5165–87.

Adzick NS, Longaker MT. Scarless fetal healing. Therapeutic implications. *Ann Surg* 1992;**215**:3–7.

Akagi Y, Isaka Y, Arai M *et al.* Inhibition of TGF-β1 expression by antisense oligonucleotides suppressed extracellular matrix accumulation in

experimental glomerulonephritis. *Kidney Int* 1996;**50**:148–55.

Alexander RM. Tendon elasticity and muscle function. *Comp Biochem Physiol* 2002;**133**:1001–11.

Aliprantis AO, Wang J, Fathman JW *et al.* Transcription factor T-bet regulates skin sclerosis through its function in innate immunity and via IL-13. *Proc Natl Acad Sci U S A* 2007;**104**:2827–30.

Andres BM, Murrell GAC. Treatment of tendinopathy: what works, what does not, and what is on the horizon. *Clin Orthop Relat Res* 2008;**466**:1539–54.

Andrew JG, Andrew SM, Ash A *et al.* An investigation into the role of inflammatory cells in Dupuytren's disease. *J Hand Surg Am* 1991;**16**:267–71.

Armstrong JR, Ferguson MW. Ontogeny of the skin and the transition from scar-free to scarring phenotype during wound healing in the pouch young of a marsupial, Monodelphis domestica. *Dev Biol* 1995;**169**:242–60.

Ashcroft GS, Yang X, Glick AB *et al.* Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1999;**1**:260–6.

Aström M, Rausing A. Chronic Achilles tendinopathy. A survey of surgical and histopathologic findings. *Clin Orthop Relat Res* 1995;**316**:151–64.

Aström M. Laser Doppler flowmetry in the assessment of tendon blood flow. *Scand J Med Sci Sports* 2000;**10**:365–7.

Aurora A, McCarron J, Iannotti JP *et al.* Commercially available extracellular matrix materials for rotator cuff repairs: state of the art and future trends. *J Shoulder Elb Surg* 2007;**16**:S171–8.

References

Backman LJ, Fong G, Andersson G *et al.* Substance P is a mechanoresponsive, autocrine regulator of human tenocyte proliferation. *PLoS One* 2011;**6**:e27209.

Barbe MF, Barr AE, Gorzelany I *et al.* Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 2003;**21**:167–76.

Bates SJ, Morrow E, Zhang AY *et al.* Mannose-6-phosphate, an inhibitor of transforming growth factor-beta, improves range of motion after flexor tendon repair. *J Bone Jt Surgery Am* 2006;**88**:2465–72.

Batson EL, Paramour, R. J.Smith TJ, Birch HL *et al.* Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? *Equine Vet J* 2010;**35**:314–8.

Battegay EJ, Raines EW, Seifert RA *et al.* TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. *Cell* 1990;**63**:515–24.

Beanes SR, Hu F-Y, Soo C *et al.* Confocal microscopic analysis of scarless repair in the fetal rat: defining the transition. *Plast Reconstr Surg* 2002;**109**:160–70.

Birch HL, Bailey J V, Bailey AJ *et al.* Age-related changes to the molecular and cellular components of equine flexor tendons. *Equine Vet J* 1999;**31**:391–6.

Birch HL. Tendon matrix composition and turnover in relation to functional requirements. *Int J Exp Pathol* 2007;**88**:241–8.

Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N*

Engl J Med 1994;**331**:1286–92.

Borthwick LA, Gardner A, De Soyza A *et al.* Transforming Growth Factor-β1 (TGF-β1) Driven Epithelial to Mesenchymal Transition (EMT) is Accentuated by Tumour Necrosis Factor α (TNFα) via Crosstalk Between the SMAD and NF-κB Pathways. *Cancer Microenviron* 2012;**5**:45–57.

Boudreault J, Desmeules F, Roy J-S *et al.* The efficacy of oral non-steroidal anti-inflammatory drugs for rotator cuff tendinopathy: a systematic review and meta-analysis. *J Rehabil Med* 2014;**46**:294–306.

Branton MH, Kopp JB. TGF-β and fibrosis. *Microbes Infect* 1999;**1**:1349–65.

Brewer BJ. Aging of the rotator cuff. *Am J Sports Med* 1979;**7**:102–10.

Brickley-Parsons D, Glimcher MJ, Smith RJ *et al.* Biochemical changes in the collagen of the palmar fascia in patients with Dupuytren's disease. *J Bone Jt Surgery Am* 1981;**63**:787–97.

Brox JI, Gjengedal E, Uppheim G *et al.* Arthroscopic surgery versus supervised exercises in patients with rotator cuff disease (stage II impingement syndrome): a prospective, randomized, controlled study in 125 patients with a 2 1/2-year follow-up. *J Shoulder Elb Surg* 1999;**8**:102–11.

Brox JI, Staff PH, Ljunggren AE *et al.* Arthroscopic surgery compared with supervised exercises in patients with rotator cuff disease (stage II impingement syndrome). *BMJ* 1993;**307**:899–903.

Bucala R, Spiegel LA, Chesney J *et al.* Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1994;**1**:71–81.

References

Butler DL, Grood ES, Noyes FR *et al.* Biomechanics of ligaments and tendons. *Exerc Sport Sci Rev* 1978;**6**:125–81.

Carr AJ, Norris SH. The blood supply of the calcaneal tendon. *J bone Jt surgery Br* 1989;**71**:100–1.

