RE-EVALUATING TENDON DISEASE:
AN EMERGING ROLE FOR INFLAMMATION AND ALARMINS

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF OXFORD FOR
THE DEGREE OF MASTER OF SCIENCE



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TRINITY TERM 2016

Abstract

Re-evaluating Tendon Disease:
An Emerging Role for Inflammation and Alarmins

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Balliol College — Trinity Term 2016

The importance of inflammation in the pathogenesis of tendon disease (TD) is a subject of ongoing debate. During the past two decades, TD has been principally characterized as a degenerative process devoid of inflammation. This pathological paradigm may disregard a complex role for inflammation in TD, barring inflammatory cells, mediators, and pathways on the basis that an 'inflammatory infiltrate' is absent in diseased tendons. Improved understanding of inflammation's cellular and molecular roles in other musculoskeletal diseases prompted a re-emergence of interest in the potential contribution of inflammation to TD. Recent investigations regarding the role of inflammation in TD fuelled the theory that alarmins, endogenous molecules released upon tissue damage, may be instrumental in the pathogenesis of TD.

The specific aims of this research were to 1) examine potential explanations for scientific equipoise regarding inflammation's role in TD through a systematic review of the literature and 2) investigate the role of alarmins in the initiation and propagation of TD. We hypothesized that support for an inflammatory component to TD has increased during the last decade, likely due to more thorough interrogations of immune cells and mediators in TD. Additionally, we hypothesized that TD is an alarmin-mediated pathology, with resident tendon cells and macrophages being vital to disease processes.

A systematic literature search and quantitative analysis of published reviews whose primary purpose was to examine the pathogenesis of TD was conducted. Reviews' methodological quality, definitions of the 'inflammatory component', and several other article characteristics were evaluated regarding reviews' conclusions about the role of inflammation in TD. Logistic regression was performed to determine significant correlates of the conclusion that inflammation plays a role in the pathogenesis of TD. Results revealed growing support for an inflammatory component to TD, particularly during the past 3-6 years. The adoption of a causal theory was significantly associated with greater methodological quality, inclusion of monocytes, macrophages, or lymphocytes, and/or inclusion of signalling molecules, effectors, or growth factors as part of the 'inflammatory component' to TD.

Immunopositive staining was determined for a set of alarmins (S100A9, HMGB1, and IL-33) and HIF-1α, an oxygen sensitive transcription factor, in three groups of human supraspinatus tissue: healthy, persistently painful diseased, and treated pain-free tendon samples. Results showed HIF-1α and S100A9 proteins were increased in diseased tissue compared to healthy and post-treatment pain-free tissue; IL-33 was decreased in diseased tissue compared to healthy and post-treatment pain-free tissue. HMGB1 was up regulated in post-treatment pain-free tendon tissue compared to healthy and diseased tissue.

This body of work supports a role of inflammation in TD and suggests an alarmin-mediated mechanism. Pathological activation of resident tendon cells and macrophages through alarmin involvement may be clinically significant, resulting in sustained inflammation leading to fibrosis. These findings provide insight into molecular pathways that may lead to novel targets and expand therapeutic options for patients with TD.

Acknowledgements

I'm very grateful for the opportunity I had to study at the Botnar Research Centre which was made possible by post-graduate scholarships awarded to me by the United States National Collegiate Athletic Association and the Harvard University Francis H. Burr, Class of 1909 Prize Fund. I am also indebted to Dr. Scott Rodeo of the Hospital for Special Surgery in New York City who prepared me well for the rigors of this research and encouraged me to pursue studies at Oxford.

This experience would not have been possible without the support of Dr. Andrew Carr who graciously welcomed me into his exceptional group. His mentorship has been invaluable to me and his leadership is inspirational as I pursue my career as an academic surgeon. My laboratory supervisors Dr. Stephanie Dakin and Dr. Sarah Snelling were instrumental in my integration and success at Botnar. They provided me with the guidance, knowledge and support I needed to learn the intricacies of inflammation and tendon disease, and to succeed as a graduate student. The time they dedicated to review this thesis and to provide insightful comments to help prepare the work for publication is very much appreciated. I am also thankful to the many other members of the Carr group and Botnar who helped me throughout my time at Oxford, especially Mustafa Rashid and Shona Kirtley. Shona provided expert assistance in designing and performing the systematic literature search, and Mustafa aided in screening abstracts for the systematic review.

Finally, I am extremely thankful to the patients who agreed to have surgical biopsies taken that made this, and much other research possible.

List of Abbreviations

Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
CGRP	Calcitonin gene-related peptide
Cox	Cyclooxygenase
ECM	Extracellular Matrix
EE	Eccentric exercises
ESWT	Extracorporeal shock wave therapy
GAG	Glycosaminoglycan
HIF-1	Hypoxia inducible factor 1
HMGB1	High mobility group box-1
HSPs	Heat shock proteins
IL	Interleukin
IFN	Interferon
iNOS	Inducible nitric oxide synthase
MMP	Matrix metalloproteinase
MTJ	Myotendinous junction
NF-κβ	Nuclear Factor-κβ
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
OSS	Oxford shoulder score
OTJ	Osteotendinous junction
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PGE_1	Prostaglandin-E_1
PGE_2	Prostaglandin-E_2
PMNs	Polymorphonuclear leukocytes
PRP	Platelet-rich plasma
RA	Rheumatoid Arthritis
RAGE	Receptor for advanced glycation end products
RCD	Rotator cuff disease
SAD	Subacromial decompression
TD	Tendon disease
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNF-α	Tumor-necrosis factor-α
UKUFF	United Kingdom Rotator Cuff Trail
VEGF	Vascular endothelial growth factor

Table of Contents

Abstract……2
Acknowledgements……..3
List of Abbreviations……...4
List of Figures and Tables……8

Chapter 1: Introduction, Background, and Aims……10

1.1 Overview of Healthy Tendons……..……11
1.1.1 Function……..……..…11
1.1.2 Structure……..…..…12
1.1.3 Composition……13
1.1.4 Cellularity……...……13
1.1.5 Blood supply……...…14
1.1.6 Nerve innervation……15
1.1.7 Mechanical properties……..……15

1.2 Characteristic Changes in Diseased Tendons……..……16
1.2.1 Structural changes……...……17
1.2.2 Compositional changes……...……18
1.2.3 Cellular changes……...……18
1.2.4 Vascular changes……19
1.2.5 Neuronal changes & pain……19
1.2.6 Mechanical properties……..……20
1.3 Paradigms of Tendon Disease……..……22
1.3.1 Terminology……22
1.3.2 Historical paradigm #1 – The ‘tendinitis’ era……..……23
1.3.3 Historical paradigm #2 – The ‘tendinosis’ era……23
1.3.4 Contemporary paradigm – The ‘tendinopathy’ era…..……24
1.4 Pathology of Tendon Disease: Key Players……..……26
1.4.1 The Peripheral Neuronal Phenotype ……..……26
1.4.2 Hypoxia inducible factor 1 (HIF-1) ……...……28
1.4.3 Cyclooxygenases and Prostaglandins……..……33
1.4.4 Nuclear Factor κβ and Interferons. ……34
1.4.5 Cytokines……37
1.4.6 Heat Shock Proteins……38
1.4.7 Matrix Metalloproteinases (Matrix Enzymes) …...……39
1.5 Inflammation and its Role in Tendon Disease……..……41
1.5.1 Inflammatory Cells Present in Tendon Diseases……41
1.5.2 Inflammatory Mediators/Effectors Present in Tendon Diseases……42
1.5.3 Normal Tissue Healing ……..……43
1.5.4 Proposed Model of Failed Healing/Resolution in Tendon Disease……44
1.6 Alarmins and their Potential Roles in Pathogenesis/Pain……46
1.6.1 Alarmins: What do we know? ……46
1.6.2 S100A9……...……48

1.6.3 High-mobility group box-1 (HMGB1) ……..……51
1.6.4 Interleukin-33 (IL-33) ……54
1.7 Summary……..……57
1.8 Objectives and Hypotheses……..……58

Chapter 2: Trends in the Theory that Inflammation Plays a Causal Role in Tendon Disease: A Systematic Review and Analysis of Published Mechanistic Reviews

2.1 Overview…….……60
2.1.1 Background……60
2.1.2 Purpose……..……60
2.1.3 Definition of terms used…….……61
2.1.4 Specific objectives and hypotheses……62
2.1.5 Rationale/novelty……...……63
2.1.6 Potential significance…….……63
2.2 Methods……64
2.2.1 Information sources and search strategies…….……64
2.2.2 Screening, inclusion, and exclusion criteria……..…..……67
2.2.3 Data items, collection process, and assessment of reproducibility……....68
2.2.4 Synthesis of results and statistical analyses……...……70
2.3 Results……..……71
2.3.1 Study selection……...……71
2.3.2 Characteristics of included reviews……...…73
2.3.3 Temporal trends: the role of inflammation in TD, discussion of specific immune cells, and the role of signalling molecules/effectors in TD. ……...…..…76
2.3.4 Potential correlates of the conclusion that inflammation has a role in tendon disease: cells and molecules incorporated into discussions. ……..…80
2.3.5 Potential correlates of the conclusion that inflammation has a role in tendon disease: quality indicators. ……..…82
2.3.6 Potential correlates of the conclusion that inflammation has a role in tendon disease: journal impact factor and number of citations. ……85
2.4 Limitations……...……87
2.5 Discussion……...…87

Chapter 3: The Role of Alarmins and HIF-1α in the Pathogenesis of Tendon Disease and Resolution of Pain after Treatment.

3.1 Overview……..……89
3.1.1 Background……89
3.1.2 Purpose……..……....90
3.1.3 Objectives……..……90
3.1.4 Hypotheses…….……90
3.1.5 Rationale/novelty……...……91
3.1.6 Potential significance…….……91
3.2 Methods/Methods
3.2.1 Patient cohort and tissue collection……92

3.2.2 Study approval and ethics……..……94
3.2.3 Tissue processing……..……..……94
3.2.4 Immunohistochemistry protocol……95
3.2.5 Image acquisition and analysis for quantitative immunohistochemistry...……97
3.2.6 Immunofluorescence……..98
3.2.7 Immunofluorescence image acquisition and analysis……..…100
3.2.8 Statistical analyses……...……100
3.3 Results……101
3.3.1 Histological assessment of diseased supraspinatus tendons……101
3.3.2 HIF-1α and S100A9 proteins increase in diseased tendons……..……101
3.3.3 IL-33 protein expression is reduced in diseased tendons……..……105
3.3.4 HMGB1 protein is increased in pain-free post-treatment tendons……..……107
3.3.5 Immunofluorescence……109
3.4 Limitations……..……111
3.5 Discussion……..……111

Chapter 4: Re-assessing the Pathological Paradigm: A New Era for Inflammation?

4.1 Discussion……...…117
4.2 Summarized findings……..……124
4.3 Conclusion……..……125
4.4 Future work……125

Appendices:

Appendix A: Data extraction sheet for mechanistic reviews……..……128
Appendix B: Methodological quality/scientific rigor of included mechanistic reviews.130
Appendix C: Results of Reproducibility Sample……132
Appendix D: List of Included Reviews……...……133
Appendix E: Summary of systematic reviews included in review……..……142
Appendix F: Positive control tissues: representative images……..145

References……..……..……146

List of Figures and Tables

Figure 1.1 The hierarchical organization of tendon tissue……12

Figure 1.2 Tendon microstructure of healthy tendons……..14

Figure 1.3 Stress/Strain curve for healthy tendons……...16

Figure 1.4 Changes in tendon structure and composition after chronic pathology……...17

Figure 1.5 Differences in Stress/Strain curve between healthy and diseased tendons…….21

Figure 1.6 Differences in Force/Elongation curve between healthy and diseased tendons…..….21

Figure 1.7 Cellular components to normal healing and inflammation…….43

Table 2.1 Search strategies for each database.……..65

Figure 2.2 PRISMA flowchart for identification and screening of reviews…….72

Figure 2.3 Quality of included reviews over time……74

Figure 2.4 Type of review and levels of evidence used in discussion to form conclusions…….75

Figure 2.5 Conclusions about the role of inflammation in TD over time…….……....77

Figure 2.6 Types of immune cells included in discussions on the role of inflammation in TD...78

Figure 2.7 Conclusions about the role of signalling molecules, etc. over time……79

Figure 2.8 Conclusions about the role of inflammation according to immune cells discussed…80

Figure 2.9 Conclusions about the role of inflammation according to molecules discussed.……81

Figure 2.10 Conclusions about the role of inflammation in TD according to evidence used…...83

Figure 2.11 Conclusions about the role of inflammation in TD according to review type……..83

Figure 2.12 Conclusions about the role of inflammation in TD by quality of the review……....84

Figure 2.13 Conclusions about the role of inflammation in TD according to journal type……..86

Table 3.1 Patient cohort details for tendon tissue samples……...94

Table 3.2 Primary antibodies used for immunohistochemistry/immunofluorescence…...…….95

Figure 3.3 Isotype control staining of diseased human supraspinatus tendons……96

Table 3.4 Immunofluorescence staining channels for negative control and diseased samples….98

Figure 3.5 Photomicrographs of immunohistochemistry staining for HIF-1α.…….……..103

Figure 3.6 Quantitative analysis of immunopositive staining for HIF-1α……..103

Figure 3.7 Photomicrographs of immunohistochemistry staining for S100A9 ……...……..104

Figure 3.8 Quantitative analysis of immunopositive staining for S100A9……104

Figure 3.9 Photomicrographs of immunohistochemistry staining for IL-33……..106

Figure 3.10 Quantitative analysis of immunopositive staining for IL-33……...106

Figure 3.11 Photomicrographs of immunohistochemistry staining for HMGB1……...108

Figure 3.12 Quantitative analysis of immunopositive staining for HMGB1……..108

Figure 3.13 Confocal images of fluorescent staining of HIF-1α and IL-33……...110

Figure 3.14 Confocal images of fluorescent staining of HMGB1 and S100A9…….110

Figure 4.1 Development of chronic inflammation in tendon tissues……..122

Figure 4.2 Development of chronic inflammation in tendon tissues continued……...…..123

Chapter 1: Introduction, Background and Aims

The role of inflammation in the pathogenesis of tendon disease (TD) is a topic of ongoing debate. Prior to the turn of the century, inflammation was believed to contribute to the pain and pathology associated with non-acute, non-ruptured diseased tendons. During the late 1990s and into the early 2000s, the medical and scientific paradigm shifted from an inflammatory etiology to TD being considered a primarily degenerative process. The sea change in scientific theory was largely due to 1) the lack of efficacy of anti-inflammatory regimens on tendon dysfunction and 2) findings that polymorphonuclear leukocytes (PMNs) were absent in diseased tendon tissues [Rees 2014]. An increased understanding of inflammation's role in other musculoskeletal diseases has helped forge a re-emergence of interest in its role in the initiation and propagation of TD [Buckley 2001, Rees 2014]. Most recently, macrophages have been detected in TD and genes associated with their activation profiles have shown differential expression dependent on the severity and stage of TD [Dakin 2015].

The overarching goal of this thesis was to examine the role of inflammation in the pathogenesis of TD. The *specific aims* of the research conducted were to: 1) examine paradigm shifts and potential explanations for scientific equipoise regarding the role of inflammation in TD through a quantitative analysis of published reviews and 2) to determine the protein expression of a set of inflammatory markers known as 'alarmins' in three groups of human tissue: healthy supraspinatus tendon tissue, persistently painful diseased supraspinatus tendon tissue, and treated pain-free supraspinatus tendon tissues. Results from the two scientific aims are synthesized to help elucidate the role of inflammation, with a particular focus on alarmins in the pathogenesis of TD. These data provide insight into an emerging theory that TD results from a failure of inflammation to resolve, similar to chronic inflammatory disorders [Buckley 2001,

Lawrence 2007, Dakin 2015]. The findings may also help to explain dissonance in the scientific community regarding the role of inflammation, and provide future direction for potential therapeutic targets for the prevention and treatment of TD.

1.1 Overview of Healthy Tendons

Healthy tendons perform essential anatomical functions in locomotion and protecting bone structure from excessive strain. A healthy tendon has a hierarchical structure of collagen fibers with low cellularity and metabolic function. Blood supply is sparse and enters the substance of the tendon through connective tissue sheaths. There is no nerve innervation in the main substance of healthy tendons; however, specialized nerves play key mechanical and physiological roles at the connections between a tendon and its associated muscle/bone.

1.1.1 Function

A tendon is a connective tissue interposed between a muscle and bone; it facilitates joint movement by transmitting force generated in muscle to the associated bone [Kannus 2000]. The point at which a muscle connects to tendon is known as the myotendinous junction (MTJ); the point where tendon attaches to bone is the osteotendinous junction (OTJ). The connective tissue between a tendon and bone is known as the enthesis. The tendon medium between muscle and bone allows for passive modulation of forces during movement. Some tendons are used for positioning and moving limbs in locomotion (positional tendons), while others act as springs to dampen forces on the connected bone during movement (elastic/energy storing tendons) [Thorpe 2013]. Positional tendons typically undergo low levels of strain; elastic tendons undergo moderate to higher levels of strain in order to maximize the efficiency of energy transfer during locomotion [Alexander 2002].

1.1.2 Structure

Tendons can range in both shape and attachment area depending on their anatomical location and function; some are cylindrical while others are wide and flat. Regardless of their gross structure, all healthy tendons have a highly ordered and hierarchical assembly of fibers bundled together [Kastelic 1987, Kannus 2000, Silver 2003].

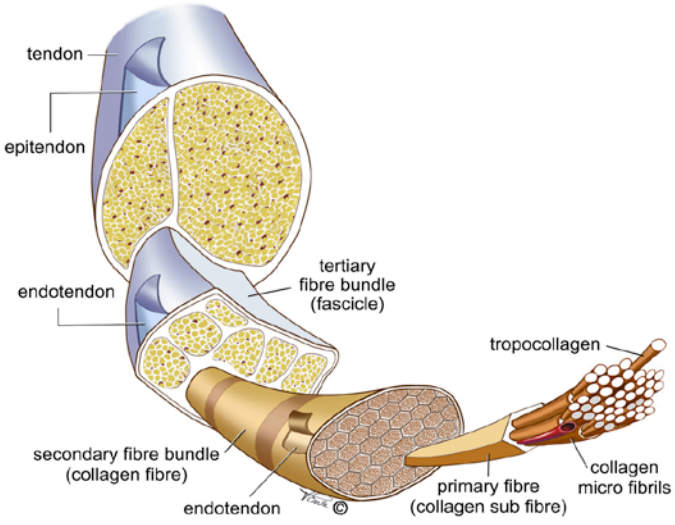


Figure 1.1 The hierarchical organization of tendon tissue [https://vearlemedicalart.com/gallery-2/structure-of-tendons/].

Tendon tissue is comprised of multiple fiber bundles bunched together and surrounded by the endotendon, a loose connective tissue [Kannus 2000]. The fiber bundles, known as fascicles, are bunched together to form the macro-structure of a tendon, which is surrounded by another connective tissue called the epitendon (Figure 1.1). The endo- and epitendon house the vascular, nerve, and lymphatic structures, providing blood supply access to the tendon and its cells [Lundborg 1980]. The fascicle contains both basic fibril unit, collagen fibres, and fibroblasts-like tenocytes, which are resident cells that produce the components of the tendon. These collagen fibrils are comprised of additional hierarchical structures of collagen and are primarily oriented longitudinally, in the direction of stress. Some of these fibrils are oriented transversely or

horizontally and often cross over the longitudinal fibrils forming spirals and plaits [Chansky 1991, Jozsa 1991]. The multi-directional array of fibrils provides a necessary buffer for potential transverse, horizontal, and rotational forces on a tendon.

A tendon's epitendon is often surrounded by a paratenon, a loose areolar connective tissue made up of synovial cells that acts as a lubricating sheath, allowing the tendon to move freely against surrounding tissues [Hess 1989].

1.1.3 Composition

Healthy tendons are white in color and have a fibrous-elastic texture [Kannus 2000]. A tendon's mass is 70% water with the remaining 30% comprised of the extracellular matrix (ECM). Type I collagen makes up around 85% of a tendon's dry mass; type III collagen and proteoglycans each comprise ~1-5%; elastin is ~1-2% of a tendon's dry mass. The remaining mass is comprised of other types of collagen, resident tendon cells, and ground substances that surround collagen. There is type II collagen in the cartilaginous zones of the tendon; type III, IV & V in the vascular zones; type X collagen is present near the entheses in the mineralized fibrocartilage [Weinreb 2014]. Additional components include elastin, glycosaminoglycans (GAG), structural glycoproteins, and inorganic substances primarily composed of calcium [Jozsa 1997].

1.1.4 Cellularity

In a healthy tendon, tenoblasts and tenocytes make up ~90-95% of resident cells. Tenoblasts are immature tendon cells that elongate over time into tenocytes, mature tendon cells that have a reduced nucleus-cytoplasm ratio and metabolic activity [Sharma 2006]. The role of tenocytes in healthy tissue is to secrete and maintain the collagen, GAGs, and glycoprotein levels within the tendon ECM. In and around the tendon tissue additional cell types are found: synovial

cells on the tendon sheath/surface, vascular cells within the epi- and endotendons, and chondrocytes around the insertion and pressure sites [Kannus 2000].

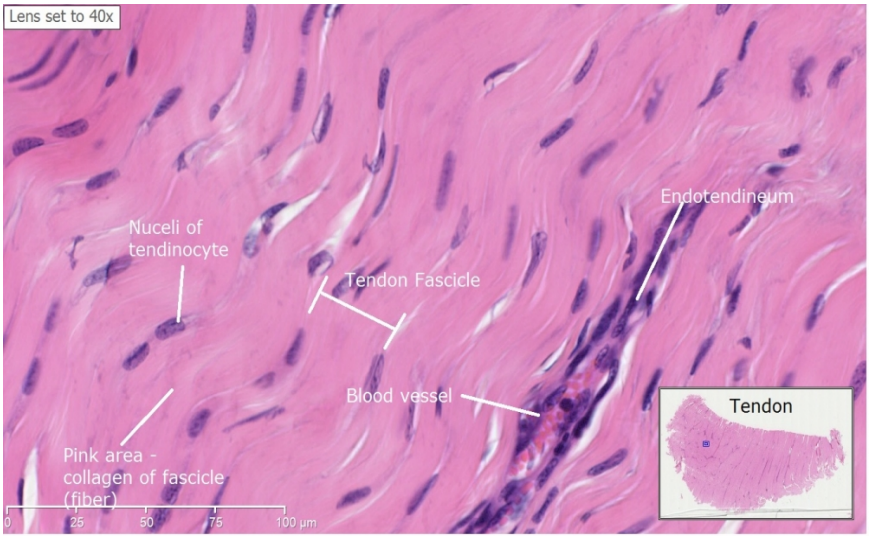


Figure 1.2 Tendon microstructure of healthy tendons: nuclei of tenocytes are darker in color and vascularization does not disrupt collagen arrays [http://www.onlineveterinaryanatomy.net/content /tendon-histology-labelled].

1.1.5 Blood Supply

Due to the lowered metabolic activity of tenocytes and the passive functional role of tendons, oxygen consumption is much lower than other tissues; healthy tendons can remain in use for extended bouts without becoming ischemic. Tendons consume ~7x less oxygen than skeletal muscle so there is less need for an abundance of blood vessels in the tissue [Vailas 1978]. For the vasculature that is present, however, it arrives to the tendon from three main sources: 1) the MTJ 2) the OTJ and 3) the surrounding paratenon or the synovial sheath [Carr 1989]. When tendons are injured, the vascular lining increases access to blood supply to facilitate timely infiltration of inflammatory and wound-healing cells to the tissue [Gelberman 1981, Kvist 1987, Astrom 1995]. In many human tendons there are regions of hypovascularity where inflammatory mediators and cells cannot adequately respond to stressful stimuli, predisposing

these areas to an increased risk of rupture due to mechanical loading, overuse, age and other pathological factors [Carr 1989].

1.1.6 Nerve Innervation

Healthy tendons have little to no nerve innervation in the bulk midsubstance of the tissue however there is significant innervation between tendons and their associated muscles at the MTJ: these nerves play an important role in tendon health, response to mechanical stimuli, and the processing of pain [Sharma 2006]. Tendon innervation arises from peritendinous, cutaneous, and muscular nerve trunks. Nerve fibers at the MTJ form vast arrays in the paratenon and branch out into the epitendon but do not enter the midsubstance of the tendon. At the MTJ there is a large group of myelinated fibers/mechanoreceptors called the Golgi tendon organs that detect changes in the pressure or tension of the tendon. The Golgi tendon organs provide information about the mechanical environment of the tendon so the nervous system can control muscle contraction to prevent injury [Lephart 1997]. There are additional unmyelinated nerve endings at the MTJ that act as nocioceptors: these sense and transmit pain through sympathetic and para-sympathetic fibers [Ackermann 2001].

The peripheral neuronal system plays a significant role in the homeostasis and the healing response to injury [Ackermann 2009]. Sensory nerves, the autonomic, and glutaminergic systems control the proliferative and remodeling responses to acute and chronic injuries.

1.1.7 Mechanical Properties

In order to act as a buffer and transmit force between muscle and bone without comprising tissue integrity, tendons have acquired unique viscoelastic properties [Viidik 1996]. Tendons balance flexibility and mechanical strength to perform their elastic functions within wide range of mechanical stimuli and situations [O'Brien 1992].

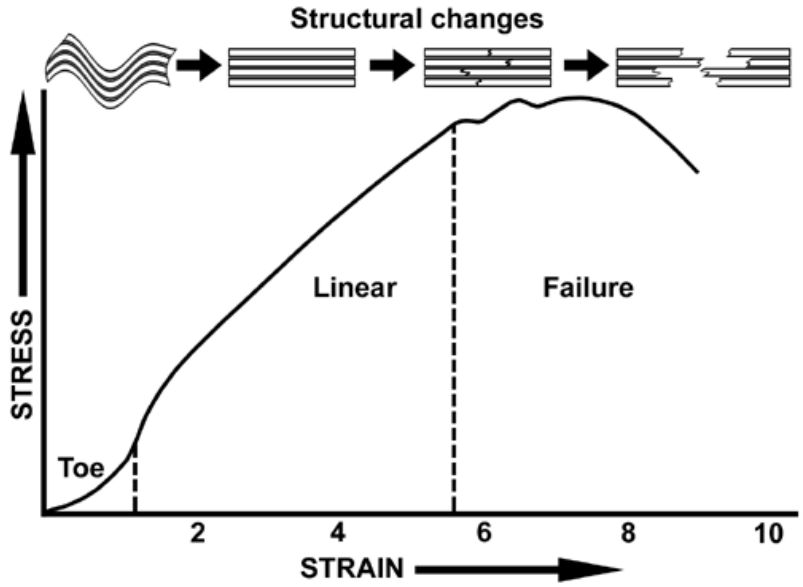


Figure 1.3 Stress/Strain curve for healthy tendons [http://www.pitchingnow.com/kinesiology/basic-biomechanics-of-tendons-and-ligaments/].

While unloaded, a tendon's fibrils have a crimped pattern that flattens as strain within the tendon rises to ~1-2% - shown as the initial concave 'toe' region in Figure 3 [Diamant 1972, Butler 1978]. The subsequent portion of the graph from strain ~2-5% represents linear elastic tendon deformation due to intra-molecular sliding of collagen fibers and inter-fascicular sliding [Butler 1978, Viidik 1973]. If the strain remains below ~5% the tendon will retain its original elastic properties and return to its original length and crimp after being unloaded [Curwin 1984]. However, if the strain exceeds ~4-5%, microscopic failure and plastic deformation occurs resulting in elongated and damaged collagen fibers [Sasaki 1999]. At or above ~8-10% strain the gap between fibers and their adjoining molecules rapidly increases resulting in complete failure of the tissue and possible rupture of the tendon [Best 1994].

1.2 Characteristic Changes in Diseased Tendons

Diseased tendons exhibit multiple histologic and structural changes from the characteristics of healthy tendons. Although the exact pathology and specific compositional changes often varies according to anatomical site and nature of injury, there are characteristics of

TD consistently seen in tendon pathologies. These are: 1) disruption and degeneration of collagen fibers 2) changes in collagen and ECM composition 3) fibrotic scar tissue 4) hypercellularity and 5) neovascularization with neoinnervation [Khan 1999]. The presence and role of inflammatory cells/mediators in diseased tendons is discussed in sections 1.3-1.5.

1.2.1 Structural Changes

Diseased tendons have typically lost their glistening white appearance, hierarchical assembly, and thick collagen fibers: they have a grey/brown appearance, their fibers are thin, some are hemorrhagic, and their hierarchical structure is disrupted [Maffulli 1998].

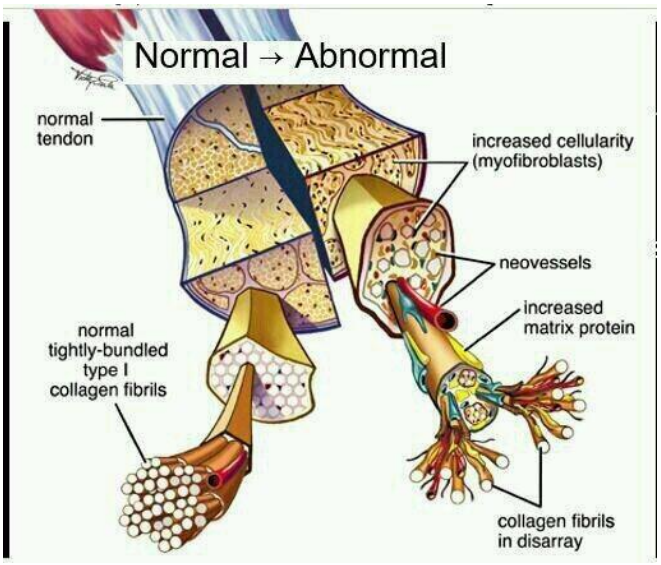


Figure 1.4 Changes in tendon structure and composition after chronic pathology [https://totally physio.wordpress.com/2014/08/22/evidence-based-review-on-achilles-tendinopathy-what-it-is-pathophysiology-diagnoses-and-management].

A reduction in crimp angle is typically seen, resulting from the stiffening of tendons, ECM compositional and organizational changes, and the infiltration of vessels/cells/substances [Kjaer 2004]. Formation of scar tissue through fibrosis, increased vascularity, and abnormal infiltration of mucoid patches, vacuoles, and lipids results in crowding and disarray within the tissue [Merkel 1982, Puddu 1976, Burry 1971, Burry 1973, Tuoheti Y 2005]. An increase in

collagen crosslinking is also present [Bank 1999]. These changes disrupt the integrity of the hierarchical structure on a macro scale, resulting in the abnormal morphology of tendinopathic tissue (Figure 1.4).

1.2.2 Compositional Changes

Total type I collagen content decreases in diseased tendon tissue as the ECM alters in composition, resulting in a pathological chondroplasia, where tissue that normally exhibits an elastic phenotype is replaced by fibrocartilage morphology with hyaline degeneration [Dean 2012]. The dense and essential type I collagen content decreases as types II, III, VI, and X increase in percentage [Gigante 2004, Panni 1996, Bank 1999, Joseph 2009, Attia 2012, Archambault 2007, Jelinsky 2008, Tomonaga 2000]. The alteration in collagen content is one of the key factors in the loss of organized fibrils seen in tendinopathic tissues. Matrix changes and increased levels of catabolic mediators, which are classic signs of a failed healing response, are seen throughout pathological tendon tissues and result in progressively weak scar tissue [Dean 2012]. ECM components with fibrocartilaginous properties, such as proteoglycans fibronectin, aggrecan, tenascin-C, and biglycan, are increased while others contributing to the fibroelastic phenotype of healthy tendon tissue are decreased, primarily elastin [Gigante 2004, Attia 2012, Tillander 2002, Archambault 2007, Jelinsky 2008].

1.2.3 Cellular Changes

The non-inflammatory cellular component of diseased tissue is well described: there is increased fibroblast cellularity, rounding of tenocytes from their elongated state, increased apoptosis, and chondrocyte-like cells [Archer 1993, Soslowsky 2000, Benson 2009, Tuoheti 2005, Uhthoff 1978]. Diseased tendon tissues have a large and pathologically active myofibroblast population that contributes to dysregulated ECM composition and collagen

disrepair [Verrecchia 2007]. These myofibroblasts are activated during wound healing and are derived from local tissue or recruited from circulating fibroblast-like stem cells [Bucala 1994, Kalluri 2003, Forbes 2004, Quan 2006].

Tenocytes in diseased tendons express mediators indicative of a tissue continually attempting but failing to heal. A recent systematic review on the histological and molecular changes in rotator cuff disease (RCD) showed that a number of cytokines released to promote survival and matrix were up regulated in rotator cuff disease (RCD) compared to healthy tissue [Dean 2012]. Some of these cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), hypoxia inducible factor 1-alpha (HIF-1α), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and prostaglandin-E^2 (PGE_2), as well as additional fibrotic, apoptotic, and neuronal mediators will be key plays in the upcoming discussion on the complex role of inflammation in the pathogenesis of TD. These are discussed in detail in sections 1.4-1.6.

1.2.4 Vascular Changes

Tendon neovascularization occurs in response to acute injuries to facilitate timely wound healing [Gelberman 1981, Kvist 1987, Astrom 1995]. The new blood vessels should form in parallel to the collagen fibrils to prevent disruption of its hierarchical structure. However, in diseased tendon tissue, after numerous injuries and micro-ruptures have occurred, this is typically not seen. Instead, vascularization is randomly oriented and blood vessels can even form perpendicular to collagen fibers [Williams 1986, Kalebo 1990, Merkel 1982, Nelen 1989, Burry 1971, Clancy 1976].

1.2.5 Neuronal Changes & Pain

During the initial inflammatory phase of tendon healing there is nerve ingrowth alongside neovascularization; the amount of innervation in tendinopathic tissue is significantly larger than

in healthy tissue [Alfredson 2003, Lian 2006, Tamai 2000, Xu 2011]. In addition to this innervation, the peripheral neuronal phenotype, the specific characteristics of nerves, neuronal mediators, and their receptors, is typically altered and become highly sensitized/up regulated [Dean 2013a]. Strong evidence exists for the up regulation of the glutaminergic system in human tendinopathy; substantial but not yet conclusive evidence has shown that sensory neuropeptides are up regulated as well [Lian 2006, Schubert 2005, Tamai 2000]. Although pain is a commonly encountered clinical problem for those with chronic tendon pathologies, the presence of histopathologically and radiologically abnormal tissue does not always result in pain [Cook 2004, Dean 2013b, Yamaguchi 2006]. Reasons for the inconsistency between neuronal changes, pathology, and pain for TD are poorly understood [Dean 2013b]. It has been suggested that the presence of pain in TD not only implicates neuronal innervation and sensitization but also requires that resident tissue cells react to these changes by activating nociceptive pathways to higher perception centers [Dean 2013b].

1.2.6 Mechanical Properties

Mechanical and material properties of diseased tendon tissue are weakened, resulting in loss of elastic properties, increased risk of rupture and re-injury. Due to the compositional accumulation of water and ground substances, the cross-sectional area of tendinopathic tissues is increased compared to healthy tendons [Ayra 2010]. Although an increased surface area typically implicates a higher mechanical strength, alterations in the tendon structure and composition result in a lower stiffness and Young's modulus than healthy tendons.

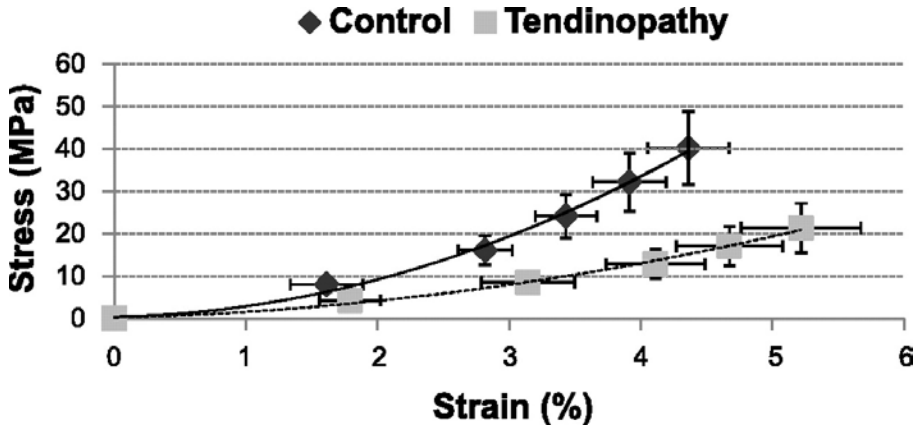


Figure 1.5 Differences in Stress/Strain curve between healthy and diseased tendons [Figure from Ayra 2010]. Diseased tendons experience higher strains at lower stress compared to control.

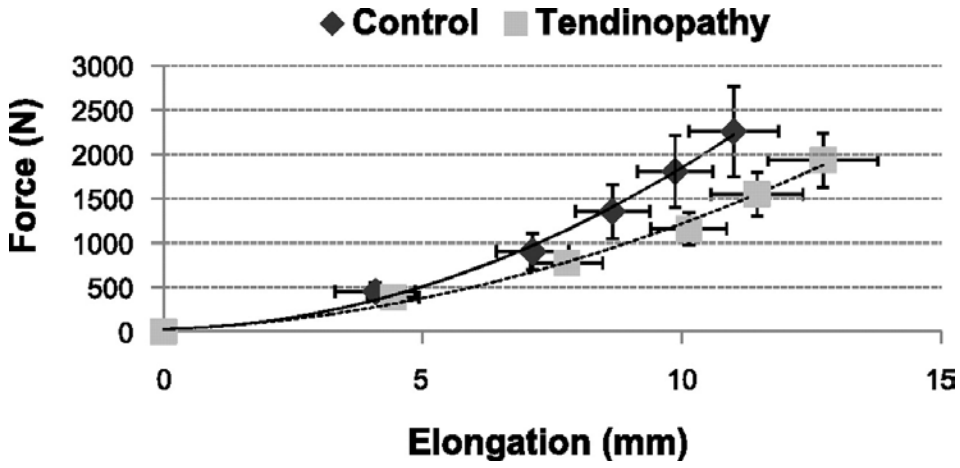


Figure 1.6 Differences in Force/Elongation curve between healthy and diseased tendons [Figure from Ayra 2010]. Less force is required to stretch a diseased tendon to the same length as a healthy one.

As evidenced in figures 1.5/1.6, less stress is required in tendinopathic tissue to achieve similar levels of strain in healthy tendons; tendinopathic tendons exhibit less resistance to elongation compared to healthy ones. It has been hypothesized that the lower stiffness in diseased tendons causes excess strain in muscle fibers, limiting their ability to function within an optimal region of the force-length curve [Ayra 2010]. Additionally, low tendon stiffness negatively impacts force transmission and leads to prolonged bouts of increased strain within the tissue. Consistently high strains within tendon tissue may increase risk of deleterious micro-

ruptures that can accumulate over time, predisposing the pathologic tissue to rupture and re-injury.

1.3 Paradigms of Tendon Disease

Throughout the last 10-15 years the prevailing pathological paradigm of tendon disease (TD) has been that it is a degenerative condition devoid of inflammation [Rees 2014]. This belief has shaped the use of treatments over time: more traditional anti-inflammatory treatments have become replaced with novel biologic ones (e.g. shock wave therapies, platelet-rich plasma, etc.). Unfortunately, there has been little to no observed efficacy for the majority of these newer treatments despite an improved understanding of tendon pathology and healing. The perception that TD is non-inflammatory in nature has not always been the case: the pathological paradigm surrounding inflammation and TD shifted throughout the late 20th into the early 21st century and is has recently entered a new era of re-assessment.

1.3.1 Terminology

Both acute injuries and chronic tendon diseases have been referred to using various terms over the years. The three most commonly used terms have been: 1) tendinitis/tendonitis 2) tendinosis and 3) tendinopathy. The use of these terms varies over time according to type of injury described, acute, chronic, or rupture, and the prevailing consensus on the role of inflammation (or lack thereof) in the condition being described [Rees 2014].

Tendinitis refers to an injury of a tendon, acute or chronic, accompanied by an inflammatory response [Dakin 2014]. Tendinosis refers to chronic degeneration of tendon tissue resulting from an accumulation of micro-trauma/ruptures over time devoid of inflammation. Tendinopathy refers to all disorders affecting tendons, acute, pathological, and

ruptures, that does not imply any underlying knowledge about pathological causes for the condition described.

For the purpose of this thesis we use the terms 'tendon disease' (TD) or 'diseased tendons' to specifically refer to the subset of tendinopathies that encompass non-acute and non-ruptured pathological tendons. Although using the term 'tendinopathy' will necessarily be used to accurately represent the conclusions of other scientists in the field, we will primarily use TD in an effort to prevent confusion on the specific entity we are evaluating: the causes and burden of chronically injured, often painful, non-ruptured pathological tendons.

1.3.2 Historical paradigm #1 – The 'tendinitis' era

Up to and around the mid-1990s, the pain associated with diseased tendons was widely believed to be due to inflammation – acute or chronic; these conditions were referred to as tendinitis [Khan 1998, Khan 1999, Khan 2002]. This was deeply entrenched in the medical literature and manifested by use of anti-inflammatory treatment modalities such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids [Rees 2006, Rees 2009].

1.3.3 Historical paradigm #2 – The 'tendinosis' era

During the late-1990s into the early 21st century, a sea change in the pathological paradigm occurred as histological data, showing absence of acute inflammatory cells in diseased tendons permeated the conversation among practitioners, scientists, and educators [Rees 2014]. In 1976, Puddu *et al* were the first to show a lack of acute inflammatory cells in the load-bearing regions of diseased tendons; they concluded:

> "The tendon does not represent the characteristics of a tissue predisposed to inflammatory processes. Inflammation can, on the contrary, easily take place in the peritendon sheath. In fact, the sheath is constituted by a connective tissue full of cells and vessels. These statements are confirmed by the histological observations in the tendon tissue and in the sheath. We therefore consider it precise to distinguish pure peritendinitis, peritendinitis with tendinosis, and tendinosis." [Puddu 1976]

After several other studies showed similar histological data in the tendon tissue (an absence of 'inflammatory cells') it became increasingly accepted that the term 'tendinitis' might have been misleading [Astrom 1995, Jozsa 1997, Khan 1999]. As the evidence mounted that an inflammatory infiltrate was seemingly absent in chronically painful tendons the pathological paradigm of tendinitis came to a head. Ultimately, the pathological explanation of TD being a 'tendinitis' was largely discredited following an editorial in the British Medical Journal that urged the medical and scientific community "to accept the irrefutable evidence the term tendinitis must be abandoned to highlight a new perspective on tendon disorders" [Khan 2002].

It has been argued that the stark change in pathological paradigm was not just driven by histological findings but was also a result of growing frustration over the lack of efficacy of traditional anti-inflammatories (e.g. non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids) and their sometimes deleterious consequences on tendon health [Rees 2014]. Additionally, it was increasingly apparent that TD was different in etiology from the classically inflammatory disease rheumatoid arthritis (RA). The sudden change in pathological paradigm away from tendinitis largely on the basis of an absence of infiltrating neutrophils in tendon tissue has arguably been an oversimplification of inflammation's possible role in TD [Rees 2014, Dean 2015]. The result may have been that novel inflammatory discoveries in TD did not garner much momentum within the medical literature.

1.3.4 Contemporary paradigm – The 'tendinopathy' era

Since the turn of the century various pathological paradigms for TD have been explored, the majority of which fall under the purview of non-inflammatory/degenerative hypotheses [Rees 2014]. The term tendinopathy has been popularized and widely used alongside or in place of 'tendinosis' to reinforce the idea that the underlying pathology of TD is multifactorial, not well

defined, and likely influenced by a combination of extrinsic, intrinsic, and environmental factors. Intrinsic factors include age, degeneration, systematic disease, and genetics; extrinsic factors include impingement, overuse, occupational injuries, and trauma [Lewis 2009].

Deleterious effects of these factors often accumulate, manifesting in both structural deformities and biological dysregulation of mechanisms for protection and repair. Various non-inflammatory models have been proposed over the years to explain the histological changes and biological dysregulation that fall into three general categories: 1) cumulative damage/vascular deficiency models explaining why tendons fail 2) models on why tendons fail to repair themselves – typically referred to as 'the failed healing response' and 3) continuum theories of TD [Rees 2006, Clancy 1989, Cook 2009, Rees 2014]. The continuum theory of TD suggests tendons progress through three major phases in response to injuries/micro trauma that result in the characteristic changes in pathological tendons. First, it is proposed that thickening of tendon tissue occurs in response to acute overload/trauma, second there is a failed healing response and disrepair of the ECM, and third there is degeneration and dysregulation of healing, resulting in major compositional changes and loss of function, predisposing the tendon to further injury and rupture [Cook 2009].