Chang J, Most D, Stelnicki E *et al.* Gene expression of transforming growth factor beta-1 in rabbit zone II flexor tendon wound healing: evidence for dual mechanisms of repair. *Plast Reconstr Surg* 1997;**100**:937–44.

Chang J, Thunder R, Most D *et al.* Studies in flexor tendon wound healing: neutralizing antibody to TGF-beta1 increases postoperative range of motion. *Plast Reconstr Surg* 2000;**105**:148–55.

Chansky HA, Iannotti JP. The vascularity of the rotator cuff. *Clin Sports Med* 1991;**10**:807–22.

Chard MD, Hazleman R, Hazleman BL *et al.* Shoulder disorders in the elderly: a community survey. *Arthritis Rheum* 1991;**34**:766–9.

Chaudhury S, Gwilym SE, Moser J *et al.* Surgical options for patients with shoulder pain. *Nat Rev Rheumatol* 2010;**6**:217–26.

Chen MR, Dragoo JL. The effect of nonsteroidal anti-inflammatory drugs on tissue healing. *Knee surgery, Sport Traumatol Arthrosc* 2013;**21**:540–9.

Chen SJ, Yuan W, Mori Y *et al.* Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. *J Invest Dermatol* 1999;**112**:49–57.

Chen W, Kirkbride KC, How T *et al.* Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-regulation of its signaling. *Science (80-)* 2003;**301**:1394–7.

References

Chiaramonte MG, Donaldson DD, Cheever AW *et al.* An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest* 1999;**104**:777–85.

Chilosi M, Poletti V, Zamò A *et al.* Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol* 2003;**162**:1495–502.

Chipchase LS, O'Connor DA, Costi JJ *et al.* Shoulder impingement syndrome: preoperative health status. *J shoulder Elb Surg* 2000;**9**:12–5.

Choo A, McCarthy M, Pichika R *et al.* Muscle gene expression patterns in human rotator cuff pathology. *J Bone Jt Surgery Am* 2014;**96**:1558–65.

Christensen B, Dandanell S, Kjaer M *et al.* Effect of anti-inflammatory medication on the running-induced rise in patella tendon collagen synthesis in humans. *J Appl Physiol* 2011;**110**:137–41.

Clark SC, Jones MW, Choudhury RR *et al.* Bilateral patellar tendon rupture secondary to repeated local steroid injections. *Emerg Med J* 1995;**12**:300–1.

Cohen DB, Kawamura S, Ehteshami JR *et al.* Indomethacin and celecoxib impair rotator cuff tendon-to-bone healing. *Am J Sports Med* 2006;**34**:362–9.

Connor TB, Roberts AB, Sporn MB *et al.* Correlation of fibrosis and transforming growth factor-beta type 2 levels in the eye. *J Clin Invest* 1989;**83**:1661–6.

Cook KF, Roddey TS, Olson SL *et al.* Reliability by surgical status of self-reported outcomes in patients who have shoulder pathologies. *J Orthop*

Sports Phys Ther 2002;**32**:336–46.

Cooper RR, Misol S. Tendon and ligament insertion. A light and electron microscopic study. *J bone Jt surgery Am* 1970;**52**:1–20.

Cowin AJ, Brosnan MP, Holmes TM *et al.* Endogenous inflammatory response to dermal wound healing in the fetal and adult mouse. *Dev Dyn* 1998;**212**:385–93.

Crofford LJ. COX-1 and COX-2 tissue expression: implications and predictions. *J Rheumatol Suppl* 1997;**49**:15–9.

Cui W, Fowlis DJ, Cousins FM *et al.* Concerted action of TGF-beta 1 and its type II receptor in control of epidermal homeostasis in transgenic mice. *Genes Dev* 1995;**9**:945–55.

Dakin SG, Dudhia J, Smith RKW. Resolving an inflammatory concept: the importance of inflammation and resolution in tendinopathy. *Vet Immunol Immunopathol* 2014;**158**:121–7.

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

Dakin SG, Smith RKW, Heinegård D *et al.* Proteomic analysis of tendon extracellular matrix reveals disease stage-specific fragmentation and differential cleavage of COMP (cartilage oligomeric matrix protein). *J Biol Chem* 2014;**289**:4919–27.

Dakin SG, Werling D, Hibbert A *et al.* Macrophage sub-populations and the lipoxin A4 receptor implicate active inflammation during equine tendon repair. *PLoS One* 2012;**7**:e32333.

Dawson J, Fitzpatrick R, Carr A. Questionnaire On The Perceptions Of

Patients About Shoulder Surgery. *J bone Jt surgery Br* 1996;**78-B**:593–600.

Dolmans GH, Werker PM, Hennies HC *et al.* Wnt signaling and Dupuytren's disease. *N Engl J Med* 2011;**365**:307–17.

Dudhia J, Scott CM, Draper ERC *et al.* Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. *Aging Cell* 2007;**6**:547–56.

Duffield JS, Forbes SJ, Constandinou CM *et al.* Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005;**115**:56–65.

Duncan MR, Frazier KS, Abramson S *et al.* Connective tissue growth factor mediates transforming growth factor beta-induced collagen synthesis: down-regulation by cAMP. *FASEB J* 1999;**13**:1774–86.

Ebihara Y, Masuya M, Larue AC *et al.* Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. *Exp Hematol* 2006;**34**:219–29.

Elder CL, Dahners LE, Weinhold PS. A cyclooxygenase-2 inhibitor impairs ligament healing in the rat. *Am J Sports Med* 2001;**29**:801–5.