The prominence of degenerative hypotheses during this time coupled with the past failures of traditional anti-inflammatories in treating TD led to new therapies that did not focus on affecting inflammation directly. Beginning in the mid-1990s and throughout the early 2000s, these treatments broadly fit into 4 categories: 1) rehabilitation and physical exercises, including eccentric exercises (EE) 2) treatments for reducing the pain associated with tendon pathologies, such as sclerosant/high-volume injections 3) treatments using blood products to augment the healing process, such as Platelet-rich Plasma (PRP), and 4) other non-invasive therapies, such as

extracorporeal shock wave therapy (ESWT) and laser therapies [Rees 2014]. Unfortunately there has been little to no observed efficacy in patient outcomes from these novel treatments [Itoi 1992**;** Tanaka 2010].

1.4 Pathology of Tendon Disease: Key Players

As noted previously, TD is a complex multifactorial pathology that results from a variety of extrinsic, intrinsic and environmental factors. These factors manifest in the characteristic structural and histological changes of diseased tendons discussed in section 1.2. In this section we outline the key biological players believed to initiate and propagate those changes in diseased human tendons. Many of these factors are integral components of the inflammatory cascade themselves or are regulated by pro- and anti-inflammatory mediators. Reasons for the historical rejection or acceptance of a causal link between inflammation and TD are a primary topic of analysis for this thesis and will be introduced in section 1.5.

1.4.1 The Peripheral Neuronal Phenotype

The role of nerve cell phenotypes and neuronal mediators in initiating and propagating pain and disease in tendon tissues [Dean 2013a, Dean 2013b, Dean 2015a]. Sensory innervation and neuropeptide up regulation are vital components of the normal healing process and play important roles in tissue homeostasis [Ivie 2002]. Sensory nerves, the autonomic and glutaminergic systems regulate proliferative and remodeling phases of healing post-injury [Ackermann 2009]. Dysregulation of these systems may be associated with TD pathogenesis. Animal models have shown that sensory neuropeptide expression is associated with pain, failure of healing, and increased histological/structural characteristics of pathological tendons [Anderson 2011, Backmann 2011, Lui 2010]. Although it is not known exactly how these neuronal mediators influence the development of tendon pathologies, there is considerable

evidence that sensory and glutaminergic systems are up regulated in human tendinopathy, with or without pain [Lian 2006, Schubert 2005, Tamai 2000]. Interestingly, changes in neuronal phenotype and neuropeptide sensitization are not only found in nerve fibers/receptors of diseased tendons but are also found in resident tenocytes and epithelial cells [Andersson 2008, Schizas 2010].

Glutamate, a key metabolite and neurotransmitter for the transmission of pain, has been shown to be up regulated in various painful musculoskeletal pathologies; most recently glutamate and its receptors were associated with the resolution of shoulder pain in rotator cuff tendinopathy [Kreiner 2011, Rosendal 2004, Bleakman 2006, Newsholme 2003, Dean 2015]. Glutamate also plays a role in cellular metabolism and collagen synthesis, so its up regulation in tendinopathic tissue could also a contributor to pathological mechanisms [Fu 2010, Dean 2012].

Substance P and Calcitonin gene-related peptide (CGRP), mediators for the sensory system, have exhibited up regulation in diseased tendons [Lian 2006, Schubert 2005, Tamai 2000]. Substance P, a neurotransmitter/neuromodulator and first responder to physiological stresses, plays key roles in vasodilation, pain signalling, and neurogenic inflammation [Carlsson, Li 2007]. CGRP, an amino acid peptide, is also a potent vasodilator and pain neurotransmitter [Rosenfeld 1983]. It has been postulated that these mediators exert proliferative effects on tenocytes, causing them to adopt a myofibroblast-like phenotype [Fong 2013]. This change results in increased tenocyte expression of type III collagen mRNA over type I collagen mRNA, and may contribute to the formation of mechanically inferior and disorganized collagen fibers.

The consistent finding of altered expression of sensory peptides in diseased tendons suggests changes in the peripheral neuronal phenotype may be a key factor in both the initiation and/or propagation of the failed healing response in TD.

1.4.2 Hypoxia inducible factor 1 alpha (HIF-1α)

As mentioned in section 1.2.4, neovascularization and increased blood flow are commonly found in diseased tendons: neovascularization may be due to aberrant expression of hypoxia-dependent factors and may contribute to tendon pathology [Rees 2007]. Until recently it was difficult to establish which mechanisms initiated and propagated abnormal angiogenesis in injured tendon tissue, whether it was hypoxia-independent or hypoxia-dependent, and what effects it had on pathogenesis aside from disrupting structural assembly of fibers [Rees 2014, Millar 2013]. Hypoxia, a deficiency in the amount of oxygen available to cells, has been suggested to be important in the pathogenesis of TD since the mid-1990s but has remained controversial due to the seemingly paradoxical paradigm it implicates: pathological tendons have increased access to blood supply so it appears contradictory to conclude that a lack of oxygen contributes to pathogenesis [Kannus 1997].

However, studies in chronic inflammatory, autoimmune, and fibrotic diseases have shown that increased access to blood supply does not necessarily equate to an absence of hypoxia [Deng 2016]. Ongoing immune responses have been associated with increased oxygen consumption and subsequent anaerobic metabolism of cells within the inflammatory lesion [Kominsky 2010, Eltzschig 2011]. Localized tissue hypoxia from ongoing immune responses has been described as ‘inflammatory hypoxia’ and hypoxia inducible factor 1 (HIF-1) mediated metabolic shift has been implicated in facilitating immune and resident cells to adapt to these hypoxic conditions [Deng 2016]. The connection between HIF-1, inflammation, and hypoxia has

explained the hypoxia paradox in autoimmune disorders, suggesting proof of concept for analogous mechanisms in TD.

A drop in oxygen concentration has two major effects at the cellular level: 1) activation of the oxygen-sensitive transcription factor hypoxia inducible factor 1 (HIF-1) and 2) tendon cell necrosis, releasing cell contents into the surrounding tissue, sending an alarm signal about the abnormal biological environment [Millar 2013, Deng 2016]. Some released contents, including S100A9, high-mobility group box-1 (HMGB1) and interleukin-33 IL-33 (section 1.6), go on to activate Nuclear Factor-κβ (section 1.4.4), triggering various inflammatory responses, potentially preventing timely resolution and contributing to degeneration [Vallabhapurpu 2009]. Studies in chronic inflammatory, autoimmune, and fibrotic diseases have implicated HIF-1 in the initiation and propagation of angiogenesis, fibrosis, and non-resolving inflammation through inflammatory hypoxia; these concepts are explored below to explain findings/hypotheses of HIF-1 in TD [Deng 2016].

HIF-1, a heterodimeric transcription factor with subunits α and β, is largely inactive under normal oxygen concentrations (normoxia) and is activated in low-oxygen conditions where it facilitates adaption of tissue to the hypoxic environment [Semenza 2009]. HIF-1α is constantly degraded by the ubiquitin-proteasome system in the cytoplasm of most cell types, however, the degradation of HIF-1α ceases under hypoxic conditions. HIF-1β is constitutively present in the nucleus and activation HIF-1 depends on the oxygen-dependent regulation of the α subunit. Once stabilized, HIF-1α translocates to the nucleus where it binds to HIF-1β, forming the active HIF-1 transcription factor [Semenza 2009, Millar 2013]. Upon activation, HIF-1 upregulates expression of vascular endothelial growth factor (VEGF) and its associated receptors, stimulating the formation of new blood vessels (vasculogenesis) in addition to the

extension of preexisting ones (angiogenesis) [Peyssonnaux 2005]. HIF-1 also directly increases expression of pro-fibrotic mediators, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and plasminogen activator inhibitor-1 (PAI-1) [Rosmorduc 2010].

Under normal circumstances of acute-resolving injuries, HIF-1 activation promotes wound healing and return to normoxia; however, if inflammatory cells remain active and do not properly resolve inflammatory hypoxia can occur [Deng 2016]. In inflammatory hypoxia, HIF-1α is not only stabilized by the low-oxygen environment: induction by pro-inflammatory cytokines has also been shown to inhibit HIF-1α degradation, thus HIF-1 can remain active even if oxygen levels return to normal. These mechanisms have been implicated in the pathogenesis of autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis [Konisti 2012, Karhausen 2004, Rosenberger 2007, Distler 2004, Yao 2008].

Activated HIF-1 has important effects on immune cells that could implicate it in an inflammatory paradigm of TD, specifically that of non-resolving inflammation resulting in phenotypic alterations of resident tendon cells. Up regulation of HIF-1α protects myeloid cells from apoptosis and enables them to increase their activity and infiltration through glycolytic metabolism [Cramer 2003]. Hypoxia drives macrophages to a M1 phenotype, releasing classic inflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and inducible nitric oxide synthase (iNOS) [Sitkovsky 2004, Vanderkooi 1991, Roos 1973]. Increased presence of reactive oxygen species (ROS) via iNOS in inflammatory hypoxia leads to increased up regulation of NF-κβ activation pathways, which further promote HIF-1α stabilization and propagation of hypoxic inflammation [Hume 1978, Greiner 1994]. Neutrophils have increased survival under low oxygen conditions due to HIF-1α suppression of NF-κβ

signalling [Walmsley 2005]. HIF-1α deficient dendritic cells are less able to drive T-cell proliferation, specifically of the T_h2 response, which may prevent the necessarily balance of inflammatory mediators and factors needed for proper resolution (discussed further in 1.4.3-5) [Lederer 1999**,** Jantsch 2008, Bhandari 2013].

Since HIF-1 activation leads to increased expression of pro-fibrotic mediators, hypoxia has also been implicated in fibrosis through constitutive stabilization of HIF-1α [Cannito 2014]. Fibrosis may be stimulated by HIF-1 not only through direction transcriptional regulation of fibrotic mediators but also through potential connections of HIF-1 with transforming growth factor-β (TGF-β) signalling [Ruthenborg 2014, Sun 2009, Xu 2015, Lee 2012]. TGF-β signalling is important in various cellular functions including homeostasis, differentiation, and ECM regulation [Wynn 2008, Wu 2009]. Dysregulation of TGF-β signalling, a potentially protective mechanism against aberrant TNF-α expression, was most recently implicated in the pathogenesis of fibrosis in tendon disease [Goodier 2016]. HIF-1 may be a critical regulator of this process in TD but has yet to be elucidated.

The various factors up regulated by stabilization of HIF-1α leads to further activation of HIF-1, resulting in the physiological and pathological state of inflammatory hypoxia [Deng 2016]. HIF-1α is believed to propagate inflammation under these abnormal conditions but could also be a key player in altering myeloid and resident cell phenotype to react more intensely to stimulation in future injuries or stressful environments, predisposing tissue for pathological inflammation even in cases where inflammation resolves and oxygen-levels return to normal. The hypothesis that HIF-1 activity drives chronic inflammation is supported by recent animal and *in vitro* models of diminished HIF-1 activity: studies have shown that inhibition of HIF-1α in hypoxic tissues is strongly anti-inflammatory [Cramer 2003, Kong 2004]. In addition, acute

inflammatory episodes are significantly diminished in models of HIF-1α gene deletion: observed results are believed to result from an inadequate energy production via glycolytic mechanisms facilitated by HIF-1α in hypoxic conditions [Stikovsky 2005, Deng 2016]. HIF-1's role in altering cell phenotype has not been directly studied in tendon tissue, however, recent studies have shown that abnormal exposure to pro-inflammatory mediators, primes tenocytes and resident macrophages for future inflammatory episodes [Dakin 2015].

A hypoxia-dependent pathway via HIF-1 as critical to the onset and development of early tendinopathy has been previously described and further substantiated through models of early human tendon pathology [Barreto 2003, Millar 2012]. In these studies, significantly increased expression of HIF-1α, clusterin and B-cell lymphoma 2 (Bcl-2), regulators of apoptosis and clearance of cellular debris, were found in early tendon tissue when compared to matched torn samples and non-matched control samples. Additionally, hypoxic tenocytes had significantly increased expression of pro-inflammatory cytokines and an increased production of inelastic type III collagen. The vascular, fibrotic, and necrotic effects of a HIF-1 in tendon tissue exhibit its potential as a key initiator and propagator of TD. One limitation of these studies is the comparison of torn supraspinatus tendons, representing late-stage disease, to matched subscapularis tendons, representing early stage disease [Millar 2012]. This potentially confounds conclusions due to variation in type (rupture vs. non-ruptured) and timing of diseased tissue (early- , medium- , or late-stage) in the late disease samples. Additionally, it is potentially problematic to identify the matched subscapularis as 'tendinopathic' tissue as rupture in one tendon may not necessarily implicate diseased tissue in adjacent ones, especially across patients.

The role of HIF-1α stabilization in late-stage tendon disease (pre-rupture) and resolution of pain post-treatment have not yet been investigated and compared to control tissue of the same tendon: this is explored using a human supraspinatus tendon model in chapter 3 of this thesis.

1.4.3 Cyclooxygenases and Prostaglandins

Cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2), enzymes located within blood vessels, stomach and kidneys, are responsible for synthesizing various prostaglandins via prostaglandin E synthase (PGE synthase); increased expression of cyclooxygenase genes has been associated with tendon pathology [Sullo 2001, Zhang 2010, Khan 2005]. Prostaglandins are physiologically active lipids that play a wide range of roles in the regulation of inflammation, cell growth, and sensitization to pain. End products of the cyclooxygenase pathways implicated in tendon pathology are prostaglandin E1 (PGE_1) and prostaglandin E2 (PGE_2). PGE_1 and PGE_2 are secreted into the bloodstream by the endothelium upon up regulation of Cox pathways where they act directly as vasodilators, facilitating the inflammatory response. PGE_2 also plays an important role in the resolution of inflammation through the suppression of T-cell receptors and signalling [Wiemer 2011].

Studies on the Cox pathway in tendon disease models have shown that peritendinous administration of PGE_1 induces chronic tendon pathology [Sullo 2001]. The introduction of PGE_1 into healthy rat Achilles tendons produces an acute inflammatory reaction, which over time can result in pronounced degenerative changes consistent with chronically diseased tendons. Changes seen from over-administration of PGE_1 are 1) decreased collagen fiber diameter and irregular crimping patter 2) decreased overall collagen density 3) micro-tearing and 4) increased tenocyte cellularity and rounding.

Elevated levels of PGE_2 have been implicated in the classic histological changes seen in chronically diseased tendons [Zhang 2010, Khan 2005]. In animal models, injection of PGE_2 into tendon tissue caused increased hypercellularity, loss of normal tissue architecture, tendon disorganization, and degeneration when compared to control groups [Khan 2005]. These tendons also showed significant reduction in collagen fibril diameter and a more loosely organized fibril structure compared to controls.

Increased activity of the Cox cascade has also been shown *in vivo*: overuse animal models and human tissue studies show significant up regulation of Cox-1, Cox-2, PGE_1, and PGE_2 compared proteins to controls [Zhang 2010, Blaine 2005, Perry 2005, Shindle 2011, Voloshin 2005]. Studies have also correlated up regulation of these pathways with tendon stem cell differentiation into non-tenocyte cell types, including towards adipogenic and osteogenic lineages [Zhang 2010, Dean 2012].

1.4.4 Nuclear Factor- κβ and Interferons

Nuclear Factor-κβ (NF-κβ), a protein complex that acts as a transcription factor, controls cytokine production and cell survival. Interferons (IFNs), a group of signalling proteins released by resident tissue cells in response to stress and pathogens, are key pro-inflammatory modulators and downstream signalling activators [De Andrea 2002]. These pathways have been implicated in the development of many chronic inflammatory diseases, including arthritis, inflammatory bowel disease, sepsis, asthma, and atherosclerosis [Gilmore 2006, Gilmore 1999, Monaco 2004, De Andrea 2002, Cohen 2001]. Recently genes and proteins associated with up regulation of NF-κβ and IFN pathways have been associated with early to intermediate stage tendon disease [Dakin 2015].

Under normal circumstances, NF-κβ transcription factors remain inactive in the cytoplasm of host cells, suppressed by a host of inhibitors: the NF-κβ pathway is activated upon degradation of these inhibitors [Jacobs 1989]. Upon activation, NF-κβ enters the nucleus and modulates expression of its target genes [Nelson 2004]. NF-κβ is often referred to as a 'master regulator' of the immune response due to a host of downstream affects its activity has on cell proliferation, phenotype, signalling, and survival [Smith 2006]. Activation of NF-kβ in tendon tissue has shown to be accompanied with catabolic processes and up regulation of several downstream mediators and pro-inflammatory cytokines by human tenocytes [Buhrmann 2011]. These mediators, which include Cox-2, IL-1β, TNF-α, and various matrix metalloproteinases (MMPs), have direct effects on the structural and histological properties of tendon tissue, apoptosis, and inflammation [Archambault 2002, Tsuzaki 2003]. Pharmacological reduction of NF-κβ expression reduces the expression and degenerative effects of these downstream factors in tendon tissue, suggesting that NF-κβ may be a key therapeutic target due to its regulatory effects on downstream mediators [Buhrmann 2011].

Chronic inflammatory conditions previously associated with NF-κβ activation showed that feedback responses develop between circulating NF-κβ activated immune cells/mediators and resident tissue cells, altering tissue cell phenotypes and reprogramming their active functions [Vidal 2013, Vlahopoulos 2015]. If similar NF-κβ mechanisms exist in tendon tissue, its activity could be a key pathogenic actor in a non-resolving inflammatory paradigm of TD (discussed further in section 1.5.4) [Feldmann 2002, Wright 2009, Bowness 2011, Sherlock 2012]. Recent studies in human supraspinatus tendinopathy have begun to illuminate the connection of NF-κβ activation pathways in the pathogenesis of TD, but further studies are required to confirm and expand on these findings [Dakin 2015].

Interferon signalling has also been associated with the pathogenesis of chronic inflammatory/autoimmune diseases, including rheumatoid arthritis and systemic lupus erythematosus, through their role in the activation and modulation of inflammation [Tsoko 2000, Crispin 2010]. The IFN signalling family is broken into three categories, I, II, & III, according to their origin, mechanisms of activation, and specific activities [De Andrea 2002]. Once activated, IFNs upregulate a broad range of genes with antiviral, immunomodulatory, and antiproliferative functions [Pestka 1987, DeMaeyer 1988, Landolfo 1995]. Interaction of IFNs with their specific receptors activates signal transducer and activator of transcription (STAT) complexes, a family of transcription factors which regulate the expression of genes associated with immunity, apoptosis, proliferation, and differentiation [Lin 2004]. A balance between the activities of all types of IFNs is crucial to timely initiation and resolution of all inflammatory responses due to their pro- and anti-inflammatory cascading effects on downstream transcription factors [Ghosh 2006]. An imbalance between the effects of type I IFNs and type II IFNs is particularly important to autoimmune and inflammatory diseases.

IFNα is produced by peripheral leukocytes, IFNβ is produced by epithelial cells and fibroblasts: both stimulate natural killer cells and macrophages to elicit anti-viral and sterile inflammatory responses [De Andrea 2002, Choubey 2011]. Aberrant expression of type I IFNs has been associated with the development of autoimmune diseases in various animal models [Marshak-Rothstein 2006, Aringer 2004, Apostolidis 2011, Niewold 2011].

IFNγ is the only type II IFN and is treated separately because it is structurally unrelated to type I IFNs, it binds to a different receptors/STAT complexes, and is encoded at a different chromosomal location [Schroder 2004]. IFNγ is released by pro-inflammatory T-cells; its expression activates macrophages, lymphocytes, and endothelial cells: these activated cells then

increase secretion of cytokines and fibrotic mediators [Ghosh 2006]. Abnormal expression of IFNγ has been implicated in various chronic inflammatory and autoimmune disorders for sensitizing resident tissue cells and macrophages to inflammatory mediators and has most recently been implicated in early stage TD [Shroder 2004, Dakin 2015]. IFNγ has been shown to increase cellular sensitivity to apoptosis and promote apoptotic effects through sensitization to TNF-α, IL-1β [Xu 1998, Zheng 2002, Tsujimoto 1986]. Some studies have shown that therapeutic use of type I IFNs can inhibit sensitizing effects on resident tissue cells by inhibiting production of IFNγ and increasing production of anti-inflammatory cytokines, although this has not been explicitly examined in tendon pathology [Ghosh 2006].

IFNs have been postulated to have similar pathogenic consequences to NF-κβ activation: their dysregulation and/or abnormal activation upregulates downstream inflammatory mediators. The abnormal level of pro-inflammatory mediators affects the phenotype of tenocytes and immune cells, priming them for chronic inflammation [Dakin 2015]. In the case of IFNs, recent research suggests that decreased activity of type I IFNs and/or increased activity of IFNγ leads to dysfunction and aberrant expression of downstream mediators, resulting in histological and phenotypic changes of tendon tissue/cells [Ghosh 2006].

1.4.5 Cytokines

Cytokines, cell signalling molecules that stimulate biologic effects and cell movement towards site of injury, are key effectors in the initiation and propagation of all immune and inflammatory responses [Millar 2009]. Cytokines can be roughly divided into several groups based on their function, pro-inflammatory, anti-inflammatory, pro-fibrotic, and anti-fibrotic although some have several functions depending on tissue location and target cell [Berger 2000]. Cytokines, both pro- and anti-inflammatory, have been implicated in the regulation of matrix

turnover and their relative expression levels may have direct effects on the activity and phenotype of tenocytes in tendon pathology [Riley 2005, Qi 2006]. Chronic exposure to pro-inflammatory and fibrotic cytokines can alter the expression of tendon matrix genes (MMPs) and lead to dysregulation of extracellular matrix and collagen turnover [Sun 2008]. These pro-inflammatory/fibrotic cytokines are stress-induced effectors are have also been shown to activate proteolytic/apoptotic enzymes [Yuan 2002]. Interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor-necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) are associated with these effects and are consistently up regulated in TD [Dean 2012]. Studies have shown that injection of these effectors into tendon tissue results in characteristic histological signs of tendon pathology [Stone 1999, Tsuzaki and Guyton 2003, Lin 2006, Nakama 2006]. Cyclical strain in animal models up regulated expression of these factors, resulting in inferior mechanical and organizational tissue properties.