Fenwick SA, Curry V, Harrall RL *et al.* Expression of transforming growth factor-beta isoforms and their receptors in chronic tendinosis. *J Anat* 2001;**199**:231–40.

Fichtner-Feigl S, Strober W, Kawakami K *et al.* IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* 2006;**12**:99–106.

References

Flanders KC, Sullivan CD, Fujii M *et al.* Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. *Am J Pathol* 2002;**160**:1057–68.

Flurin P-H, Hardy P, Abadie P *et al.* Rotator cuff tears after 70 years of age: a prospective, randomized, comparative study between decompression and arthroscopic repair in 154 patients. *Orthop Traumatol Surg Res* 2013;**99**:S371–8.

Forbes SJ, Russo FP, Rey V *et al.* A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004;**126**:955–63.

Fu FH, Harner CD, Klein AH. Shoulder impingement syndrome. A critical review. *Clin Orthop Relat Res* 1991;**269**:162–73.

Gangadharan V, Wang R, Ulzhöfer B *et al.* Peripheral calcium-permeable AMPA receptors regulate chronic inflammatory pain in mice. *J Clin Invest* 2011;**121**:1608–23.

Gao HGL, Fisher PW, Lambi AG *et al.* Increased serum and musculotendinous fibrogenic proteins following persistent low-grade inflammation in a rat model of long-term upper extremity overuse. *PLoS One* 2013;**8**:e71875.

Gelberman RH, Amifl D, Gonsalves M *et al.* The influence of protected passive mobilization on the healing of flexor tendons: a biochemical and microangiographic study. *Hand* 1981;**13**:120–8.

Ghahary A, Shen YJ, Scott PG *et al.* Enhanced expression of mRNA for transforming growth factor-beta, type I and type III procollagen in human

post-burn hypertrophic scar tissues. *J Lab Clin Med* 1993;**122**:465–73.

Gong D, Shi W, Yi S *et al.* TGFβ signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol* 2012;**13**:31.

Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2002;**2**:46–53.

Green S, Buchbinder R, Glazier R *et al.* Interventions for shoulder pain. *Cochrane database Syst Rev* 2000;**2**:CD001156.

Gressner OA, Lahme B, Rehbein K *et al.* Pharmacological application of caffeine inhibits TGF-β-stimulated connective tissue growth factor expression in hepatocytes via PPARγ and SMAD2/3-dependent pathways. *J Hepatol* 2008;**49**:758–67.

Gwilym SE, Oag HCL, Tracey I *et al.* Evidence that central sensitisation is present in patients with shoulder impingement syndrome and influences the outcome after surgery. *J bone Jt surgery Br* 2011;**93**:498–502.

Gwilym SE, Watkins B, Cooper CD *et al.* Genetic influences in the progression of tears of the rotator cuff. *J bone Jt surgery Br* 2009;**91**:915–7.

Haraldsson BT, Langberg H, Aagaard P *et al.* Corticosteroids reduce the tensile strength of isolated collagen fascicles. *Am J Sports Med* 2006;**34**:1992–7.

Hays PL, Kawamura S, Deng X-H *et al.* The role of macrophages in early healing of a tendon graft in a bone tunnel. *J bone Jt surgery Am* 2008;**90**:565–79.

Heinemeier K, Langberg H, Olesen JL *et al.* Role of TGF-beta1 in relation to

exercise-induced type I collagen synthesis in human tendinous tissue. *J Appl Physiol* 2003;**95**:2390–7.

Hess GW. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot Ankle Spec* 2010;**3**:29–32.

Hesse M, Modolell M, La Flamme AC *et al.* Differential Regulation of Nitric Oxide Synthase-2 and Arginase-1 by Type 1/Type 2 Cytokines In Vivo: Granulomatous Pathology Is Shaped by the Pattern of L-Arginine Metabolism. *J Immunol* 2001;**167**:6533–44.

Hills CE, Siamantouras E, Smith SW *et al.* TGFβ modulates cell-to-cell communication in early epithelial-to-mesenchymal transition. *Diabetologia* 2012;**55**:812–24.

Hirokawa S, Hasezaki H. Model analysis to investigate the contribution of ground substance to ligament stiffening. *Med Eng Phys* 2010;**32**:610–6.

Holmes GB, Lin J. Etiologic factors associated with symptomatic achilles tendinopathy. *Foot ankle Int* 2006;**27**:952–9.

Hopkinson-Woolley J, Hughes D, Gordon S *et al.* Macrophage recruitment during limb development and wound healing in the embryonic and foetal mouse. *J Cell Sci* 1994;**107**:1159–67.

Hsu M, Peled ZM, Chin GS *et al.* Ontogeny of expression of transforming growth factor-beta 1 (TGF-beta 1), TGF-beta 3, and TGF-beta receptors I and II in fetal rat fibroblasts and skin. *Plast Reconstr Surg* 2001;**107**:1787–94.

Huang T, David L, Mendoza V *et al.* TGF-β signalling is mediated by two autonomously functioning TβRI:TβRII pairs. *EMBO J* 2011;**30**:1263–76.

References

Humphreys BD, Lin S-L, Kobayashi A *et al.* Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am J Pathol* 2010;**176**:85–97.

Imamura M, Ogawa T, Sasaguri Y *et al.* Suppression of macrophage infiltration inhibits activation of hepatic stellate cells and liver fibrogenesis in rats. *Gastroenterology* 2005;**128**:138–46.