T-cells are a major source of cytokines and helper T-cells are especially important to cytokine production. Helper T-cells can be further divided into Type1 and Type2 helper T-cells (T_h1/T_h2) based on their effector and cytokine functions. T_h1 cells produce IFNγ and pro-inflammatory cytokines that and responsible for cell-mediated immunity and phagocyte-dependent inflammation [Romagnani 2000]. T_h2 cells produce various cytokines that evoke antibody responses that inhibit several functions of phagocytic cells and have an overall anti-inflammatory response. A well balanced regulation of help T-cells is vital to a proper immune response: excess T_h1 responses have been implicated in the pathogenesis of various autoimmune and chronic inflammatory disorders [Romagnani 2000, Berger 2000]. Up regulation of anti-inflammatory cytokines such as interleukin-10 (IL-10) can inhibit the pathogenic effects of pro-inflammatory/fibrotic ones [Torres-Silva 2015]. IL-10 has been shown to inhibit IL-1, IL-6, and

TNF-α, which in turn inhibits their activating effects on MMPs that have negative effects on tissue repair and ECM remodeling [Skutek 2001].

1.4.6 Heat Shock Proteins

Heat shock proteins (HSPs), intracellular stress proteins found in all cell types that are rapidly released following non-programed cell death, are important effectors of the immune system. The primary functions of HSPs are 1) protein folding 2) cell survival and protection against apoptosis and 3) maintaining tissue homeostasis through stress signalling and reconstruction of injured tissue [Yoshioka 2012, Bruey 2000, Srivastava 2002, Quintana 2005, Nicchitta 2003]. HSPs are constitutively expressed but are significantly up regulated in response to stressful stimuli, including environmental, physiological, and pathological stimuli [Jaattela 1999]. HSPs are released passively from necrotic cells and actively in response to these stimuli [Barreto 2003, Edington 1989]. HSP activation induces cytokine and chemokine release in addition to activation of natural killer cells, implicating them as primary danger signals for tissue stress in various pathologies and potential regulators of inflammation-mediated tendon pathology [Gao 2003, Millar 2012b]. Although there is a paucity of literature on the effects of HSPs in tendon diseases, studies from rheumatoid arthritis suggest that up regulation of HSPs cause significant up regulation in cytokines, growth factors and changes in extracellular matrix production through the differential activation of MMPs [Lindquist 1988, Tsan 2009, Srivastava 1994, Snyder 1992, Kluger 1997, Pan 2003, Jagodzinski 2006].

1.4.7 Matrix Metalloproteinases (Matrix Enzymes)

Matrix Metalloproteinases (MMPs) are potent proteolytic enzymes that, upon activation, play key roles in the degradation of connective tissue components and modification of extracellular matrix (ECM) composition [Del Buono 2011]. MMPs form a family of 24

proteolytic peptidases whose active forms degrade all components of the ECM [Birkedal-Hansen 1993, Brown 1997, Kahari 1997, Nagase 1999, Ravanti 2000, Vincenti 2001]. In healthy connective tissue resident cells control the activity of these proteases by producing endogenous inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs), which bind to MMPs and prevent excess ECM degeneration. The proper balance of MMP and TIMP activity allows for suitable collagen remodeling in response to natural degeneration over time; an improper balance of MMPs leads to collagen dysregulation and ECM composition alteration [Bond 2002, Gomez 1997]. Chronic dysregulation of MMP activity is believed to be a key pathogenic factor in the development of tendon diseases in addition to its role in similar inflammatory diseases [Dalton 1995, Brinckerhoff 1992, Enomoto 2003, Firestein 1992, Salminen 2002, Lin 2002, Testa 1994, Tetlow 2002, Yasuda 2003].

ECM turnover is a dynamic process of synthesis and degradation regulated in large part to the activity of MMPs and their inhibitors [Del Buono 2011]. The concentrations and activity levels of several MMPs are well documented in tendon tissues [Riley 2002. Riley 2004]. Up regulation of several MMPs has been consistently associated with degenerative changes seen in chronic tendon diseases [Magra 2005, Fong 2013, Dean 2013b]. Inflammatory cytokines have important roles in the activity of MMPs and TIMPs: IL-1 and TNF-α have been shown to enhance the activity and production of MMPs while TGF-β and IL-6 have been shown to increase the activity of TIMPs [Dayer 1994, Dayer 1997, Mauviel 1993, Wright 1991, Lotz 1991]. Substance P has also been implicated in regulating the expression of MMP and TIMPs in fibroblasts [Cury 2008]. A recent systematic review on the histological changes in rotator cuff disease (RCD) showed that there is up regulation of MMPs associated with degradation of type I collagen and other important ECM components (MMP-1, 2, 9, 13) and down regulation of

MMPs associated with maintenance and remodeling of healthy tendons (MMP-3) [Dean 2012]. There is also significant evidence that TIMPs associated with inhibiting excessive degeneration of ECM by MMP-2 (TIMP-1, 2, 30) are down regulated in RCD. In sum, dysregulation of MMPs and their tissue inhibitors in response to activation via pro-inflammatory cascades has been consistently linked to development of RCD and other chronic tendon diseases [Riley 2004]. Since their activity is integral to inflammatory activation pathways, it is plausible that a failure of resolution would contribute to MMP dysregulation and lead to increased ECM compositional changes.

1.5 Inflammation and its Role in Tendon Disease

As discussed in sections 1.3.2-1.3.4, the pathological paradigm and terms used to describe tendon pathologies have shifted considerably within the past three decades. These changes reflect dissonance within the scientific and clinical community about the role of inflammation in the pathogenesis of TD; the controversy surrounding inflammation in tendon disease may, in part, be due to a lack of consistency in defining the cells, mediators, and effectors that compose the 'inflammatory component' of TD. If consistency existed in the inflammatory cells and mediators included as part of the inflammatory component to TD, then the apparent discordance with more contemporary inflammatory paradigms of TD might be explained. This notion, that conclusions about the role of inflammation in TD are reflective of the cells and mediators assessed, has not previously been systematically evaluated.

In this section, recent findings on the presence and/or absence of leukocytes and inflammatory mediators in TD are described. Additionally, proposed models of normal and dysregulated healing in TD are presented.

1.5.1 Inflammatory Cells Present in Tendon Diseases

As Puddu *et al* first concluded that "[diseased tendons] do not represent the characteristics of tissue predisposed to inflammatory processes" and subsequent studies confirmed a lack of an inflammatory infiltrate in pathological tendon tissue, 'tendinitis' was increasingly seen as misleading [Puddu 1976, Astrom 1995, Jozsa 1997, Khan 1999, Rehg2012]. These descriptive histological studies often referred to the absence of acute inflammatory polymorphonuclear leukocytes (PMNs), primarily neutrophils, and concluded there was a complete lack of inflammatory cells within the tendon tissue [Dean 2015]. Recent studies in diseased tendon tissues have interrogated the 'inflammatory component' to TD more closely using cell markers for specific immune cell types and inflammatory cytokines. The most recent systematic review on the presence of inflammatory cells in human tendinopathic tissue illuminated that there are increased numbers of macrophages and mast cells in tendinopathic tissue versus controls [Dean 2015]. One study has shown an increased number of T-cells in diseased tissue while other studies have detected little to no change in T-cells between disease and control tissue [Kragsnaes 2014, Millar 2010, Scott 2008, Gotoh 1997]. There is an absence of published research documenting increased levels of PMNs/neutrophils in diseased tissue, confirming the discovery by Puddu *et al* [Matthews 2006]. Although PMNs are not present in diseased tendon tissues, this does not equate to an absence of chronic inflammation from other leukocytes [Dean 2015].

1.5.2 Inflammatory Mediators/Effectors Present in Tendon Diseases

There has only been one review to systematically assess the histological changes of tendon pathologies that also includes a critical assessment of cytokines in pathogenesis [Dean 2012]. The review, which assessed the histological changes of rotator cuff disease (RCD) in human and animal models, found that cytokines, peptides, and growth factors associated with

loss of matrix organization, fatty degeneration/infiltration, vascularity, chondroid metaplasia, and fibrosis were all up regulated in diseased tissue compared to controls. Cytokines and growth factors produced by tenocytes/macrophages in response to tissue strain that were up regulated in RCD included IL-1α, IL-1β, IL-6, VEGF, HIF-1α, TGF-β, and PG-E2 [Legerlotz 2011, Qi 2011, Yang 2005, Petersen 2004]. Other mediators and activators up regulated in RCD were COX-1, COX-2, NOS, TNF-α, and bFGF [Wang and Iso 2006]. Many of these mediators are increased during normal tendon healing and their up regulation in RCD is reflective of a tendon attempting to heal [Dean 2012].

1.5.3 Normal Tissue Healing

When a tendon injury, trauma, or micro-rupture occurs, blood vessel damage results in the aggregation of platelets, release of pro-inflammatory mediators, and recruitment of leukocytes to the damaged tissue [Hope 2007].

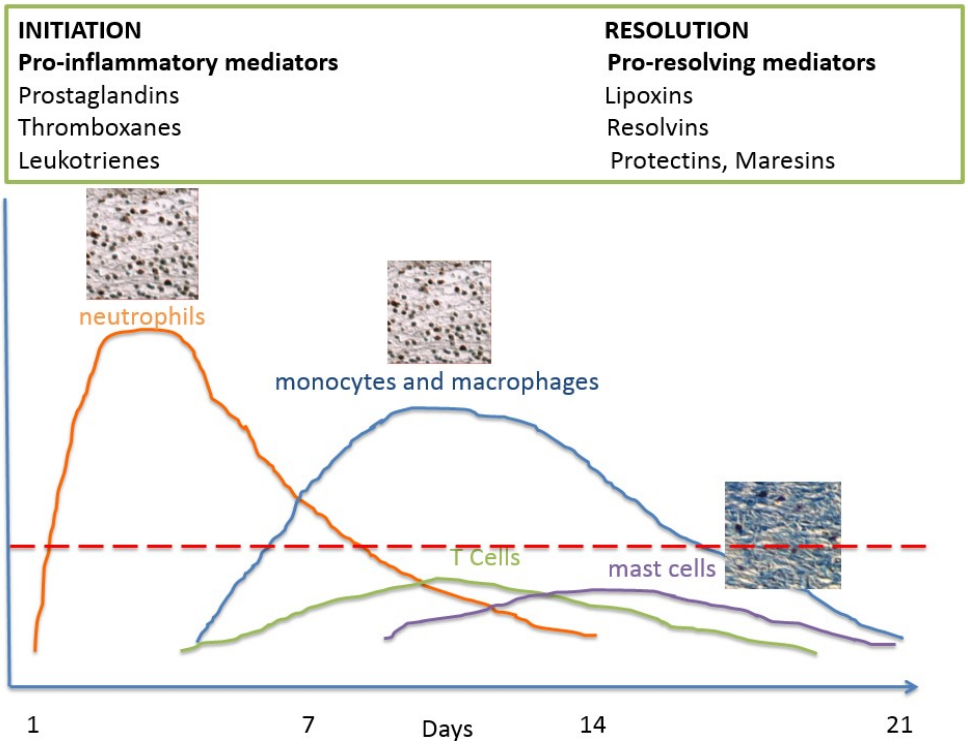


Figure 1.7 Cellular components to normal healing and inflammation [Adapted from Fredberg *et al*]

As illustrated in Figure 1.7, phagocytic neutrophils are the first leukocytes recruited to the site of injury; these neutrophils release additional cytokines that attract monocytes, which mature into inflammatory macrophages [Marsolais 2001, Sheran 2005]. The activated macrophages remove necrotic materials in addition to releasing cytokines and growth factors, which induce fibroblast proliferation and ECM formation [Fukasawa 1987, Saxby 2007]. The proliferating immune macrophages also affect the function of resident tissue macrophages, causing them to release additional growth factors and cytokines. During normal healing, immune cells will infiltrate, perform their functions, and leave the tissue within a few weeks of the onset of the injury.

Inflammation is considered 'resolved' when "granulocytes are eliminated and the tissue mononuclear cell population (macrophages and lymphocytes) returns to normal pre-inflammation numbers and phenotypes" [Gallin 1999, Serhan 2005]. Resolution is the result of a timely switch between the expression of arachidonic acid-derived prostaglandins and leukotrienes to lipoxins, resolvins, protectins, and maresins by infiltrating granulocytes [Serhan 2005]. Lipoxins initiate this termination sequence shortly after neutrophil cell numbers reach their maximum. If this switch away from pro-inflammatory to pro-resolving mediators occurs correctly an up regulation of cell messengers associated with leukocyte apoptosis results. After immune cells undergo apoptosis they are removed via phagocytes, which then leave the previously-inflamed tissue through lymphatics [Serhan 2007, Serhan 2008].

1.5.4 Proposed Model of Failed Healing/Resolution in Tendon Pathologies

Although the resolution of inflammation is initiated early in leukocyte recruitment, a timely end to healing is an active and highly regulated physiological process. Failure to regulate

this process has been implicated in many chronic inflammatory conditions. Similar mechanisms have recently been proposed for TD and may provide the bridge between degenerative and inflammatory theories [Serhan 2007**,** Serhan 2008]. In various chronic inflammatory/autoimmune pathologies the resolution sequence becomes dysregulated, leading to the persistence of activated leukocytes in the tissue, hyperplasia, and subsequent tissue destruction and fibrotic scarring via the various pathological mechanisms described in sections 1.4.1-1.4.7 [Akbar 1997]. This failure of acute inflammation to resolve has been shown to be, in large part, due to pathological fibroblast activation and subsequent dysregulation of inflammatory cytokines, chemokines, and prostaglandins [Buckley 2001]. Studies have consistently shown that fibroblasts taken from pathological arthritic and autoimmune tissues have a significantly altered phenotype compared to those taken from healthy tissue of the same type [Brouty-Boye 2000, Hogaboam 1998]. Although the mechanisms that lead to alteration of fibroblasts phenotypes remain somewhat speculative, there is substantial evidence that fibroblasts actively define immune cell behavior and tissue microenvironments [Buckley 2001].

The altered fibroblast phenotype found in arthritic and autoimmune diseases has been implicated in the consistent up regulation of pro-inflammatory cytokines and pathways including PG-E2, NF-κβ, Cox-2, IL-1(α/β) and TGF-β [Smith 1997, Zhang 1998, Zhou 1999]. These factors have been shown to determine the pattern and persistence of leukocytes in *in vivo* models and often result in degeneration through fibrosis.

Tenocyte and resident macrophage plasticity may play a similar role in tendon pathology: it has been proposed that alterations of these tendon stromal cells towards an NF-κβ or IFN activation profile could play a central role in regulating the switch from acute to persistent inflammation [Dakin 2015]. As described in section 1.4, the retention of inflammatory cells and

fibrotic stimuli would result in stark compositional and structural changes to tendon tissue, predisposing it to further injury. Models of tendon disease have shown that fibrotic scarring and degeneration is commonly associated with persistent inflammatory stimuli, production of cytokines, growth factors, and proteolytic enzymes known to be up regulated in TD [Wynn 2004, Dakin 2014].

The totality of evidence suggests that the pathological activation of tenocytes through these mechanisms, similar to fibroblasts in other inflammatory diseases, can transform an acute resolving immune response into chronic inflammatory reactions [Buckley 2007, Dakin 2014, Dakin 2015].

1.6 Alarmins and their Potential Roles in Pathogenesis of TD

Alarmins, endogenous molecules released upon tissue damage, stress, and infection, are key effectors in the activation of the immune system and homeostasis that may play important roles in the pathogenesis of TD [Millar 2013]. The term ‘alarmin,’ coined by J. Oppenhiem (Frederick, MD, USA), refers to an array of structurally diverse multifunctional host proteins with intracellular/intranuclear functions that also act extracellularly to mobilize/activate cells with host-defense and tissue repair functions [Harris 2006]. Studies from autoimmune/inflammatory conditions have implicated excessive release of alarmins with the failure of acute inflammatory resolution, resulting in dysregulated processes, pathogenesis, and degeneration [Chan 2012]. Understanding alarmin function and/or dysregulation in tendons may illuminate similar mechanisms in TD and could invoke novel therapeutic options.

This section includes a brief overview of alarmins, their roles in immune function, their intra- and extracellular functions, and their pathogenic and/or regenerative roles in

musculoskeletal diseases. Three alarmins: S100A9, interleukin-33 (IL-33), and high-mobility group box-1 (HMGB1) will be featured.

1.6.1 Alarmins: What do we know?

There is a growing list of possible alarmins in the literature. The most studied include: high-mobility group box-1 (HMGB1), the S100 protein family, some cytokines in the IL-1 family, and heat shock proteins (HSPs) [Chan 2012]. These molecules are found in a variety of organelles in all cell types including the nucleus as transcription factors (HMGB1/IL-33), exosomes as chaperones (HSPs), and the cytoplasm as calcium regulators (S100s) [Chen 2007, Millar 2013]. There is increasing evidence that activation of the immune system after tissue trauma, stress, or injury is in large part due to the interactions of alarmins released from necrotic cells with their associated receptors [Yoshioka 2012]. Findings that these molecules 'alarm' the immune system into action has resulted in the increased use of the term 'alarmin' over the original term endokine [Harris 2006]. The majority of alarmins are recognized by toll-like receptors (TLRs) on innate and acquired immunity cells: activation of these cells upregulates several signalling pathways, including NF-κβ, IFNs, and cyclooxygenases [Harris 2006]. As previously discussed, activation of these regulatory pathways (NF-κβ, IFNs, and Cox) can lead to degenerative changes within tendon tissue over time. Since alarmins are increasingly released in stressed tissue and their interactions with leukocyte receptors cause cascading effects on tissue morphology and resident cell phenotype, it is plausible that tendinopathy is an alarmin-mediated pathology [Millar 2013]. Understanding the role of alarmins in tendon disease may bridge the gap between inflammatory and degenerative paradigms if pathological mechanisms are similar to alarmin-mediated pathologies established in autoimmune and chronic inflammatory diseases.

There is a paucity of research on alarmins in tendon pathologies; what research exists is primarily limited to assessments of differences in alarmin expression between ruptured supraspinatus tendons and matched subscapularis tendons representing 'early-stage' pathology [Millar 2012a, Millar 2012b, Millar 2009]. To our knowledge, this thesis we will be first to assess the levels of alarmin expression in non-ruptured late-stage pathological tendons compared to control and treated tissue of the same tendon (supraspinatus). Additionally, these data are the first to examine if changes in expression of alarmins (HMGB1, S100A9 and IL-33) and HIF-1α correlate with resolution of pain post-treatment (subacromial decompression).

1.6.2 S100A9

The S100 superfamily is comprised of calcium-binding proteins that have multiple intracellular and extracellular activities, dysregulation of these functions has been implicated in various chronic inflammatory disorders [Donato 2001]. The intracellular roles of the S100 family include regulation of Ca^{2+} homeostasis, protein phosphorylation, enzyme activity, transcription factor activity, and cellular proliferation/differentiation. Extracellular S100 proteins modulate the activity of inflammatory cells; large quantities can cause cell death via apoptosis. S100A9 is one many S100 proteins that are typically found as a heterodimer: S100A9 associates with S100A8 and is the most abundant naturally occurring S100 complex [Edgeworth 1991, Teigelkamp 1991, Foell 2004, Hunter 1998]. The S100A8/S100A9 (S100A8/A9) heterodimer, previously referred to as calprotectin, was first thought to have primarily anti-microbial activities via PMN secretion but recently has been implicated as an initiator and propagator of inflammation in various pathologies through several leukocyte cell types [Dale 1983, Steinbakk 1990, Gebhardt 2006].

S100A8 and S100A9 originate in the cytosol of epithelial and myeloid cells, primarily phagocytes; their intracellular functions are calcium regulation and regulation of differentiation

through modulation of enzyme casein kinase I and II activity [Murao 1989, Lagasse 1988, Ehrchen 2009, Chan 2012]. S100A8 and S100A9 can be secreted into the extracellular space two ways: actively by epithelial and immune cells or passively by necrotic cells [Rammes 1997, Gebhardt 2006]. Active secretion occurs in response to increased Ca^{2+} cytoplasm levels in response to stress/injury or by induction via pro-inflammatory cytokines, primarily TNF-α and IL-1 [Yen 1997, Xu 2000, Roth 1993]. Once in the extracellular space, S100A8/A9 induce pro-inflammatory responses, neutrophil release from bone marrow/migration/adhesion and monocyte infiltration/differentiation [Foell 2004, Roth 2003].

In early stages of injury/inflammation PMNs expressing S100A8/A9 alarm circulating monocytes and phagocytes to differentiate and undergo phagocytosis, release proteolytic enzymes as well as arachidonic acid-derived eicosanoids (e.g. PGE_1/PGE_2) [Zwadlo 1988]. The increased levels of prostaglandins amplifies and perpetuates the acute inflammatory response. S100A8/A9 also has a direct effect on the expression of cytokines in both infiltrating and resident cells. S100A8/A9 interacts with its receptors toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE), leading to the production of TNF-α and other pro-inflammatory cytokines [Vogl 2007]. These interactions typically upregulate NF-κβ expression from resident and infiltrating cells, resulting in further downstream activation of cytokines and adhesion receptors [Roth 2003]. S100A8/9 expression in stressed tissues can result in a positive feedback mechanism if not properly resolved: the acute inflammatory responses activate downstream expression of TNF-α, a potent stimulator of S100A8/9 expression. Even if resolution occurs, continued insults to tissues can cause cell phenotype changes over time and predispose tissues to failed healing: S100A8/9 from myeloid cells promotes further leukocyte recruitment, priming myeloid cells for more intense inflammatory reactions in following injuries

[Roth 2003, Gebhardt 2006]. This has been shown as an essential part to the development of autoreactive CD8+ T cells in systemic immunity as well as other chronic inflammatory disorders [Loser 2010].

Several S100 proteins (S100A1, S100A2, S100A4, S1008 S100A9, S100A11 and S100B) are up regulated in diseased human articular cartilage; the S100A8/A9 complex has pathologic roles in other inflammatory conditions [Clos 2001]. Patients with rheumatoid arthritis have highly active phagocytes in joint tissues that express S100A8, S100A9, and S100A12 [Frosch 2000, Odink 1987, Frosch 2008]. These proteins have been shown to elicit catabolic signalling via RAGE in cartilage tissues, initiating and propagating degeneration and arthritis [Queitsch 2000]. Patients with late-stage arthritic diseases have altered activation and phenotypes of resident cells/macrophages: these cells, which do not express S100A8 or S100A9 under normal conditions, overexpress these proteins under inflammatory conditions. Secretion of S100A8/A9 has been associated with impaired healing/regeneration in muscle diseases and higher levels of the complex are found in the exudate of chronic non-healing wounds than in traumatic ones [Seeliger 2003, Eming 2010].

Strategies that target the levels of expression of S100 proteins may provide a novel approach to immunotherapy in chronic pathologies because decreased expression of these proteins could reduce the activity of NF-κβ, thus reducing downstream release of TNF-α and pro-inflammatory cytokines. Promising results for this theory have been demonstrated in animal studies of hypersensitivity and chronic bowel inflammation [Schmidt 2001]. Other studies have shown that blockage of the S100A8/A9 receptor RAGE restored effective healing and resolution in diabetic mice with overexpression of S100s [Goova 2010]. Additionally, direct blocking of

S100A8 and S100A9 suppressed endotoxin-induced pro-inflammatory activities by suppressing neutrophil migration [Vandal 2003].

Due to their significant role in the pathogenesis of chronic inflammatory disorders, including rheumatoid arthritis, cystic fibrosis atherosclerosis, and inflammatory muscle diseases, S100A8 and S100A9 may play a role in alarmin-mediated tendon pathology [Goebeler 1994, Roth 1992, Brun 1994, Millar 2013]. There is a paucity of literature on S100 proteins in tendon tissues, and there is no study that has evaluated expression of S100A8 and/or S100A9 during any stage of tendon pathology.

1.6.3 High-mobility group box-1 (HMGB1)

High mobility group box-1 (HMGB1), a nuclear protein whose intracellular role is to organize and regulate transcription of DNA, was first implicated as an endogenous signalling molecule by Wang *et al*, who showed that cultured macrophages secrete HMGB1 as a cytokine mediator of inflammation in response to endotoxin [Wang 1999, Chan 2012]. Over time HMGB1 has become categorized as an alarmin due to its involvement in mobilization and activation of immune cells in both infectious and sterile immune processes [Klune 2008]. Extracellular HMGB1 signals through various receptors to simulate immune cells and has been shown to be a key player in many acute and chronic inflammatory diseases [Chan 2012]. However, HMGB1 has also demonstrated significant regenerative potential in cardiac and musculoskeletal diseased tissue. It has been postulated that its pathogenic effects or therapeutic potential may hinge on environmental conditions and its interactions with specific receptors, although these mechanisms have not yet been elucidated. Although more should be learned about these mechanisms, there is considerable evidence from similar musculoskeletal diseases that

HMGB1 likely plays a role in pathogenesis, healing, and/or resolution of pain [Schierbeck 2011, Ostberg 2010, Pisetsky 2008, Andersson A 2008, De Mori 2007].

HMGB1 is a highly mobile nuclear protein that interacts with nucleosomes, histones, and transcription factors to regulate the transcription of DNA in all cell types [Chan 2012]. It is passively released by necrotic cells to signal neighboring cells to damage, sequestered during apoptosis, and secreted by activated immune cells to stimulate further proliferation and differentiation of immune and precursor cells [Yang 2004 Scaffidi 2002]. Stimuli for the secretion of HMGB1 from monocytes, macrophages, and dendritic cells include cellular stress, pathogen-associated molecular patterns (PAMPs), and cytokines, including TNF-α, IL-1, and IFNγ [Wang 1999, Youn 2006, Jiang 2007, Jiang 2006, Wang 2006]. Other studies have shown that HMGB1 can be actively secreted from resident tissue cells in response to these stimuli and sometimes during hypoxic conditions, including pituicytes, enterocytes, and hepatocytes [Wang/Vish 1999, Liu 2006, Tsung 2007].

The primary receptors for extracellular HMGB1 are RAGE, TLR2, and TLR4 [Hori 1995, Chan 2012]. Activation of RAGE by HMGB1 promotes chemotaxis and cytokine production through the up regulation NF-κβ pathways [Palumbo 2007, Park 2003]. Additional RAGE-dependent mechanisms are maturation/migration of immune cells and up regulation of their cell surface receptors [Messmer 2004, Dumitriu 2005, Telusma 2006, Yang 2007, Dumitriu 2007, Fiuza 2003, Dumitriu 2005, Telusma 2006, Yang 2007]. Binding of HMGB1 to TLR2/4 has also shown up regulation of NF-κβ and pro-inflammatory responses [Kokkola 2005, Park 2004]. However, some studies have shown that HMGB1 can anti-inflammatory effects if it is a part of a DNA complex, HMGB1-DNA, upon secretion [Klune 2008]. One such study showed that HMGB1-DNA complex interaction with another toll-like receptor, TLR9, led to suppression

of immune responses in some cell types [Popovic 2006]. Other studies have shown that this complex can also initiate pro-inflammatory responses through TLR9, although it is still unclear if this implicates a potential pathogenic or therapeutic role [Tian 2007, Ivanov 2007, Sha 2008].

The pro-inflammatory nature of extracellular HMGB1 has been shown to contribute to the pathogenesis of chronic inflammatory diseases [Klune 2008]. HMGB1 interactions have multiple cell-specific effects through receptor activation that leads to overexpression of signalling molecules and possible cell phenotype plasticity. Examples include: 1) monocytes stimulated by HMGB1 have increase adhesion capacity and upregulate cytokine expression 2) neutrophils stimulated by HMGB1 increase their sensitivity to RAGE-dependent mechanisms, resulting in increased recruitment of leukocytes 3) T-cells stimulated by HMGB1 show increased Th_1 polarization and upregulate proliferative and survival responses of other immune cells 4) dendritic cells stimulated by HMGB1 increase expression of their cell-surface markers and secretion of NF-κβ pathway dependent cytokines and 5) endothelial cells stimulated by HMGB1 release TNF-α and IL-8, which enhance the local inflammatory response [Messmer 2004, Rouhiainen 2004, Park 2003, Dumitriu 2005, Dumitriu 2007, Yang 2007, Fiuza 2003, Treutiger 2003, Kokkola 2005, Park 2004, Fan 2007].

These pro-inflammatory mechanisms, stimulated by HMGB1 release, have been implicated in both acute trauma and chronic inflammatory disorders [Chan 2012]. HMGB1 is significantly associated with autoimmune disorders: it is overexpressed in synovial tissue of patients with rheumatoid arthritis and animal models have shown that direct intra-articular injection of HMGB1 induces arthritis while injection of antagonists ameliorates arthritic conditions [Kokkola 2002, Taniguchi 2003, Kokkola 2003, Pullerits 2003]. Other conditions

have shown similar results including systemic lupus erythematosus and multiple sclerosis [Wittemann 1990, Ayer 1994, Andersson A 2008].

Not all HMGB1 expression leads to inflammatory pathogenesis: many studies have shown there are significant regenerative effects of HMGB1 [Bianchi 2007]. HMGB1 has shown to induce migration of stem cells towards inflamed regions, promoting repair and regeneration [Palumbo 2004]. Myoblasts migrating to stressed tissues to stimulate repair have been shown to be more effective through HMGB1-stimulated transit of these progenitors across the endothelial layer [Mitola 2006, De Mori 2007, Sorci 2004]. Exogenous HMGB1 was first shown to enhance wound healing through resident stem-cell stimulation in a murine model of heart disease: administration of HMGB1 led to increased functional outcomes after infarction [Limana 2005, Germani 2007, Limana 2011]. Further studies showed that overexpression of HMGB1 resulted in tissue damage protection and improved cardiac-function while HMGB1 antagonists exacerbated degeneration [Kitahara 2008, Oozawa 2008]. In smooth muscle, HMGB1 induces proliferation and positive cellular architecture changes in diseased tissue [Degryse 2001, Porto 2006]. In skeletal muscle, HMGB1 is associated with increased myogenesis/angiogenesis and functional outcomes [Palumbo 2007, Palumbo and SM 2004, De Mori 2007]. It is believed these restorative effects are mediated through the same receptors of HMGB1 in pro-inflammatory pathogenesis, RAGE, TLR2 and TLR4, although the mechanisms differentiating regeneration and pathogenesis are unknown [Mitola 2006, De Mori 2007, Sorci 2004, Degryse 2001, Chavakis 2007].

Interleukin 33 (IL-33)

IL-33 is a recently discovered member of the interleukin-1 family (IL-1): this family consists of 11 cytokines with important roles in the initiation, propagation, and suppression of innate immune responses [Dinarello 2009]. The majority of cytokines within the IL-1 family

have pleiotropic pro-inflammatory through extracellular binding of their target cells, primarily through toll-like receptors (TLRs) [Martin 2016]. In addition to orchestrating acute inflammatory responses, IL-1 cytokines shape adaptive immunity by affecting T- and B-cell differentiation. Dysregulation of some IL-1 cytokines has been implicated in systematic diseases, pathological changes in organs, and the development of chronic inflammatory conditions [Dinarello 2009]. IL-33 was first discovered as a cytokine that promotes T_h2 immunity, however, recent studies have shown that it contributes to T_h1 and CD8+ T-cell immune responses [Villarreal 2014, Yang 2011, Ngoi 2012, Bonilla 2012, Villarreal and Wise 2014]. Although overexpression of extracellular IL-33 has been implicated in various immune pathologies, its protective intracellular roles may be equally important for chronic inflammatory conditions.

A nuclear protein constitutively expressed inside endothelial cells, epithelial cells, fibroblasts, and smooth muscle cells IL-33's intracellular function is to regulate gene expression and interact with chromatin [Rider 2013, Martin 2016]. In normal conditions, nuclear IL-33 acts as a transcription factor whose activity results in downregulation of surface ST2 receptors and excreted pro-inflammatory IL-6 and IL-8 [Shao 2014, Shan 2015]. A reduction in TLR-ST2 reduces the cell's response to incoming pro-inflammatory cytokines that often promote activation of NF-κβ signalling pathways and inflammatory mediator secretion through ST2 [Miller 2011, Schmitz 2005, Funakoshi-Tago 2008]. Additionally, physical interaction of intracellular IL-33 with NF-κβ sequesters NF-κβ and curbs any incoming pro-inflammatory or fibrotic signals from other receptors [Ali 2011].

IL-33 is considered an alarmin because resident tissue cells release it upon tissue damage, stress or infection [Saluja 2015, Martin 2016]. IL-33 in mRNA and/or protein form is released into the extracellular space from resident cells in its full-length form and interacts with its

receptor ST2 on macrophages, mast cells, and dendritic cells [Chen 2007, Cayrol 2009]. These cells, particularly mast cells, process the bioactive full-length IL-33 into increasingly bioactive forms that have up to 30-fold increase in potency, which results in further expression of IL-33 [Lefrancais and Cayrol 2012, Hong 2011, Cayrol 2009, Ali 2010, Lüthi 2009]. IL-33 expression in tissues has been shown to stimulate IFNγ production under certain circumstances and its interaction with infiltrating mast cells ST2 receptors results in up regulation of NF-κβ signalling pathways. This means that low amounts of IL-33 can have large impacts on innate immune responses in tissues has been implicated in exacerbating acute inflammatory responses into chronic non-resolving ones in rheumatoid arthritis and intestinal diseases [Cascao 2010, Fournier 2012**]**

Although extracellular expression of IL-33 can lead to non-resolving inflammatory cascades via NF-κβ and IFNγ, this appears to be dependent on unexplained environmental conditions [Martin 2016]. Different tissue sites and environmental conditions have implicated extracellular IL-33 as protective and key to regenerative responses, although these mechanisms are largely unknown. Prolonged exposure to IL-33 is protective in atherosclerosis and has been shown to facilitate production of protective T_h1 and CD8 T-cells in tumor cells [Villarreal 2015**,** Theoharides 2015]. Studies have shown that injection of IL-33 in mice induces production of T_h2 cytokines, including IL-4, IL-5 and IL-13, and polarizes macrophages towards an anti-inflammatory phenotype [Schmitz 2005, Fu 2016]. Additionally, knockout mice for IL-33 have shown diminished T_h2 cytokine production under inflammatory conditions and can result in failure of resolution [Townsend 2000, Louten 2011]. This means that some chronic non-resolving pathologies could theoretically be augmented with IL-33 injection, although functional tests have not been performed in conditions where M2 polarization is seen. The pleiotropic

nature of IL-33 may also complicate such studies and potential treatments depending on the disease context.

Isoforms of IL-33 have been identified through alternative splicing of IL-33 mRNA and may explain the varied effects of its activity on immunity and disease progression [Tsuda 2012, Lefrancais 2012, Hong 2011]. Although little is known about the biological differences and mechanisms of action among these isoforms, other cytokines, including IL-2/4/6/7/10, have antagonistic and agonistic isoforms similar to the recently discovered IL-33 ones and have explained some of their variable effects on tissue homeostasis [Vudattu 2009, Whitaker 2011]. Very little is known about IL-33 expression in tendon tissue, however, there are emerging pathogenic and protective roles of fibroblast-derived IL-33 in inflammatory and fibrotic disorders [Xu 2008, Zaiss 2011, Rankin 2010].

1.7 Summary

The pathology of tendon disease (TD) exhibits multiple histologic structural and molecular changes including 1) disruption and degeneration of collagen fibers 2) changes in collagen and ECM composition 3) fibrotic scar tissue 4) hypercellularity and 5) neovascularization with neo-innervation [Khan 1999]. Despite initial enthusiasm for a significant inflammatory component in the pathogenesis of TD, the medical paradigm of TD shifted during the past 10-15 years to that of a degenerative condition devoid of an inflammatory component [Rees 2014]. This construct redirected clinical treatments away from traditional anti-inflammatory NSAIDs and corticosteroids. Despite an improved understanding of tendon pathology and healing, there has been little to no efficacy documented with these novel treatments. Recently, more thorough interrogations of inflammation's role in TD have paved the

way to consider it as a fibrotic disorder caused by the failure of inflammation to resolve [Millar 2012a, Millar 2012b, Dean 2012, Dean 2015, Dakin 2014, Dakin 2015, Goodier 2016].

TD is a complex multifactorial pathology that results from a variety of extrinsic, intrinsic and environmental factors. These factors include but are not limited to: 1) the peripheral neuronal phenotype 2) inflammatory hypoxia 3) cyclooxygenases and prostaglandins 4) cytokines 5) heat shock proteins and 6) matrix metalloproteinases. Many of these pathogenic factors are integral components of the inflammatory cascade or are activated/propagated by pro- and anti-inflammatory mediators. The resurgence of interest in inflammation in initiating and propagating TD may reflect a greater appreciation for the types of cells, mediators, and effectors that are considered inflammatory components [Dean 2015].

1.8 Objectives and Hypotheses

The overarching goal of this thesis was to examine the role of inflammation in the pathogenesis of TD. The *specific aims* of the research conducted were to:

1) Examine paradigm shifts and potential explanations for scientific equipoise regarding the role of inflammation in TD through a quantitative analysis of published reviews.
2) Determine the protein expression of alarmins in persistently painful diseased human supraspinatus tendon tissues compared to healthy tissue and treated pain-free supraspinatus tendon tissues.

It was hypothesized that:

1) There is an increasing amount and proportion of literature supporting an inflammatory component to TD and that this is likely due to increased

interrogation of monocytes, macrophages lymphocytes, and inflammatory/fibrotic mediators in pathology

2) Tendinopathy is an alarmin-mediated pathology

Results from the two projects are synthesized to help elucidate the role of inflammation in the pathogenesis of TD. These data provide insight into an emerging scientific theory that TD results from failure of inflammation to resolve, resulting in chronic inflammatory and fibrotic stimuli. The totality of evidence suggests that the pathological activation of tenocytes through alarmin-mediated mechanisms, similar to fibroblasts in other inflammatory diseases, can transform an acute resolving immune response into chronic inflammation [Buckley 2007, Dakin 2014, Dakin 2015]. If inflammatory signals are further implicated in TD through an alarmin-mediated pathology, it could arguably help to resolve the ongoing debate about the role of inflammation in TD by integrating immune cells, activation pathways, and mediators into known models of degeneration and disrepair. These findings may also provide future direction for therapeutic targets for the prevention and treatment of TD.

Chapter 2: Trends in the Theory that Inflammation Plays a Causal Role in Tendon Disease: A Systematic Review and Analysis of Published Mechanistic Reviews

2.1 Overview

2.1.1 Background

The role of inflammation in the pathogenesis of tendon disease (TD) has been a topic of debate since the mid-late 20th century. Prior to the turn of the 21st century, inflammation was believed to contribute to the pain and pathology associated with non-acute, non-ruptured diseased tendons [Khan 1998, Khan 1999, Khan 2002]. This is often referred to as the 'tendinitis era;' treatments used for TD during this time were traditional anti-inflammatories, such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids [Weiler 1992]. However, as anti-inflammatory regimens for tendon dysfunction were shown to be ineffective and histological studies of diseased tendons indicated an absence of 'inflammatory cells,' the term 'tendinitis' and its associated inflammatory paradigm were increasingly considered misleading [Puddu 1976, Astrom 1995, Jozsa 1997, Khan 1999, Khan 2002]. The orthopaedic community shifted their attention towards other explanatory paradigms and novel treatment modalities. As a result, TD has been predominantly characterized as a degenerative condition devoid of inflammation since the early 2000s [Astrom 1995, Movin 1997, Hashimoto 2003, Rees 2006, Rees 2014].

The abrupt shift change in disease paradigms may have been due to an oversimplification or acceptance of inflammation's role in TD, thus impeding the discovery of novel inflammatory mechanisms in TD [Rees 2014, Dean 2012, Dean 2015].

2.1.2 Purpose

A systematic review was conducted for published reviews whose primary purpose was to examine the pathogenesis of TD. The purpose of this review was to investigate paradigm shifts

and potential explanations for scientific equipoise regarding the role of inflammation in TD among these publications.

2.1.3 Definition of terms used

- ‘Mechanistic’ reviews: any review whose primary purpose was to discuss the pathogenesis of TD, potential causes or risk factors for TD, pathways associated with the development of TD, histological characteristics of diseased tendon tissue, or reasons for the failure of healing in diseased tendons. All reviews included in this study were determined to be mechanistic reviews.

Terms used to categorize mechanistic reviews based on their research question/specificity:

- Narrative-style review: any mechanistic review whose primary goal was to discuss and/or evaluate the pathogenesis of TD on a broad scale without an explicitly stated or implicit focus. (For example: “The pathogenesis of tendinopathy: an overview” vs. “The pathogenesis of tendinopathy: the role of metalloproteinases”).
- Specific review: any mechanistic review whose primary goal was to discuss and/or evaluate a specific subject related to the pathogenesis of TD.

Terms used to categorize reviews based on the evidence used

- Expert opinion: any review that formed its conclusions without explicitly referencing, overviewing, and presenting the findings of all relevant literature.
- Critical review: any review that explicitly analyzed the relevant literature (according to its research question and/or each topic of discussion) in depth by referencing, overviewing, and presenting the findings of all relevant literature. The relevant data or conclusions from the relevant literature must have been be an integral part of forming the review’s conclusions.

Other terms:

- Systematic review: any review that performed a systematic review and analysis of the literature relevant to their research question.
- Quality score (QS): the total score a review received in its methodological rigor assessment (Appendix B). The maximum score a review could receive was 13 points, minimum of 0

2.1.4 Specific objectives and hypotheses

Equipoise in the literature on the role of inflammation in TD may have been due to specific factors, including inconsistent definitions of inflammatory components, the quality of the review, and a lack of exposure and/or impact of high-rigor reviews supporting an inflammatory paradigm. Therefore, our specific objectives were:

1) To assess trends and paradigm shifts prior to 2015 about the perception of inflammation in TD. Search criteria were not limited by the year of publication.
    - We hypothesized that there has been an increase in the overall number of reviews on the pathogenesis of TD since 1989, the earliest date of included reviews, with an increasing percentage supporting an inflammatory component.
2) To evaluate if the adoption and/or rejection of a causal theory between inflammation and TD varied as a function of the characterization of the "inflammatory component" (what immune cell types, mechanisms, and/or molecular mediators are included in discussions)
    - We hypothesized that reviews only assessing neutrophils would be more likely to refute an inflammatory paradigm than reviews that also included monocytes, macrophages, lymphocytes, signalling molecules, or growth factors.

3) To evaluate if the adoption and/or rejection of a causal theory between inflammation and TD varied as a function of quality indicators (quality score, expert opinion vs. critical analysis, and systematic vs. non-systematic).
    - We hypothesized that reviews with higher quality score, that were critical, or were systematic would be more likely to support an inflammatory paradigm.
4) To examine other correlates of an article's conclusion about the role of inflammation in TD, including the type of journal the review was published in, the journal's impact factor, and the number of citations per year the review has received.
    - We hypothesized that a correlation exists between conclusions about the role of inflammation and other characteristics of review articles such as the level of impact and specialty orientation of the journal where review articles were published.

2.1.5 Rationale/novelty

Previous literature that has evaluated the role of inflammation in TD has consisted of original research, systematic and qualitative reviews. This is the first body of work to systematically evaluate published reviews' conclusions about the role of inflammation in TD and evaluate potential correlates of those conclusions through comprehensive data collection and statistical analysis.

2.1.6 Potential significance

The results of this research may illuminate inconsistency in terminology and scientific conclusions over time and by quality, potentially elucidating reasons for the lack of consensus among basic scientists about the role of inflammation in TD. This research may provide evidence useful for the orthopaedic community to standardize the 'inflammatory component' to TD.

Standardizing evaluations and discussions of inflammation's role in TD among basic scientists could speed the comparison, discussion, and dissemination of novel inflammatory findings in TD among all scientists and lead to faster development of effective therapeutic strategies to reduce tendon disabilities.

2.2 Methods

This systematic review used the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement and the Cochrane handbook as guidelines in the development of study protocol and the report of the study [Cochrane Handbook for Systematic Reviews of Interventions, Liberati 2009]. The inclusion criteria, data items, and methods of analysis were specified prior to the systematic search. Multiple pilot studies were used to generate, test, and refine relevant data items and collection protocol for the final systematic review. Inclusion/exclusion criteria, data items, data collection protocols, and methods of analyses were specified in a written protocol prior to the systematic search.

2.2.1 Information sources and search strategies

A systematic review was performed on the 15th of December using Medline, Embase, Cochrane, CINAHL, & SPORTDiscus databases for published literature on related to the pathogenesis of tendon disease. The search strategy was tailored for each database using appropriate search fields, filters, and Medical Subject Headings (MeSH) to provide the best balance of sensitivity and specificity. Detailed search strategies for each database are included in table 2.1. All search strategies included the terms: 'tendinitis,' OR 'tendinosis,' OR 'tendinopathy,' OR 'tendon disease / rupture / injury / rupture / tear' etc. Publications were limited to reviews in the English language where possible. This search did not include non-

indexed reviews, dissertations, etc. After abstract and full text screening, citation lists of included studies were manually searched to identify additional reviews for screening.