Itoi E, Tabata S. Conservative treatment of rotator cuff tears. *Clin Orthop Relat Res* 1992;**275**:165–73.

Itoi E. Rotator cuff tear: physical examination and conservative treatment. *J Orthop Sci* 2013;**18**:197–204.

Iwano M, Plieth D, Danoff TM *et al.* Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;**110**:341–50.

Jacquot A, Dezaly C, Goetzmann T *et al.* Is rotator cuff repair appropriate in patients older than 60 years of age? Prospective, randomised trial in 103 patients with a mean four-year follow-up. *Orthop Traumatol Surg Res* 2014;**100**:S333–8.

Jiang H, Harris MB, Rothman P. IL-4/IL-13 signaling beyond JAK/STAT. *J Allergy Clin Immunol* 2000;**105**:1063–70.

Jørgensen HG, McLellan SD, Crossan JF *et al.* Neutralisation of TGF beta or binding of VLA-4 to fibronectin prevents rat tendon adhesion following transection. *Cytokine* 2005;**30**:195–202.

Józsa L, Kannus P, Balint JB *et al.* Three-Dimensional infrastructure of Human Tendons. *Cells Tissues Organs* 1991;**142**:306–12.

Józsa L, Réffy A, Kannus P *et al.* Pathological alterations in human tendons.

Arch Orthop Trauma Surg 1990;**110**:15–21.

Judge A, Murphy RJ, Maxwell R *et al.* Temporal trends and geographical variation in the use of subacromial decompression and rotator cuff repair of the shoulder in England. *Bone Joint J* 2014;**96-B**:70–4.

Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003;**112**:1776–84.

Kannus P, Józsa L, Natri A *et al.* Effects of training, immobilization and remobilization on tendons. *Scand J Med Sci Sports* 1997;**7**:67–71.

Kannus P, Józsa L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J bone Jt surgery Am* 1991;**73**:1507–25.

Kannus P. Structure of the tendon connective tissue. *Scand J Med Sci Sports* 2000;**10**:312–20.

Kapetanos G. The effect of the local corticosteroids on the healing and biomechanical properties of the partially injured tendon. *Clin Orthop Relat Res* 1982;**163**:170–9.

Kasai H, Allen JT, Mason RM *et al.* TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res* 2005;**6**:56.

Kastelic J, Galeski A, Baer E. The multicomposite structure of tendon. *Connect Tissue Res* 1978;**6**:11–23.

Katzel EB, Wolenski M, Loiselle AE *et al.* Impact of Smad3 loss of function on scarring and adhesion formation during tendon healing. *J Orthop Res* 2011;**29**:684–93.

References

Kaviratne M, Hesse M, Leusink M *et al.* IL-13 Activates a Mechanism of Tissue Fibrosis That Is Completely TGF- Independent. *J Immunol* 2004;**173**:4020–9.

Ker RF. The implications of the adaptable fatigue quality of tendons for their construction, repair and function. *Comp Biochem Physiol A Mol Integr Physiol* 2002;**133**:987–1000.

Kijima H, Minagawa H, Nishi T *et al.* Long-term follow-up of cases of rotator cuff tear treated conservatively. *J shoulder Elb Surg* 2012;**21**:491–4.

Kim HM, Dahiya N, Teefey SA *et al.* Location and initiation of degenerative rotator cuff tears: an analysis of three hundred and sixty shoulders. *J bone Jt surgery Am* 2010;**92**:1088–96.

Kim KC, Shin HD, Lee WY *et al.* Repair integrity and functional outcome after arthroscopic rotator cuff repair: double-row versus suture-bridge technique. *Am J Sports Med* 2012;**40**:294–9.

Kirkbride KC, Townsend TA, Bruinsma MW *et al.* Bone morphogenetic proteins signal through the transforming growth factor-beta type III receptor. *J Biol Chem* 2008;**283**:7628–37.

Klein MB, Yalamanchi N, Pham H *et al.* Flexor tendon healing in vitro: Effects of TGF-β on tendon cell collagen production. *J Hand Surg Am* 2002;**27**:615–20.

Kleinman M, Gross AE. Achilles tendon rupture following steroid injection. Report of three cases. *J bone Jt surgery Am* 1983;**65**:1345–7.

Kloen P, Jennings CL, Gebhardt MC *et al.* Transforming growth factor-beta: possible roles in Dupuytren's contracture. *J Hand Surg Am* 1995;**20**:101–

8.

Knobloch K, Schreibmueller L, Meller R *et al.* Superior Achilles tendon microcirculation in tendinopathy among symptomatic female versus male patients. *Am J Sports Med* 2008;**36**:509–14.

Koesters R, Kaissling B, Lehir M *et al.* Tubular overexpression of transforming growth factor-beta1 induces autophagy and fibrosis but not mesenchymal transition of renal epithelial cells. *Am J Pathol* 2010;**177**:632–43.

Krause C, Kloen P, Ten Dijke P. Elevated transforming growth factor β and mitogen-activated protein kinase pathways mediate fibrotic traits of Dupuytren's disease fibroblasts. *Fibrogenesis Tissue Repair* 2011;**4**:14.

Krieg T, Abraham D, Lafyatis R. Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast-epithelial cell interactions. *Arthritis Res Ther* 2007;**9**:S4.

Krischak GD, Augat P, Sorg T *et al.* Effects of diclofenac on periosteal callus maturation in osteotomy healing in an animal model. *Arch Orthop Trauma Surg* 2007;**127**:3–9.

Kriz W, Kaissling B, Le Hir M. Epithelial-mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy? *J Clin Invest* 2011;**121**:468–74.

Kuhn JE, Dunn WR, Sanders R *et al.* Effectiveness of physical therapy in treating atraumatic full-thickness rotator cuff tears: a multicenter prospective cohort study. *J shoulder Elb Surg* 2013;**22**:1371–9.

Kuhn JE. Exercise in the treatment of rotator cuff impingement: a systematic review and a synthesized evidence-based rehabilitation protocol. *J shoulder Elb Surg* 2009;**18**:138–60.

References

Kvist M, Hurme T, Kannus P *et al.* Vascular density at the myotendinous junction of the rat gastrocnemius muscle after immobilization and remobilization. *Am J Sports Med* 1995;**23**:359–64.

Kvist M, Józsa L, Järvinen MJ *et al.* Chronic Achilles paratenonitis in athletes: a histological and histochemical study. *Pathology* 1987;**19**:1–11.

Kvist M, Jozsa L, Kannus P *et al.* Morphology and histochemistry of the myotendineal junction of the rat calf muscles. Histochemical, immunohistochemical and electron-microscopic study. *Acta Anat (Basel)* 1991;**141**:199–205.

Larson BJ, Longaker MT, Lorenz HP. Scarless fetal wound healing: a basic science review. *Plast Reconstr Surg* 2010;**126**:1172–80.

Lee CG, Homer RJ, Zhu Z *et al.* Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001;**194**:809–21.

Lee CH, Shah B, Moioli EK *et al.* CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model. *J Clin Invest* 2010;**120**:3340–9.

Lewis JS. Rotator cuff tendinopathy: a model for the continuum of pathology and related management. *Br J Sports Med* 2010;**44**:918–23.

Li F, Cao Y, Townsend CM *et al.* TGF-beta signaling in colon cancer cells. *World J Surg* 2005;**29**:306–11.

Li JH. Smad7 Inhibits Fibrotic Effect of TGF-β on Renal Tubular Epithelial Cells by Blocking Smad2 Activation. *J Am Soc Nephrol* 2002;**13**:1464–72.

Li L, Zepeda-Orozco D, Black R *et al.* Autophagy is a component of epithelial

cell fate in obstructive uropathy. *Am J Pathol* 2010;**176**:1767–78.

Li MO, Wan YY, Sanjabi S *et al.* Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* 2006;**24**:99–146.

Liu Y, Meyer C, Müller A *et al.* IL-13 induces connective tissue growth factor in rat hepatic stellate cells via TGF-β-independent Smad signaling. *J Immunol* 2011;**187**:2814–23.

Lo DD, Zimmermann AS, Nauta A *et al.* Scarless fetal skin wound healing update. *Birth defects Res* 2012;**96**:237–47.

Longaker MT, Whitby DJ, Ferguson MW *et al.* Adult skin wounds in the fetal environment heal with scar formation. *Ann Surg* 1994;**219**:65–72.

Longo UG, Franceschi F, Ruzzini L *et al.* Histopathology of the supraspinatus tendon in rotator cuff tears. *Am J Sports Med* 2008;**36**:533–8.

López-Casillas F, Payne HM, Andres JL *et al.* Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *J Cell Biol* 1994;**124**:557–68.

López-Casillas F, Wrana JL, Massagué J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* 1993;**73**:1435–44.

Lorenz HP, Longaker MT, Perkocha LA *et al.* Scarless wound repair: a human fetal skin model. *Development* 1992;**114**:253–9.

Lu L, Saulis AS, Liu WR *et al.* The temporal effects of anti-TGF-beta1, 2, and 3 monoclonal antibody on wound healing and hypertrophic scar formation. *J Am Coll Surg* 2005;**201**:391–7.

Lundborg G, Holm S, Myrhage R. The Role of the Synovial Fluid and Tendon

Sheath for Flexor Tendon Nutrition. *Scand J Plast Recontructive Surg* 1980;**14**:99–107.

Ma CB, Kawamura S, Deng X-H *et al.* Bone morphogenetic proteins-signaling plays a role in tendon-to-bone healing: a study of rhBMP-2 and noggin. *Am J Sports Med* 2007;**35**:597–604.

MacDermid JC, Ramos J, Drosdowech D *et al.* The impact of rotator cuff pathology on isometric and isokinetic strength, function, and quality of life. *J Shoulder Elb Surg* 2004;**13**:593–8.

Maeda T, Sakabe T, Sunaga A *et al.* Conversion of mechanical force into TGF-β-mediated biochemical signals. *Curr Biol* 2011;**21**:933–41.

Magnusson SP, Beyer N, Abrahamsen H *et al.* Increased Cross-sectional Area and Reduced Tensile Stress of the Achilles Tendon in Elderly Compared With Young Women. *J Gerontol* 2003;**58**:B123–7.

Marsolais D, Côté CH, Frenette J. Neutrophils and macrophages accumulate sequentially following Achilles tendon injury. *J Orthop Res* 2001;**19**:1203–9.

Martin M, Lefaix J-L, Delanian S. TGF-β1 and radiation fibrosis: a master switch and a specific therapeutic target? *Int J Radiat Oncol* 2000;**47**:277–90.