Table 2.1 Search strategies for each database. All searches were performed on December 15, 2015

Database	**Technique**	**Search strategy**
MEDLINE(R) 1946 to November Week 3 2015 via OVID (https://ovidsp.ovid.com/)	McMaster best balance of sensitivity and specificity review filter	1. Tendinopathy/ 2. tendinopath$.ti,ab. 3. tendinitis.ti,ab. 4. tendinosis.ti,ab. 5. (tendon adj (disease$ or injur$ or rupture or tear)).ti,ab. 6. OR/1-5 7. meta analysis.mp,pt. 8. review.pt. 9. search:.tw. 10. OR/7-9 11. 6 and 10 12. limit 11 to english language
Embase 1974 to 2015 Week 50 via OVID (https://ovidsp.ovid.com/)	McMaster best balance of sensitivity and specificity review filter	1. Tendon Disease/ 2. tendinopath$.ti,ab. 3. tendinitis.ti,ab. 4. tendinosis.ti,ab. 5. (tendon adj (disease$ or injur$ or rupture or tear)).ti,ab. 6. OR/1-5 7. meta-analys:.mp. 8. search:.tw. 9. review.pt. 10. OR/7–9 11. 6 AND 10 12. limit 11 to english language
Cochrane Library via Wiley (http://onlinelibrary.wiley.com/cochranelibrary/search/)	n/a	1. [mh Tendinopathy] in Cochrane Reviews (Reviews and Protocols) and Other Reviews 2. [mh "Tendon Injuries"] in Cochrane Reviews (Reviews and Protocols) and Other Reviews 3. tendinopath*:ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 4. tendinitis:ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 5. tendinosis:ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 6. "tendon disease":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 7. "tendon diseases":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 8. "tendon injury":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 9. "tendon injuries":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 10. "tendon rupture":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews 11. "tendon tear":ti,ab,kw in Cochrane Reviews (Reviews and Protocols) and Other Reviews

		12. #1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11
CINAHL via EBSCOHost (https://www.ebscohost.com/)	CINAHL SIGN Filter for systematic reviews	1. MH "Tendinopathy+" 2. MH "Tendon Injuries" 3. MH "Achilles Tendon Rupture" 4. MH "Tendon Injuries, Finger" 5. TI tendinopath* OR AB tendinopath* 6. TI tendinitis OR AB tendinitis 7. TI tendinosis OR AB tendinosis 8. TI (tendon N1 disease*) OR AB (tendon N1 disease*) 9. TI (tendon N1 injur*) OR AB (tendon N1 injur*) 10. TI (tendon N1 rupture) OR AB (tendon N1 rupture) 11. TI (tendon N1 tear) OR AB (tendon N1 tear) 12. S1 OR S2 OR S3 OR S4 OR S5 OR S6 OR S7 OR S8 OR S9 OR S10 OR S11 13. MH "Meta analysis" 14. TX (meta N1 analys*) 15. TX metaanaly* 16. MH "Literature review+" 17. TX (systematic N1 review) 18. TX (systematic N1 overview) 19. S13 OR S14 OR S15 OR S16 OR S17 OR S18 20. PT Commentary 21. PT Letter 22. PT Editorial 23. MH Animals 24. S20 OR S21 OR S22 OR S23 25. S19 NOT S24
SPORTDiscus via EBSCOHost (https://www.ebscohost.com/)	n/a	1. DE "TENDINOSIS" 2. DE "Tendinitis" 3. DE "Achilles tendinitis" 4. DE "TENDONS -- Wounds & injuries" 5. DE "ACHILLES tendon -- Wounds & injuries" 6. TI tendinopath* OR AB tendinopath* 7. TI tendinitis OR AB tendinitis 8. TI tendinosis OR AB tendinosis 9. TI (tendon N1 disease*) OR AB (tendon N1 disease*) 10. TI (tendon N1 injur*) OR AB (tendon N1 injur*) 11. TI (tendon N1 rupture) OR AB (tendon N1 rupture) 12. TI (tendon N1 tear) OR AB (tendon N1 tear) 13. S1 OR S2 OR S3 OR S4 OR S5 OR S6 OR S7 OR S8 OR S9 OR S10 OR S11 OR S12 14. KW Review 15. TX (systematic N1 review) 16. TX (systematic N1 overview) 17. TX (meta N1 analy*) 18. TX metaanaly* 19. ZU "meta-analysis" 20. ZU "literature reviews" 21. ZU "reviews" 22. ZU "systematic reviews (medical research)" 23. S14 OR S15 OR S16 OR S17 OR S18 OR S19 OR S20 OR S21 OR S22 24. S13 AND S23

2.2.2 Screening, inclusion, and exclusion criteria

Once the systematic search was completed, results for each database were compiled into Endnote7 for title and abstract screening. Duplicate references were removed using the 'Find Duplicates' function and manually by a single observer (Michael Mosca). Two reviewers (MM and Mustafa Rashid) independently screened titles and abstracts for inclusion/exclusion criteria. There were no unresolved disputes after abstract screening. Publications were included in our study/analysis if they met the following criteria:

- The primary purpose of the publication was to discuss the pathogenesis of TD, including potential causes or risk factors for TD, pathways associated with the development of TD, histological characteristics of tendinopathic tissue, or reasons for the failure of healing in diseased tendons.
- The publication was a review and not an original research paper
- The publication was in the English language

Publications were excluded from the study during abstract screening if:

- The publication was focused on non-pathological, acute injuries
- The publication was focused on complete ruptures/tears and not in-tact, diseased, tendon tissues
- The publication was a case study and/or series
- The publication did not have a clear intent to focus its discussion on the pathogenesis, causes, risk factors or characteristics of diseased tendons, but is rather a brief overview of TD (e.g. "what's new in tendinopathy")

Publications were excluded from the study during full-text screening if:

- The full-text could not be located

- The publication was not substantially engaging with pathogenesis (e.g. < 2 pages dedicated to discussion)
- The publication was not explicitly discussing non-acute, non-ruptured, pathological tendon tissue
- The publication lumped other musculoskeletal diseases (e.g. ligament and/or rheumatoid diseases) into the discussions and/or conclusions without a primary focus on TD.

2.2.3 Data items, collection process, and assessment of reproducibility

After abstracts and full texts were independently screened for inclusion/exclusion criteria, classification and quality data were extracted from all included reviews and documented in an excel spreadsheet. 21 data items were collected for each review, including:

- 1 categorical data item on the review's conclusions about the role of inflammation in TD
- 2 categorical data items on the types of immune cells/mediators discussed in the review to form its conclusions about inflammation
- 1 categorical data item assessing how inflammatory mediators and activation pathways were defined/discussed in regards to any 'inflammatory component' to TD (e.g. separately from the umbrella of inflammation or discussed as an integral part of inflammation's role)
- 2 categorical data items on the specificity of the review and the type of journal it was published in
- 3 numerical/temporal data items: the impact factor of the journal, the number of citations the review has received, and the year of publication

- A 12 question assessment on the methodological quality/depth of scientific rigor of each review, adjusted from the validated quality of assessment tool for systematic reviews, "Assessment of Multiple Systematic Reviews" (AMSTAR) to evaluate reviews of a wide range of quality [Shea 2007]. AMSTAR was modified to assess more robust range of reviews. AMSTAR was designed to assess only systematic reviews; this analysis included both systematic and non-systematic reviews. A detailed list of the questions and changes from the original can be found in Appendix B.

For each categorical data item, a list of responses was developed, piloted, and internally corroborated to provide an appropriate balance between specificity and generalizability. All data items were collected for included reviews using standardized extraction sheets (**Appendix A**: classification data items extraction sheet; **Appendix B**: methodological quality/depth of scientific rigor extraction sheet). During final data extraction, extraction sheets were printed and responses where determined and documented. Once data collection was complete, data were transferred from the physical extraction sheets into Microsoft Excel. Categorical variable responses were recorded in Excel using the characters (0, 1, 2, 3, 4, X, or P), each associated with a pre-defined response or category (Appendix A/B). Numeric values were copied into excel as variables for analyses. For any given question, if it was unclear what response to choose the letter "P" was written and input into Excel. Throughout data collection, any question marked "P" would be discussed by an expert panel of scientists and/or orthopedic surgeons, Dr. Stephanie Dakin (SD), Dr. Sarah Snelling (SS), and Professor Andrew Carr (AC), to determine the most appropriate response. Additionally, if no data exists for a given question (e.g. a journal without an impact factor) a "*" was input into excel, indicating missing data.

All data were collected by MM. Additionally, all data items were independently extracted by SD and SS from a random sample of included reviews (n = 10). Results from the random sample were compared among MM, SD, and SS to assess reproducibility of all outcomes. Any outcomes without sufficient reproducibility were excluded from the analysis in this review. A full report of the reproducibility sample is detailed in **Appendix C**.

2.2.4 Synthesis of results and statistical analyses

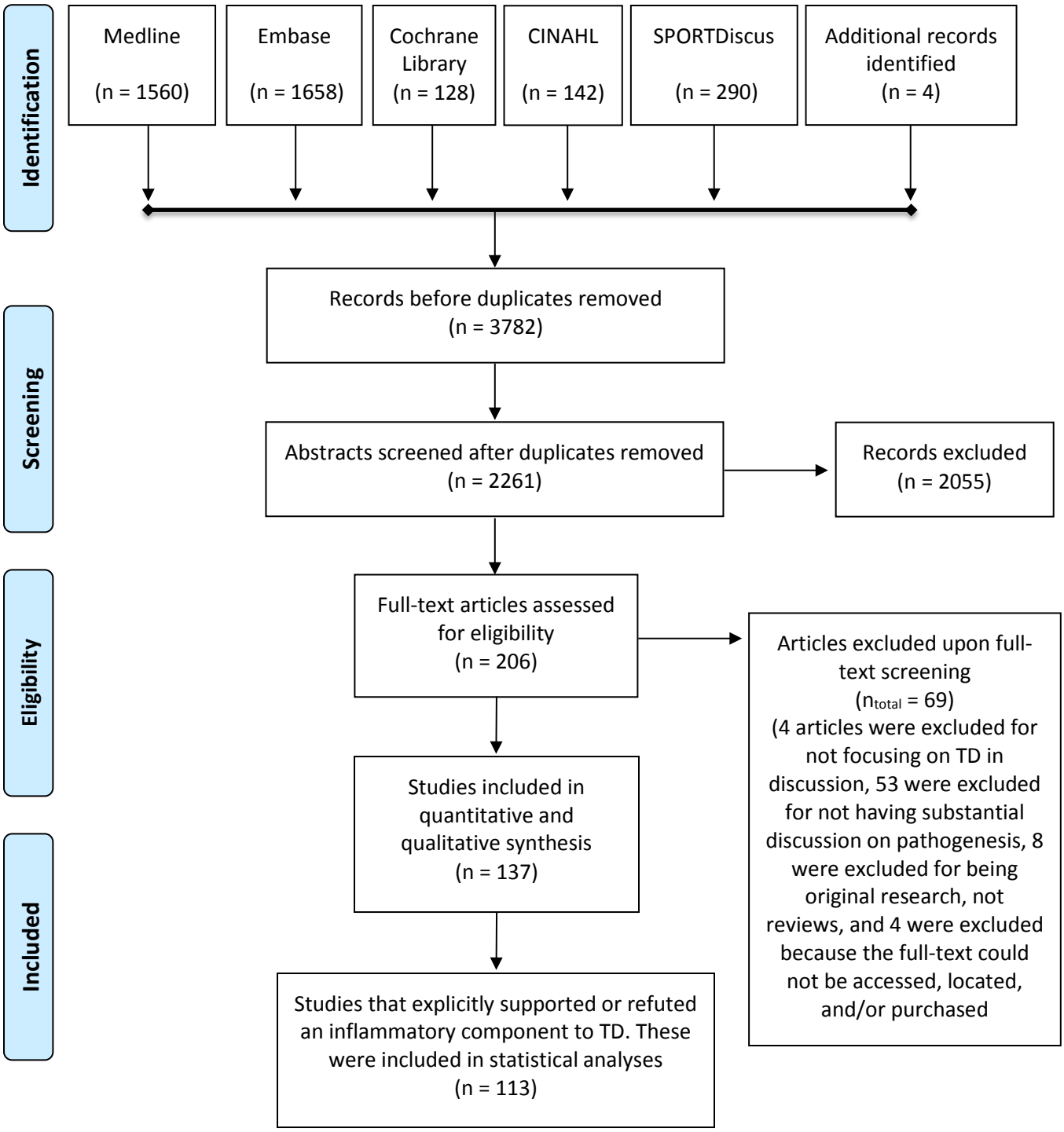
All graphs and statistical analyses were performed in SPSS or GraphPad Prism 7 (GraphPad Software). Frequency distributions were performed for all data abstracted; appropriate categories were collapsed or stratified for ease of data interpretation and display. Temporal trends in select outcomes were evaluated using basic proportions of categorization (displayed by histograms). Stratified analyses were conducted for specific questions and predictors of the conclusion that inflammation plays a causal role in TD. Associations between potential correlates and a review's conclusion about the role of inflammation in TD were tested using binomial logistic regression. Omnibus tests of model coefficients (x^2) were performed to ensure that variance in the data set was significantly greater than unexplained variance. Significant was set at $p < 0.05$.

2.3 Results

2.3.1 Study selection

The search strategy revealed a total of 3782 results (Figure 2.2). After removal of 1521 duplicates, 2261 unique publications remained. Screening of the titles and abstracts revealed 206 reviews of the pathogenesis of TD eligible for inclusion. Further assessment of eligibility through a screening of the full-texts led to the exclusion of 69 papers: 4 for having discussions centered around a range of musculoskeletal diseases rather than focusing on TD, 53 for not having substantial discussion on pathogenesis (< 2 pages of discussion and analysis), 8 were excluded for being original research and not review articles, and 4 were excluded because the full-text could not be accessed, located, and/or purchased. This left 137 reviews meeting our criteria for inclusion, a list of which is available in **Appendix D**. Only reviews that explicitly supported or refuted an inflammatory component to TD were included in statistical analyses (n = 113). All systematic reviews (n = 18) included in the review are summarized in **Appendix E**.

Figure 2.2 PRISMA flowchart for identification and screening of reviews



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

2.3.2 Characteristics of included reviews

Included reviews consisted of publications that examined human models, animal models, and reviews that examined both. Reviews were not categorized based on the type of tendon examined (e.g. elastic type flexors, positional, etc.) Of the 137 included reviews, 62% (n = 85) had specific aims and discussions on the pathogenesis TD: the remaining 52 were narrative-style overviews on the pathogenesis of TD. Specific reviews discussed range of topics, including 1) the role of mechanical factors in TD 2) matrix protein dysregulation in TD 3) inflammatory mediator involvement in TD 4) drug-induced TD 5) neuronal regulation and involvement in TD 6) vascularity and its role in TD 7) histological findings of diseased tendons and 8) the association of metabolic disorders/adiposity with TD. 13.1% (n = 18) of included reviews conducted a systematic search and analysis of published literature (**Appendix E** for a full characterization of systematic reviews). There were no reviews that conducted a meta-analysis.

37.2% (n = 51) of reviews formed conclusions based on an analysis of focused primary literature, the rest (n = 86) were expert opinions. Of the 51 reviews that analyzed focused primary literature, 50 used level 3 evidence, and a single review used level 2 evidence [Muller 2015]. 34 of these reviews informally assessed the methodological quality of the primary literature as part of their discussions. Only 9 reviews formally assessed the methodological quality of their primary literature, all of which were systematic reviews. 86.1% (n = 118) of included reviews were low-quality (quality score ≤ 4), 9.5% (n = 13) were moderate-quality ($4 <$ quality score < 9), and 4.4% (n = 6) were high-quality (quality score ≥ 9). Although the majority of included reviews were of low-quality, there has been an increase in the number of moderate- to high-quality reviews over time (Figure 2.3). Additionally, there has been an increase in the number and proportion of reviews critically assessing primary literature (Figure 2.4).

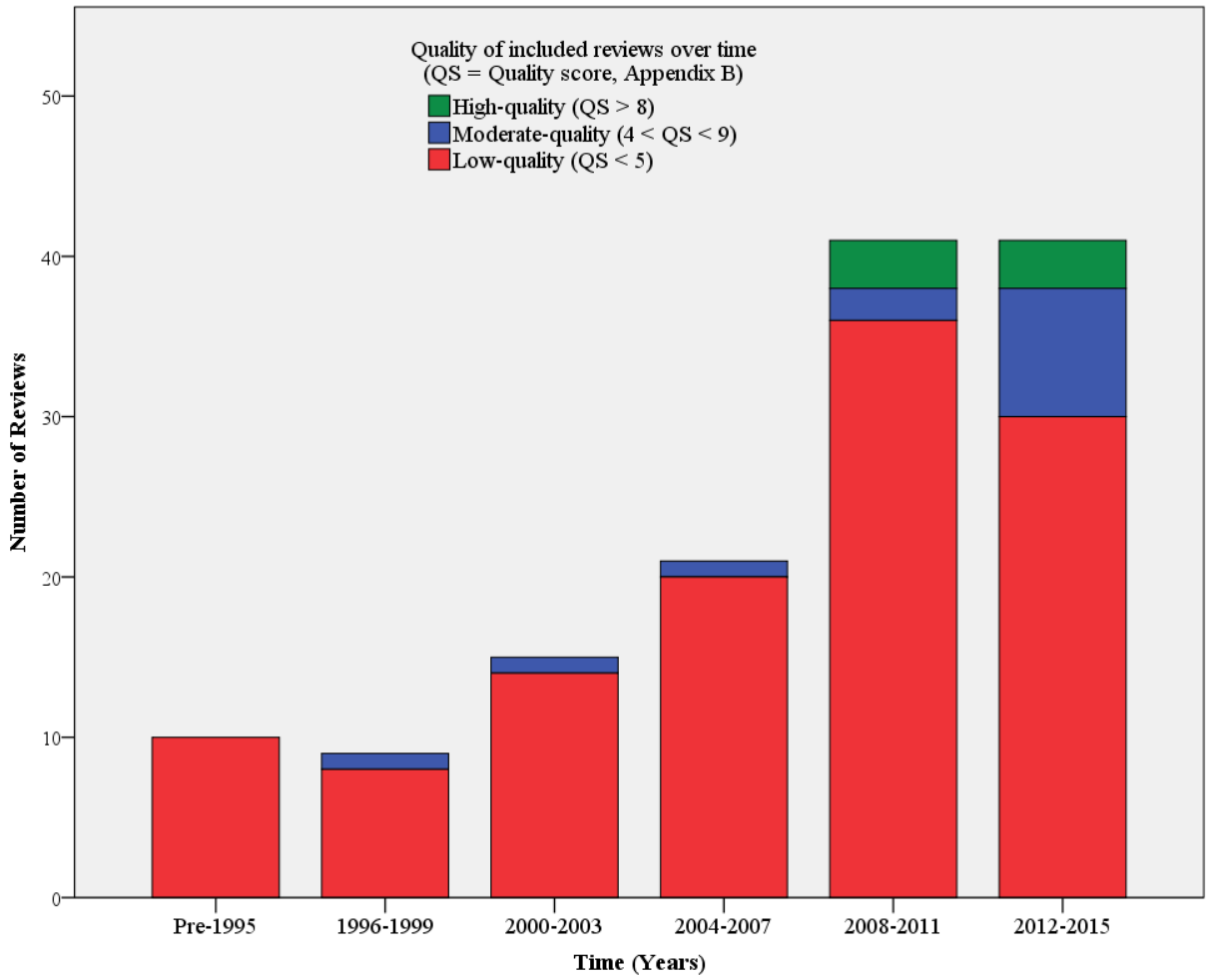


Figure 2.3 Quality of included reviews over time (intervals of 3 years). Quality score (QS), determined by 12 questions with a maximum score of 13 points of each review was used to categorize it as 'low,' 'moderate,' and 'high' quality. The past 3 years saw an increase in number and proportion of moderate- to high-quality reviews on the pathogenesis of TD: n_{total} = 41, n_{low} = 30, $n_{moderate}$ = 8, n_{high} = 3. [2008-2011]: n_{total} = 41, n_{low} = 36, $n_{moderate}$ = 2, n_{high} = 3. [2004-2007]: n_{total} = 21, n_{low} = 20, $n_{moderate}$ = 1, n_{high} = 0. [2000-2003]: n_{total} = 15, n_{low} = 14, $n_{moderate}$ = 1, n_{high} = 0. [1996-1999]: n_{total} = 9, n_{low} = 8, $n_{moderate}$ = 1, n_{high} = 0. [Pre-1995]: n_{total} =10, n_{low} = 10, $n_{moderate}$ = 0, n_{high} = 0.

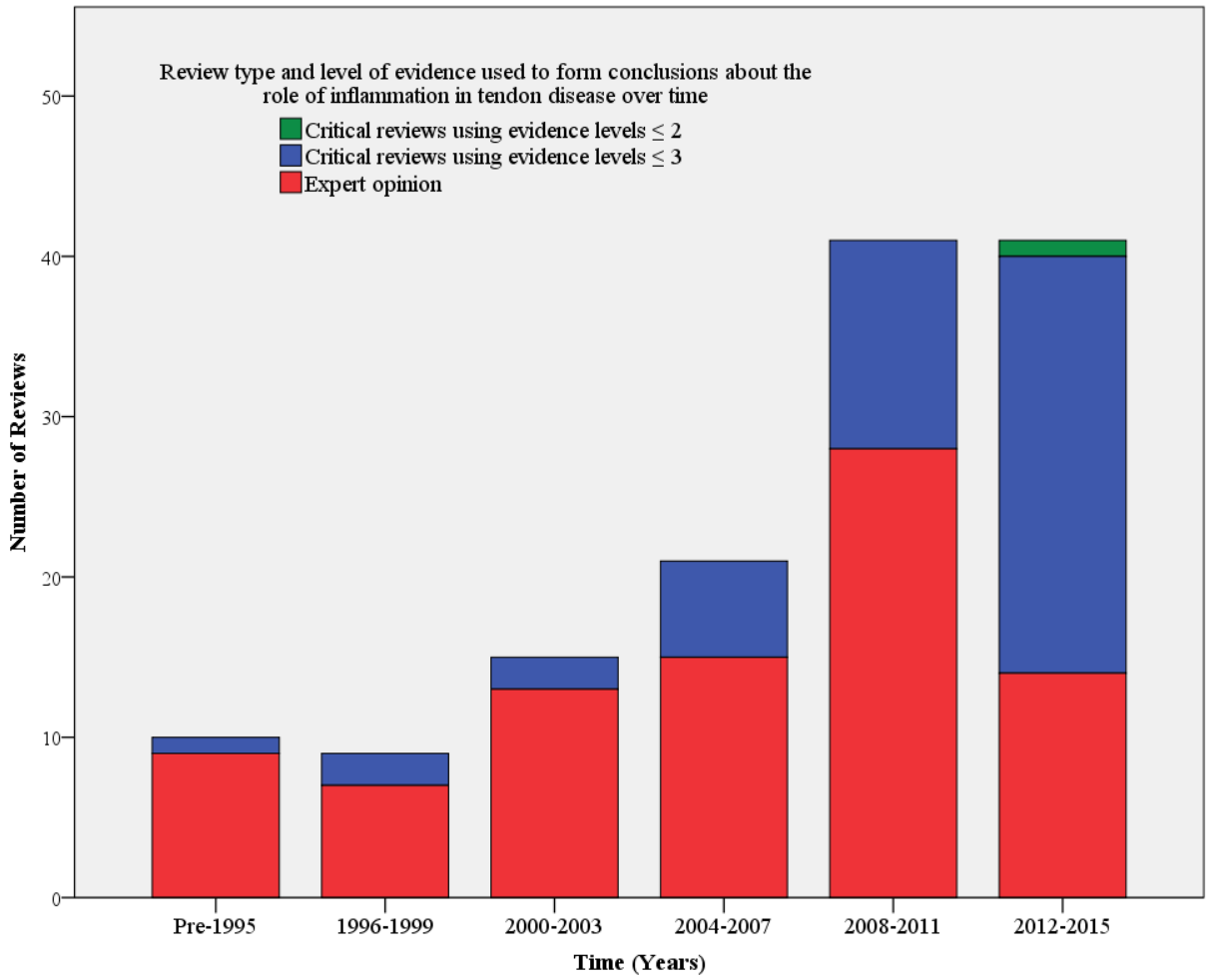


Figure 2.4 Type of review and levels of evidence used in discussion to form conclusions over time (intervals of 3 years). Reviews were categorized as critical only if they had a focused topic of discussion and interpreted substantial evidence from associated primary literature. The past 3-6 years has shown a substantial increase in the number and proportion of reviews including critical analyses of focused topics related to the pathogenesis or onset of TD. [2012-2015] n_{total} = 41, $n_{narrative}$ = 14, $n_{critical}$ = 27. [2008-2011] n_{total} = 41, $n_{narrative}$ = 28, $n_{critical}$ = 13. [2004-2007] n_{total} = 21, $n_{narrative}$ = 15, $n_{critical}$ = 6. [2000-2003] n_{total} = 15, $n_{narrative}$ = 13, $n_{critical}$ = 2. [1996-1999]: n_{total} = 9, $n_{narrative}$ = 7, $n_{critical}$ = 2. [Pre-1995] n_{total} = 10, $n_{narrative}$ = 9, $n_{critical}$ = 1.

2.3.3 Temporal trends: the role of inflammation in TD, discussion of specific immune cells, and the role of signalling molecules/effectors in TD.

Of included reviews, 47.4% (n = 65) concluded that TD is primarily degenerative with little to no chronic inflammatory component, 35.8% (n = 49) concluded that inflammation plays a role in TD. 8.8% (n = 12) discussed a potential for inflammation to contribute to the pathogenesis of TD but remained skeptical or their conclusions could not be easily determined. 8% (n = 11) of included reviews' conclusions did not fit into one of these categories: 3 discussed the pathogenesis of TD without any mention of inflammation or discussion on degenerative theories [Laseter 1991, Bureau 2013, Uhthoff 1989]. Three did not discuss pathogenesis but evaluated risk factors of TD [Blanco 2005, van der Worp 2011, Stephenson 2013]. Four reviews discussed or evaluated the association between fluoroquinolone use and tendinopathy/rupture, however, they did not substantially engage with the inflammation vs. degeneration debate [Khaliq 2003, Kaleagasioglu 2012, Lewis 2014, Melhus 2005]. The final review whose conclusions did not fit into a pre-defined category systematically assessed the association between tendinopathy and diabetes mellitus but did not discuss the inflammation vs. degeneration debate [Shi 2015].

Although a significant portion of the literature concluded no inflammatory component to TD, the proportion of reviews supporting an inflammatory component has increased substantially during the last 3-6 years (Figure 2.5). The recent surge in reviews favoring an inflammatory component to TD may be in part due to increasing investigation and interest of immune cells and inflammatory signalling pathways. Previous discussions focused on the lack of an 'inflammatory infiltrate,' typically referring to the known absence of neutrophils in diseased tendon tissue. However, more recent discussions on the pathogenesis of TD have included potential roles for monocytes, macrophages, and lymphocytes to contribute to TD (Figure 2.6). There has also been

a surge in the interest of signalling molecules, molecular effectors, and inflammatory activation pathways in initiating and propagating TD (Figure 2.7). Prior to 2004, there were little to no reviews that included these effectors as an integral part of the discussion and/or conclusion about the role of inflammation in TD.

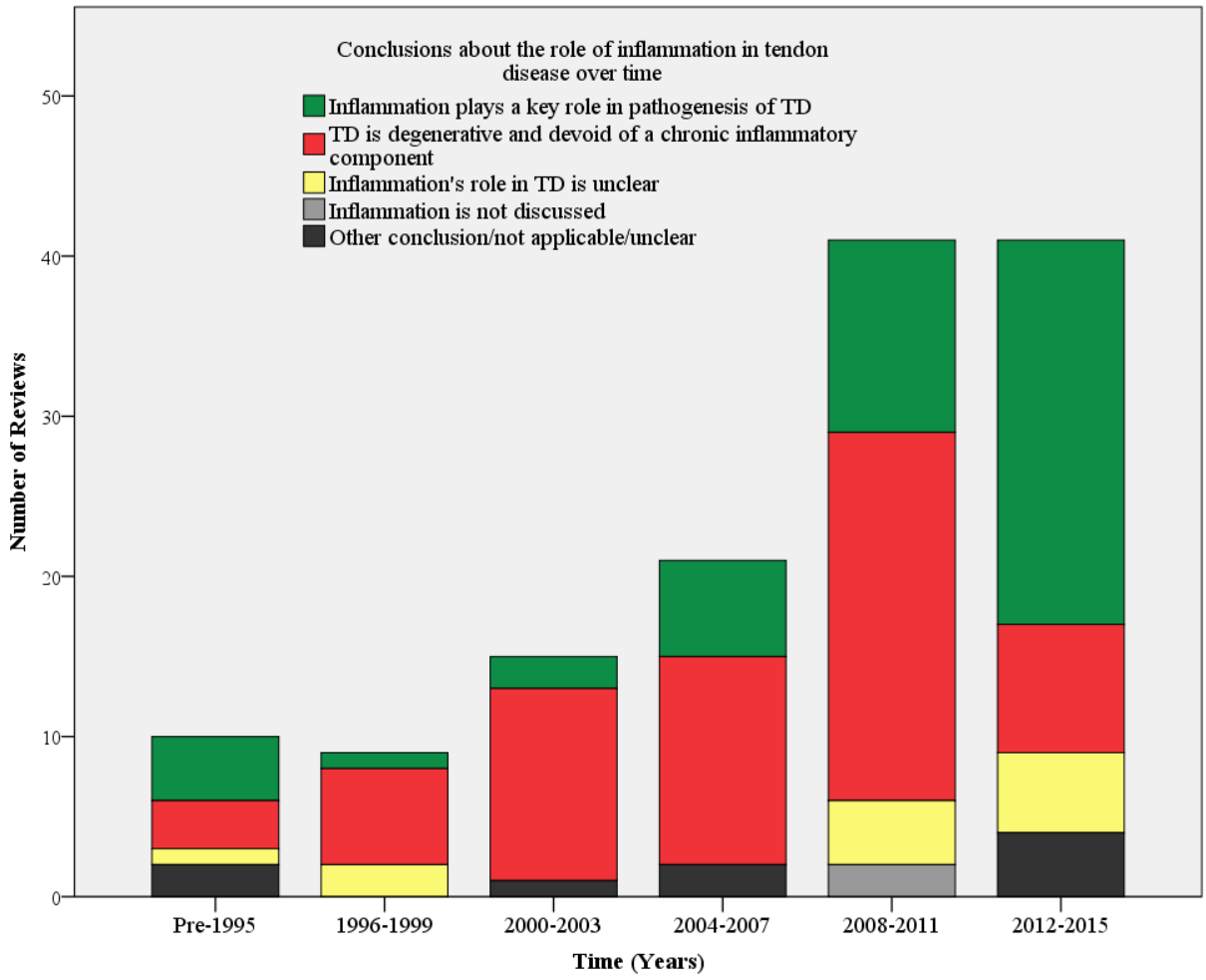


Figure 2.5 Conclusions about the role of inflammation in tendon disease over time (intervals of 3 years). Reviews were categorized based on their overall conclusion about the role of inflammation in TD, if discussed. Prior to 2012, the majority of reviews concluded that inflammation did not play a role in the pathogenesis of TD. However, during the past 3 years, there has been a substantial increase in support for an inflammatory component to TD. [2012-2015]: n_{total} = 41, $n_{degenerative}$ = 8, $n_{inflammatory}$ = 24, n_{unsure} = 5. [2008-2011]: n_{total} = 41, $n_{degenerative}$ = 23, $n_{inflammatory}$ = 12, n_{unsure} = 4. [2004-2007]: n_{total} = 21, $n_{degenerative}$ = 13, $n_{inflammatory}$ = 6, n_{unsure} = 0. [2000-2003]: n_{total} = 15, $n_{degenerative}$ = 12, $n_{inflammatory}$ = 2, n_{unsure} = 0. [1996-1999]: n_{total} = 9, $n_{degenerative}$ = 6, $n_{inflammatory}$ = 1, n_{unsure} = 2. [Pre-1996]: n_{total} = 10, $n_{degenerative}$ = 3, $n_{inflammatory}$ = 4, n_{unsure} = 1.

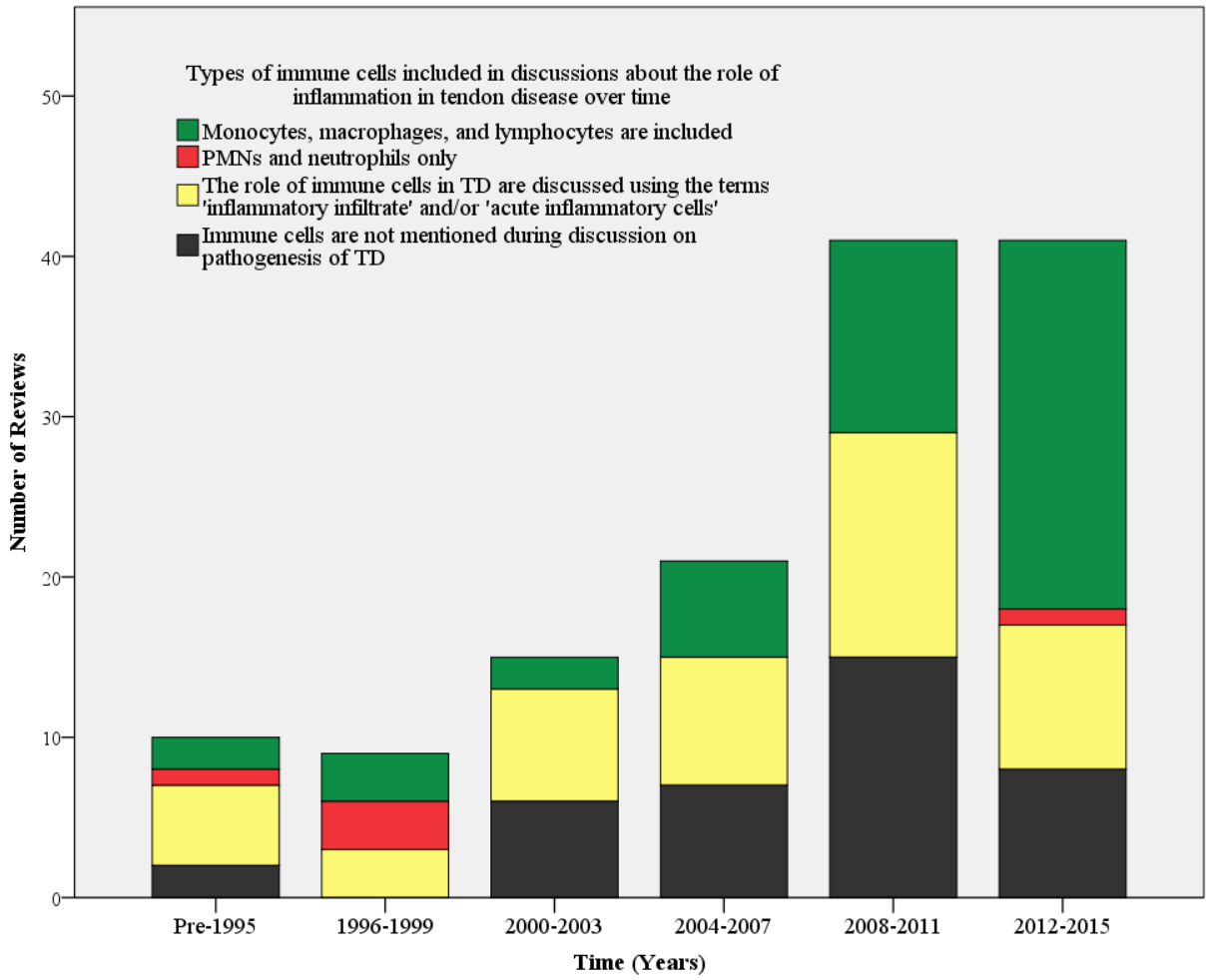


Figure 2.6 Types of immune cells included in discussions about the role of inflammation in TD over time (intervals of 3 years). Reviews were categorized based on the specific immune cells mentioned, referred to, or discussed when conclusions about the role of inflammation in TD were made. A substantial portion of studies that included a discussion on immune cells did not explicitly define the leukocytes being referenced. These reviews typically mentioned a presence or lack of 'an inflammatory infiltrate' in TD. Of the reviews discussing an 'inflammatory infiltrate' (n = 51), only 5 explicitly defined/discussed the cells of interest. Reviews that discussed the presence and/or role of monocytes/lymphocytes on tendon pathology have been consistently present but largely minimal until very recently. During the past 3 years, reviews discussing monocytes and lymphocytes have become a majority. Additionally, reviews not discussing any immune cell types during conclusions about the role of inflammation have decreased since 2011. [2012-2015]: n_{total} = 41, $n_{mono/lmyphocytes}$ = 23, $n_{infiltrate/PMN}$ = 10, $n_{neither}$ = 8. [2008-2011]: n_{total} = 41, $n_{mono/lmyphocytes}$ = 12, $n_{infiltrate/PMN}$ = 14, $n_{neither}$ = 15. [2004-2007]: n_{total} = 21, $n_{mono/lmyphocytes}$ = 6, $n_{infiltrate/PMN}$ = 8, $n_{neither}$ = 7. [2000-2003]: n_{total} = 15, $n_{mono/lmyphocytes}$ = 2, $n_{infiltrate/PMN}$ = 7, $n_{neither}$ = 6. [1996-1999]: n_{total} = 9, $n_{mono/lmyphocytes}$ = 3, $n_{infiltrate/PMN}$ = 6, $n_{neither}$ = 0. [Pre-1996]: n_{total} = 10, $n_{mono/lmyphocytes}$ = 2, $n_{infiltrate/PMN}$ = 6, $n_{neither}$ = 2.

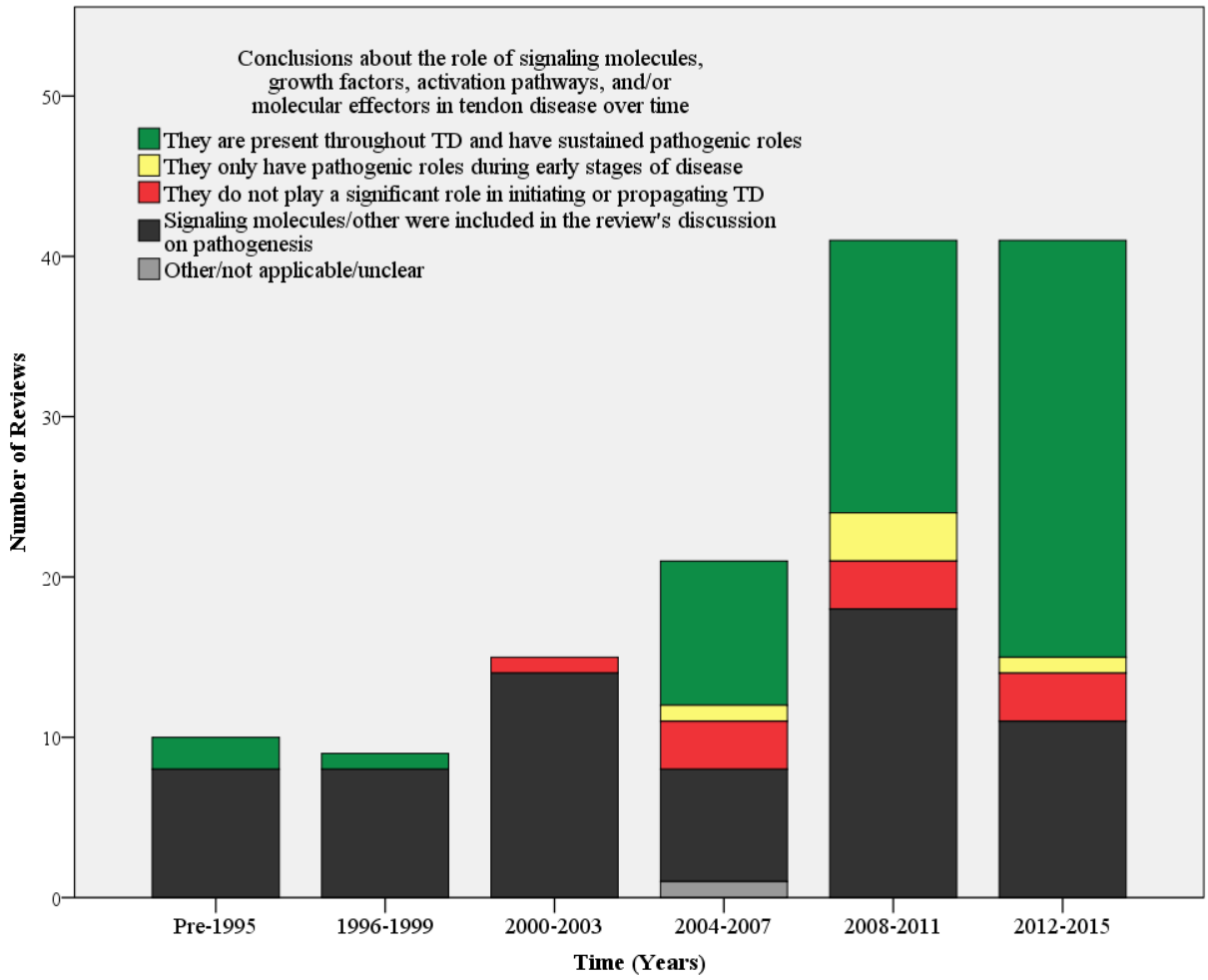


Figure 2.7 Conclusions about the role of signalling molecules and/or molecular effectors on tendon disease over time (intervals of 3 years). Reviews were categorized according to their conclusions about the role signalling molecules and/or molecular effectors (e.g. cytokines, prostaglandins, neuronal mediators) play in initiating and propagating TD. These conclusions were extracted and treated separately from the review's overall thoughts on inflammation in TD. Prior to 2004 there was little discussion of these effectors in TD. Since then there has been mixed interest and conclusions about their role in pathogenesis. However, during the past 3 years, the proportion of reviews concluding a key role for these effectors/activation pathways in initiating and propagating TD has increased to a majority. [2012-2015]: $n_{total} = 41$, $n_{sustained\text{-}roles} = 26$, $n_{early\text{-}only} = 1$, $n_{no\text{-}role} = 3$, $n_{not\text{-}included} = 11$. [2008-2011]: $n_{total} = 41$, $n_{sustained\text{-}roles} = 17$, $n_{early\text{-}only} = 3$, $n_{no\text{-}role} = 3$, $n_{not\text{-}included} = 18$. [2004-2007]: $n_{total} = 21$, $n_{sustained\text{-}roles} = 9$, $n_{early\text{-}only} = 1$, $n_{no\text{-}role} = 3$, $n_{not\text{-}included} = 7$. [2000-2003]: $n_{total} = 15$, $n_{sustained\text{-}roles} = 0$, $n_{early\text{-}only} = 0$, $n_{no\text{-}role} = 1$, $n_{not\text{-}included} = 14$. [1996-1999]: $n_{total} = 9$, $n_{sustained\text{-}roles} = 1$, $n_{early\text{-}only} = 0$, $n_{no\text{-}role} = 0$, $n_{not\text{-}included} = 8$. [Pre-1996]: $n_{total} = 10$, $n_{sustained\text{-}roles} = 2$, $n_{early\text{-}only} = 0$, $n_{no\text{-}role} = 0$, $n_{not\text{-}included} = 8$.

2.3.4 Potential correlates of the conclusion that inflammation has a role in tendon disease: cells and molecules incorporated into discussions.

Conclusions about the role of inflammation in TD were stratified by immune cell type and inclusion of signalling molecules/effectors to determine if associations were present (Figures 2.8 & 2.9). Reviews that discussed neutrophils or an 'inflammatory infiltrate' largely concluded that there is not an inflammatory component to TD. The same trend was present in reviews that did not discuss any immune cell type. However, reviews that included discussions on monocytes and lymphocytes largely concluded that there is an inflammatory component to TD. There was little difference between immune cells discussed for reviews whose conclusion about the role of inflammation was ambiguous or not clearly stated.