Martin P, D'Souza D, Martin J *et al.* Wound healing in the PU.1 null mouse--tissue repair is not dependent on inflammatory cells. *Curr Biol* 2003;**13**:1122–8.

Martinek V, Latterman C, Usas A *et al.* Enhancement of tendon-bone integration of anterior cruciate ligament grafts with bone morphogenetic

protein-2 gene transfer: a histological and biomechanical study. *J bone Jt surgery Am* 2002;**84-A**:1123–31.

Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 2014;**6**:13.

Mather RC, Koenig L, Acevedo D *et al.* The societal and economic value of rotator cuff repair. *J bone Jt surgery Am* 2013;**95**:1993–2000.

Matthews TJW, Hand GC, Rees JL *et al.* Pathology of the torn rotator cuff tendon. Reduction in potential for repair as tear size increases. *J bone Jt surgery Br* 2006;**88**:489–95.

McLennan IS, Koishi K. Cellular localisation of transforming growth factor-beta 2 and -beta 3 (TGF-beta2, TGF-beta3) in damaged and regenerating skeletal muscles. *Dev Dyn* 1997;**208**:278–89.

Melling M, Reihsner R, Pfeiler W *et al.* Comparison of palmar aponeuroses from individuals with diabetes mellitus and Dupuytren's contracture. *Anat Rec* 1999;**255**:401–6.

Mihelic R. Bone Morphogenetic Protein-7 (Osteogenic Protein-1) Promotes Tendon Graft Integration in Anterior Cruciate Ligament Reconstruction in Sheep. *Am J Sports Med* 2004;**32**:1619–25.

Milgrom C, Schaffler M, Gilbert S *et al.* Rotator-cuff changes in asymptomatic adults. The effect of age, hand dominance and gender. *J bone Jt surgery Br* 1995;**77-B**:296–8.

Minagawa H, Yamamoto N, Abe H *et al.* Prevalence of symptomatic and asymptomatic rotator cuff tears in the general population: From mass-screening in one village. *J Orthop* 2013;**10**:8–12.

Minns Lowe CJ, Moser J, Barker K. Living with a symptomatic rotator cuff tear "bad days, bad nights": a qualitative study. *BMC Musculoskelet Disord* 2014;**15**:228.

Mitchell C, Adebajo A, Hay E *et al.* Shoulder pain: diagnosis and management in primary care. *BMJ* 2005;**331**:1124–8.

Moosmayer S, Tariq R, Stiris M *et al.* The natural history of asymptomatic rotator cuff tears: a three-year follow-up of fifty cases. *J bone Jt surgery Am* 2013;**95**:1249–55.

Mosler E, Folkhard W, Knörzer E *et al.* Stress-induced molecular rearrangement in tendon collagen. *J Mol Biol* 1985;**182**:589–96.

Moustakas A, Heldin C-H. Non-Smad TGF-beta signals. *J Cell Sci* 2005;**118**:3573–84.

Movin T, Guntner P, Gad A *et al.* Ultrasonography-guided percutaneous core biopsy in Achilles tendon disorder. *Scand J Med Sci Sports* 1997;**7**:244–8.

Murphy R, Carr A. Management of shoulder pain in general practice. *InnovAiT* 2009;**2**:402–7.

Murphy RJ, Floyd Dean BJ, Wheway K *et al.* A Novel Minimally Invasive Ultrasound-Guided Technique to Biopsy Supraspinatus Tendon. *Oper Tech Orthop* 2013;**23**:56–62.

Murrell GA, Francis MJ, Bromley L. The collagen changes of Dupuytren's contracture. *J Hand Surg Am* 1991;**16**:263–6.

Nakajima D, Yamamoto A, Kobayashi T *et al.* The effects of rotator cuff tears, including shoulders without pain, on activities of daily living in the general population. *J Orthop Sci* 2012;**17**:136–40.

Nath RK, LaRegina M, Markham H *et al.* The expression of transforming growth factor type beta in fetal and adult rabbit skin wounds. *J Pediatr Surg* 1994;**29**:416–21.

Neer CS. Anterior acromioplasty for the chronic impingement syndrome in the shoulder: a preliminary report. *J bone Jt surgery Am* 1972;**54**:41–50.

Nishimura R, Kato Y, Chen D *et al.* Smad5 and DPC4 Are Key Molecules in Mediating BMP-2-induced Osteoblastic Differentiation of the Pluripotent Mesenchymal Precursor Cell Line C2C12. *J Biol Chem* 1998;**273**:1872–9.

O'Brien M. Structure and metabolism of tendons. *Scand J Med Sci Sports* 2007;**7**:55–61.

Okuno M, Akita K, Moriwaki H *et al.* Prevention of rat hepatic fibrosis by the protease inhibitor, camostat mesilate, via reduced generation of active TGF-β. *Gastroenterology* 2001;**120**:1784–800.

Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007;**22**:S79–84.

Pauly S, Klatte F, Strobel C *et al.* BMP-2 and BMP-7 affect human rotator cuff tendon cells in vitro. *J shoulder Elb Surg* 2012;**21**:464–73.

Pelton RW, Saxena B, Jones M *et al.* Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. *J Cell Biol* 1991;**115**:1091–105.

Post M, Silver R, Singh M. Rotator cuff tear. Diagnosis and treatment. *Clin Orthop Relat Res* 1983;**173**:78–91.

Quaggin SE, Kapus A. Scar wars: mapping the fate of epithelial-

mesenchymal-myofibroblast transition. *Kidney Int* 2011;**80**:41–50.

Quan TE, Cowper SE, Bucala R. The role of circulating fibrocytes in fibrosis. *Curr Rheumatol Rep* 2006;**8**:145–50.

Rathbun JB, Macnab I. The microvascular pattern of the rotator cuff. *J bone Jt surgery Br* 1970;**52-B**:540–53.

Reilly P, Macleod I, Macfarlane R *et al.* Dead men and radiologists don't lie: a review of cadaveric and radiological studies of rotator cuff tear prevalence. *Ann R Coll Surg Engl* 2006;**88**:116–21.

Rhee YG, Cho NS, Yoo JH. Clinical outcome and repair integrity after rotator cuff repair in patients older than 70 years versus patients younger than 70 years. *Arthroscopy* 2014;**30**:546–54.

Rifkin DB. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem* 2005;**280**:7409–12.

Rigby BJ. The Mechanical Properties of Rat Tail Tendon. *J Gen Physiol* 1959;**43**:265–83.

Rizzardi AE, Johnson AT, Vogel RI *et al.* Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. *Diagn Pathol* 2012;**7**:42.

Roberts AB, Sporn MB, Assoian RK *et al.* Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A* 1986;**83**:4167–71.

Rui YF, Lui PPY, Ni M *et al.* Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. *J Orthop Res* 2011;**29**:390–6.

Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. *Anal Quant Cytol Histol* 2001;**23**:291–9.

Sano H, Ishii H, Trudel G *et al.* Histologic evidence of degeneration at the insertion of 3 rotator cuff tendons: a comparative study with human cadaveric shoulders. *J shoulder Elb Surg* 1999;**8**:574–9.

Sano H, Ishii H, Yeadon A *et al.* Degeneration at the insertion weakens the tensile strength of the supraspinatus tendon: a comparative mechanical and histologic study of the bone-tendon complex. *J Orthop Res* 1997;**15**:719–26.

Sato EJ, Killian ML, Choi AJ *et al.* Skeletal muscle fibrosis and stiffness increase after rotator cuff tendon injury and neuromuscular compromise in a rat model. *J Orthop Res* 2014;**32**:1111–6.

Sato H, Lagan AL, Alexopoulou C *et al.* The TNF-863A allele strongly associates with anticentromere antibody positivity in scleroderma. *Arthritis Rheum* 2004;**50**:558–64.

Schibany N, Zehetgruber H, Kainberger F *et al.* Rotator cuff tears in asymptomatic individuals: a clinical and ultrasonographic screening study. *Eur J Radiol* 2004;**51**:263–8.

Scott PG, Dodd CM, Tredget EE *et al.* Immunohistochemical localization of the proteoglycans decorin, biglycan and versican and transforming growth factor-β in human post-burn hypertrophic and mature scars. *Histopathology* 1995;**26**:423–31.

Screen HRC, Toorani S, Shelton JC. Microstructural stress relaxation mechanics in functionally different tendons. *Med Eng Phys* 2013;**35**:96–

102.

Seibert K, Zhang Y, Leahy K *et al.* Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci U S A* 1994;**91**:12013–7.

Sein ML, Walton J, Linklater J *et al.* Shoulder pain in elite swimmers: primarily due to swim-volume-induced supraspinatus tendinopathy. *Br J Sports Med* 2010;**44**:105–13.

Selvanetti A, Cipolla M, Puddu G. Overuse tendon injuries: Basic science and classification. *Oper Tech Sports Med* 1997;**5**:110–7.

Shah M, Foreman DM, Ferguson MW. Control of scarring in adult wounds by neutralising antibody to transforming growth factor beta. *Lancet* 1992;**339**:213–4.

Shah M, Foreman DM, Ferguson MW. Neutralising antibody to TGF-beta 1,2 reduces cutaneous scarring in adult rodents. *J Cell Sci* 1994;**107**:1137–57.

Shah M, Foreman DM, Ferguson MW. Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 1995;**108**:985–1002.

Sher JS, Uribe JW, Posada A *et al.* Abnormal findings on magnetic resonance images of asymptomatic shoulders. *J bone Jt surgery Am* 1995;**77**:10–5.

Shrier I, Matheson GO, Kohl HW. Achilles tendonitis: are corticosteroid injections useful or harmful? *Clin J Sport Med* 1996;**6**:245–50.

Silver FH, Freeman JW, Seehra GP. Collagen self-assembly and the development of tendon mechanical properties. *J Biomech* 2003;**36**:1529–

53.

Sime PJ, Xing Z, Graham FL *et al.* Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;**100**:768–76.

Smith AG, Kosygan K, Williams H *et al.* Common extensor tendon rupture following corticosteroid injection for lateral tendinosis of the elbow. *Br J Sports Med* 1999;**33**:423–4.

Sporn MB, Roberts AB, Shull JH *et al.* Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. *Science (80-)* 1983;**219**:1329–31.

Stilwell DL. The innervation of tendons and aponeuroses. *Am J Anat* 1957;**100**:289–317.

Su B, O'Connor JP. NSAID therapy effects on healing of bone, tendon, and the enthesis. *J Appl Physiol* 2013;**115**:892–9.

Sugaya H, Maeda K, Matsuki K *et al.* Repair integrity and functional outcome after arthroscopic double-row rotator cuff repair. A prospective outcome study. *J bone Jt surgery Am* 2007;**89**:953–60.

Szczodry M, Zhang J, Lim C *et al.* Treadmill running exercise results in the presence of numerous myofibroblasts in mouse patellar tendons. *J Orthop Res* 2009;**27**:1373–8.

Tanaka M, Itoi E, Sato K *et al.* Factors related to successful outcome of conservative treatment for rotator cuff tears. *Ups J Med Sci* 2010;**115**:193–200.

Tempelhof S, Rupp S, Seil R. Age-related prevalence of rotator cuff tears in

asymptomatic shoulders. *J Shoulder Elb Surg* 1999;**8**:296–9.

Thorpe CT, Udeze CP, Birch HL *et al.* Specialization of tendon mechanical properties results from interfascicular differences. *J R Soc Interface* 2012;**9**:3108–17.

Urwin M, Symmons D, Allison T *et al.* Estimating the burden of musculoskeletal disorders in the community: the comparative prevalence of symptoms at different anatomical sites, and the relation to social deprivation. *Ann Rheum Dis* 1998;**57**:649–55.

Vailas AC, Tipton CM, Laughlin HL *et al.* Physical activity and hypophysectomy on the aerobic capacity of ligaments and tendons. *J Appl Physiol* 1978;**44**:542–6.

Verjee LS, Verhoekx JSN, Chan JKK *et al.* Unraveling the signaling pathways promoting fibrosis in Dupuytren's disease reveals TNF as a therapeutic target. *Proc Natl Acad Sci U S A* 2013;**110**:E928–37.

Verrecchia F, Mauviel A. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 2007;**13**:3056–62.

Viidik A. Functional properties of collagenous tissues. *Int Rev Connect Tissue Res* 1973;**6**:127–215.

Virchenko O. Parecoxib Impairs Early Tendon Repair but Improves Later Remodeling. *Am J Sports Med* 2004;**32**:1743–7.

Vitale MA, Vitale MG, Zivin JG *et al.* Rotator cuff repair: an analysis of utility scores and cost-effectiveness. *J shoulder Elb Surg* 2007;**16**:181–7.

Vos T, Barber R, Bell B *et al.* Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic

diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015;**386**:743–800.

Wang J, Hori K, Ding J *et al.* Toll-like receptors expressed by dermal fibroblasts contribute to hypertrophic scarring. *J Cell Physiol* 2011;**226**:1265–73.

Wang R, Ghahary A, Shen Q *et al.* Hypertrophic scar tissues and fibroblasts produce more transforming growth factor-beta1 mRNA and protein than normal skin and cells. *Wound Repair Regen* 2000;**8**:128–37.

Wang RN, Green J, Wang Z *et al.* Bone Morphogenetic Protein (BMP) signaling in development and human diseases. *Genes Dis* 2014;**1**:87–105.

Wei AS, Callaci JJ, Juknelis D *et al.* The effect of corticosteroid on collagen expression in injured rotator cuff tendon. *J bone Jt surgery Am* 2006;**88**:1331–8.

Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol* 2007;**293**:L525–34.

Van der Windt DA, Koes BW, Boeke AJ *et al.* Shoulder disorders in general practice: prognostic indicators of outcome. *Br J Gen Pract* 1996;**46**:519–23.

Wolf BR, Altchek DW. Techniques in Shoulder & Elbow Surgery. *Tech Shoulder Elb Surg* 2003;**4**:55–68.

Wolfgang GL. Surgical repair of tears of the rotator cuff of the shoulder. Factors influencing the result. *J bone Jt surgery Am* 1974;**56**:14–26.

References

Wong JKF, Lui YH, Kapacee Z *et al.* The cellular biology of flexor tendon adhesion formation: an old problem in a new paradigm. *Am J Pathol* 2009;**175**:1938–51.

Wren TA., Yerby SA, Beaupré GS *et al.* Mechanical properties of the human achilles tendon. *Clin Biomech* 2001;**16**:245–51.

Wu MY, Hill CS. Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* 2009;**16**:329–43.

Wynn TA. IL-13 effector functions. *Annu Rev Immunol* 2003;**21**:425–56.

Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* 2007;**117**:524–9.

Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;**214**:199–210.

Yamaguchi K, Tetro AM, Blam O *et al.* Natural history of asymptomatic rotator cuff tears: a longitudinal analysis of asymptomatic tears detected sonographically. *J shoulder Elb Surg* 2001;**10**:199–203.

Yamamoto A, Takagishi K, Osawa T *et al.* Prevalence and risk factors of a rotator cuff tear in the general population. *J shoulder Elb Surg* 2010;**19**:116–20.

Yamamoto T, Noble NA, Miller DE *et al.* Sustained expression of TGF-β1 underlies development of progressive kidney fibrosis. *Kidney Int* 1994;**45**:916–27.

Zeisberg M, Kalluri R. The role of epithelial-to-mesenchymal transition in renal fibrosis. *J Mol Med* 2004;**82**:175–81.

References

Zhang AY, Pham H, Ho F *et al.* Inhibition of TGF-beta-induced collagen production in rabbit flexor tendons. *J Hand Surg Am* 2004;**29**:230–5.

Zhang HY, Gharaee-Kermani M, Zhang K *et al.* Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1996;**148**:527–37.

Zhou X, Tan FK, Milewicz DM *et al.* Autoantibodies to fibrillin-1 activate normal human fibroblasts in culture through the TGF-beta pathway to recapitulate the “scleroderma phenotype”. *J Immunol* 2005;**175**:4555–60.