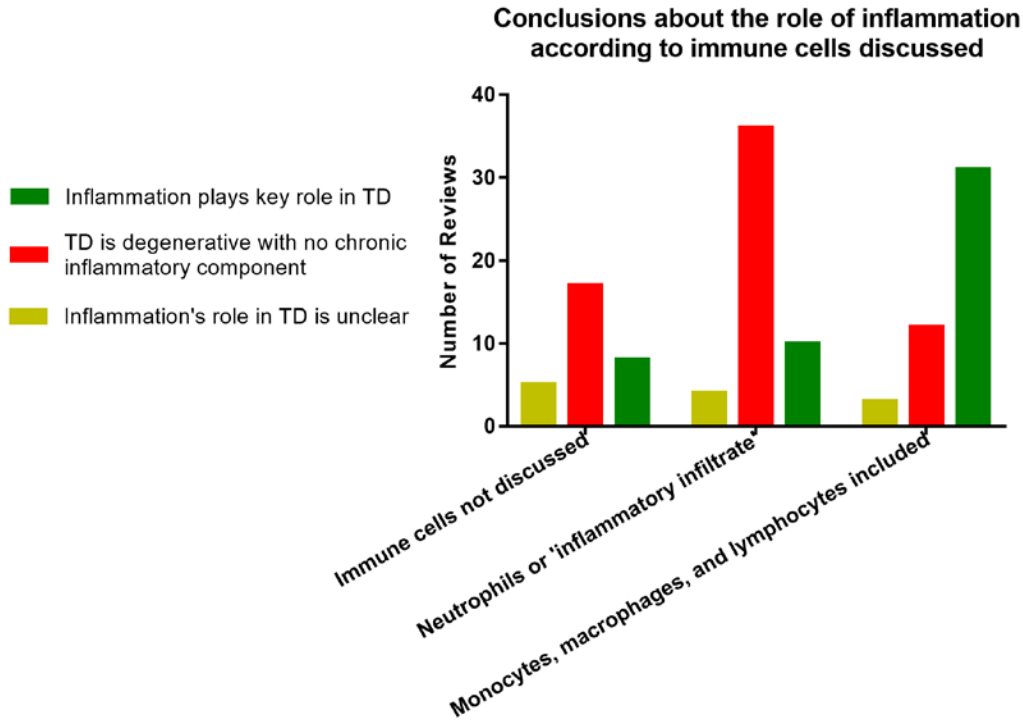


Figure 2.8 Conclusions about the role of inflammation according to immune cells discussed. A clear association is present between the inclusion of monocytes, macrophages, and/or lymphocytes in the discussion and concluding that inflammation plays a role in TD. Reviews that did not include monocytes/ lymphocytes were more likely to refute an inflammatory component, regardless of inclusion or exclusion of other leukocytes.

Similar associations exist for the inclusion or exclusion of signalling molecules/effectors and conclusions about inflammation in TD. In order to be categorized as 'including' these molecules, a review could have discussed any cytokine, activation pathway, or molecular effector with particular focus to its role in initiating and/or propagating pathology. They did not have to be pro- or anti-inflammatory pathways or molecules to be included, however, the majority of reviews that were categorized as including these in their discussions focused on inflammatory cytokines and activations pathways (e.g. Cox, NF-κβ, PGE_1, PGE_2, IL-6, IL-10, TNF-α, etc.). Reviews that did not include these molecules/effectors largely concluded no inflammatory component to TD. Reviews that did include these molecules mostly supported an inflammatory paradigm, however, a large portion of these reviews did not or were unsure.

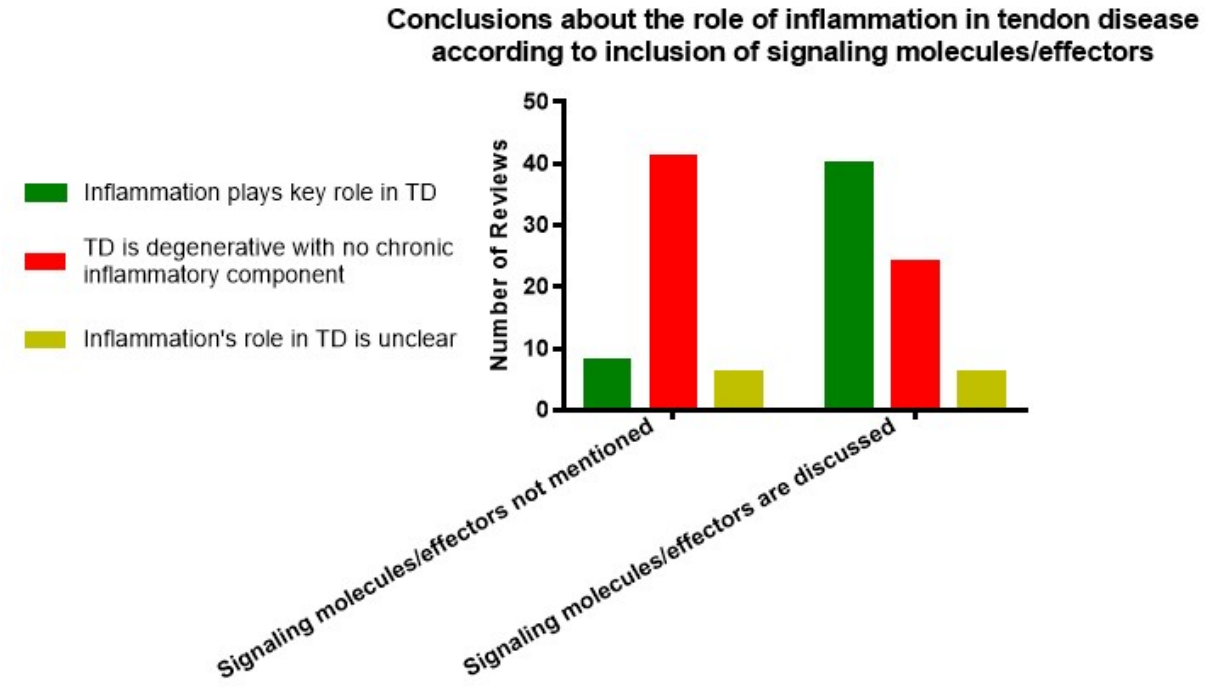


Figure 2.9 Conclusions about the role of inflammation according to the inclusion of signalling molecules, effectors, or activation pathways. An association is present between inclusion and concluding that inflammation plays a role in TD. However, dissonance among these reviews is still present. Reviews that did not discuss these molecules consistently concluded no inflammatory component to TD.

A binary logistic regression was performed to ascertain any effects inclusion of monocytes, macrophages, lymphocytes, and signalling molecules on the likelihood that a review concludes inflammation plays a role in TD. Only reviews that explicitly supported or refuted an inflammatory paradigm to TD were included in the model (n = 113). The logistic regression model was statistically significant: $x^2(2) = 35.4$, $p < 0.001$. The model explained 36.1% (Nagelkerke R^2) of the variance in conclusions about the role of inflammation in TD and correctly classified 75.2% of cases. Reviews including monocytes and lymphocytes in their discussions were 5.23 times more likely to conclude inflammation was important than reviews that did not, $p < 0.001$. Reviews that included signalling molecules, effectors and activation pathways in their discussions on pathogenesis were 4.92 times more likely to conclude inflammation is important than reviews that did not, $p = 0.003$.

2.3.5 Potential correlates of the conclusion that inflammation has a role in tendon disease: quality indicators.

Conclusions about the role of inflammation in TD were stratified by several quality indicators to determine if any associations were present. Quality indicators included in analysis were 1) whether they were critical evaluations with focused research questions 2) if the review was a systematic review of the literature and 3) the reviews total quality score

The majority of expert opinions and narrative overviews concluded that TD was degenerative (Figure 2.10). Critical reviews showed substantial support for an inflammatory component but were noticeably fewer in quantity overall. There were very few systematic reviews that met inclusion criteria (n = 18) and discussed the inflammation versus degeneration paradigm (n = 13). Of the included systematic reviews that discussed the inflammation debate, the majority supported an inflammatory component: 8 reviews supported an inflammatory

component, 2 reviews rejected it, and 3 reviews' conclusions about inflammation were ambiguous or not readily apparent (Figure 2.11).

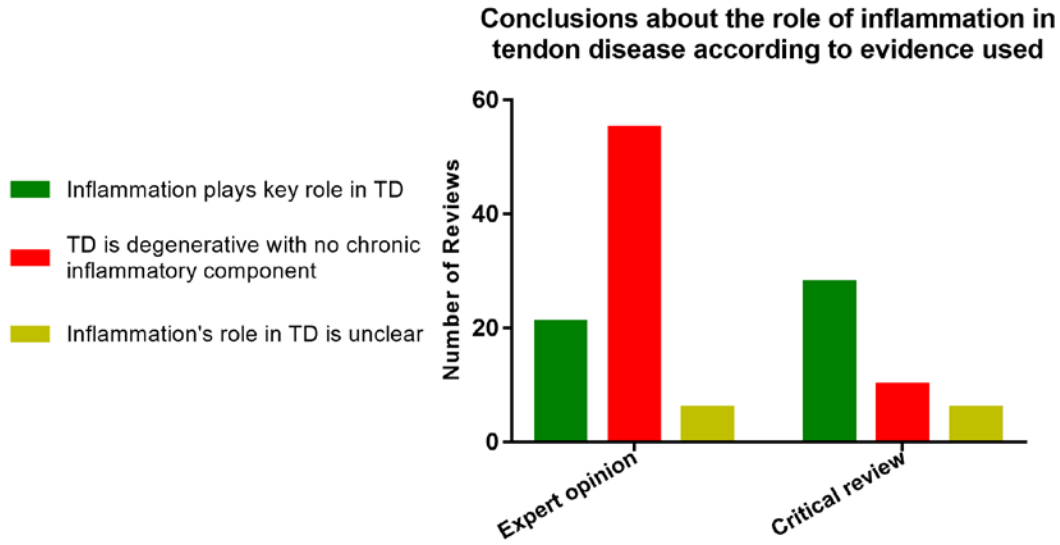


Figure 2.10 Conclusions about the role of inflammation in TD according to the evidence used: expert opinion vs. critical analysis of primary literature. Reviews with critical evaluations of primary literature on their topic of choice were more likely to support an inflammatory component to TD.

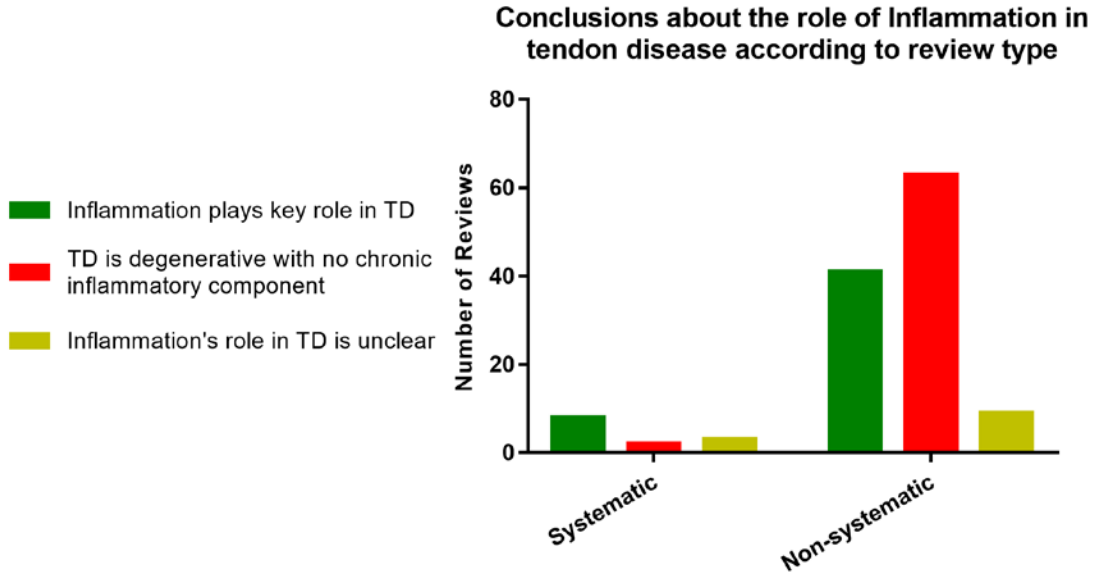


Figure 2.11 Conclusions about the role of inflammation in TD according to review type: systematic vs. not systematic. Systematic reviews are largely underrepresented among included reviews. However, the majority of systematic reviews supported an inflammatory component to TD. A full characterization of all included systematic reviews is available in **Appendix E**.

The majority of included reviews are of low methodological quality (QS < 5). Among low-quality reviews, reviews both support and refute an inflammatory component to TD (Figure 2.12). However, there we no reviews with a quality score > 3 that explicitly stated TD is degenerative and devoid of inflammation.

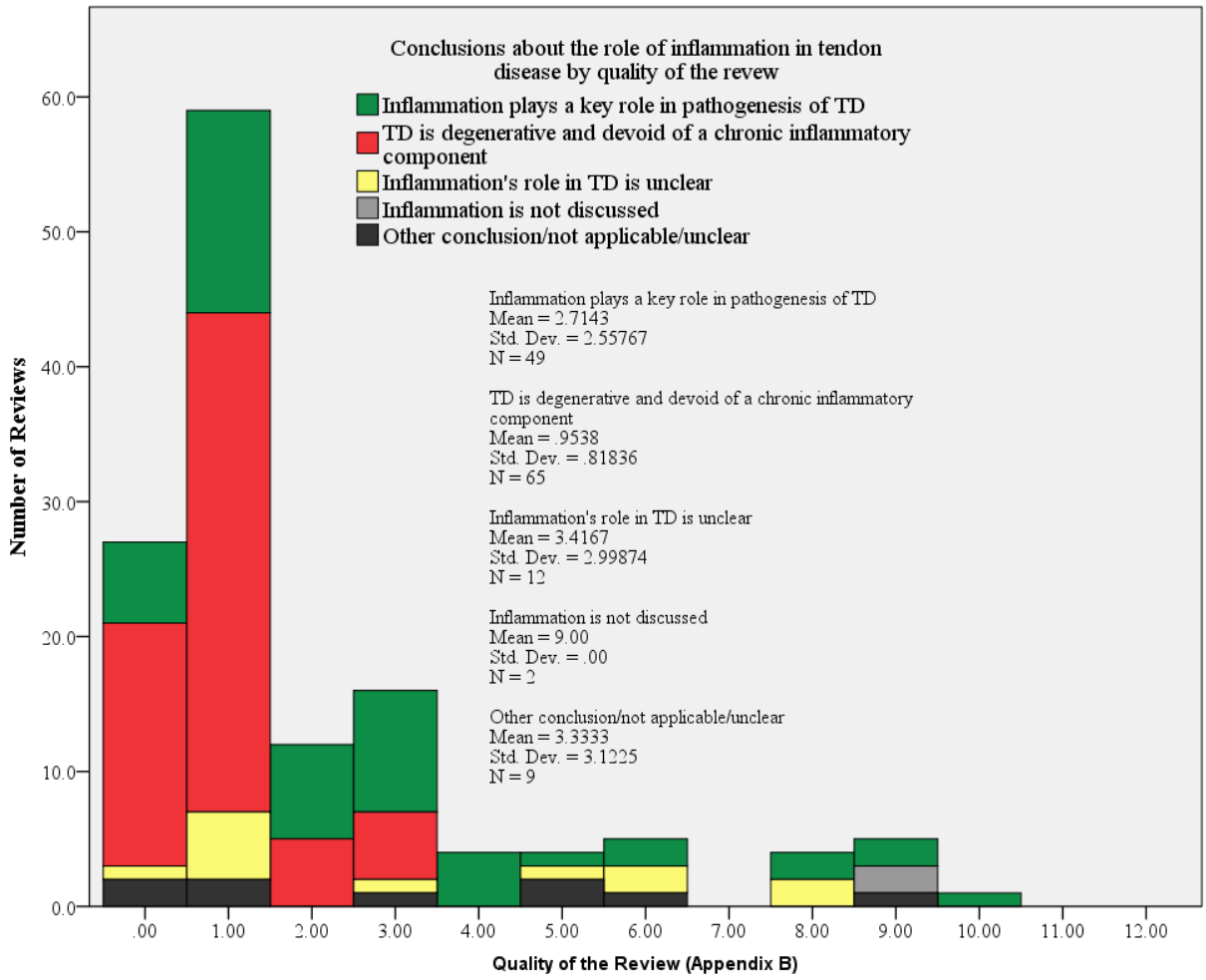


Figure 2.12 Conclusions about the role of inflammation in TD by the overall methodological quality score of the review. The maximum quality score was 13, however, the highest observed was 10.

A binary logistic regression was performed to ascertain the effects of these quality indicators on the likelihood that a review concludes inflammation plays a role in TD. Again, only reviews that explicitly supported or refuted an inflammatory paradigm to TD were included in the model (n = 113). The logistic regression model was statistically significant: $x^2(3) = 31.852$,

$p < 0.001$. The model explained 33% (Nagelkerke R^2) of the variance in conclusions about the role of inflammation in TD and correctly classified 75.2% of cases. Increasing quality score was associated with an increased likelihood to support an inflammatory component to TD, $p = 0.015$. However, if the review was systematic and/or critical it was not significantly more likely to support an inflammatory paradigm. This is likely due to a wide range in the quality of both systematic and critical reviews: [Systematic reviews: range, 3-10; median = 5].

2.3.6 Potential correlates of the conclusion that inflammation has a role in tendon disease: journal impact factor and number of citations.

Included reviews' conclusions about the role of inflammation in TD were stratified by journal type, journal impact factor, and number of citations per year to determine if any associations were present. The majority of included reviews were published in clinical (n = 50) or hybrid (n = 39) orthopaedic journals (Figure 2.13). Only 5 reviews were published in basic science orthopaedic journals. The rest of included reviews were published in non-orthopaedic journals: 19 in basic science journals, 12 in clinical journals and 12 in other types. Among most journal types no given conclusion was vastly overrepresented. However, in clinical journals (orthopaedic and non-specific), the degenerative hypothesis represented a majority of conclusions. In non-orthopaedic basic science journals the majority of published reviews support an inflammatory hypothesis. Journal impact factor does not correlate to any given conclusion. The number of citations per year does not correlate with any given conclusion, however, the top 5 reviews (>33 citations/year) all concluded there is no inflammatory component to TD.

A binary logistic regression was performed to ascertain any effects journal type, impact factor, and citations/per had on the likelihood that a review concludes inflammation plays a role in TD. Only reviews that explicitly supported or refuted an inflammatory paradigm to TD were included in the model (n = 113). The logistic regression model was statistically significant: $x^2(7)$

= 16.348, p = 0.022. The model explained 18.2% (Nagelkerke R^2) of the variance in conclusions about the role of inflammation in TD and correctly classified 65.2% of cases. If the review was published in a basic science journal it was 12 times more likely to support an inflammatory component to TD than if it was not, p = 0. 042. Journal impact factor and number of citations per year were not associated with any given conclusion about the role of inflammation in TD.

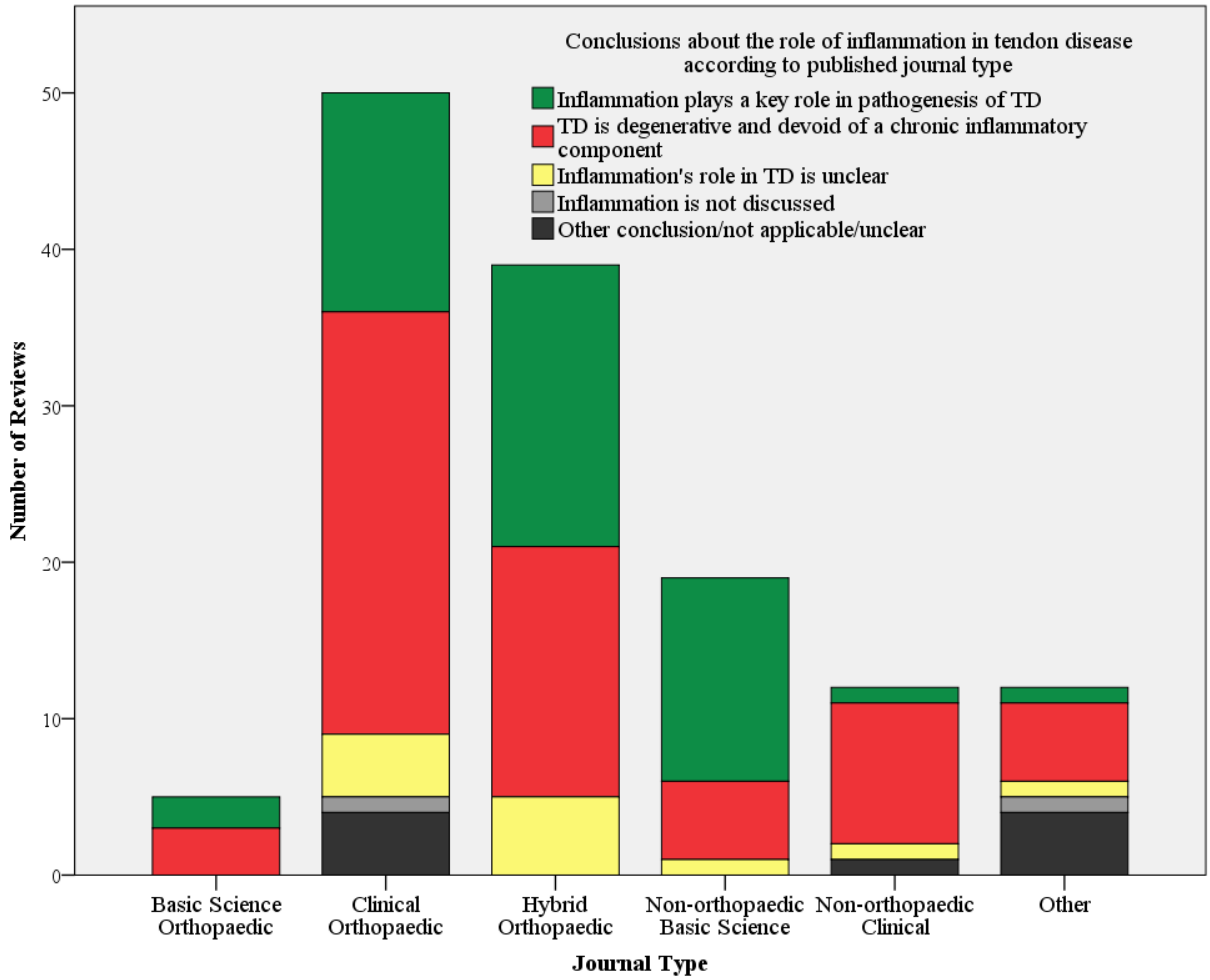


Figure 2.13 Conclusions about the role of inflammation in TD according to published journal type.

2.4 Limitations

Our review was designed to be generalizable: reviews of all levels of quality and focus were included to present an accurate representation of consensus over time. We did not take into account what type of tendon was analysed and/or whether the review was focusing on human or animal studies. Grouping both human and animal studies together could impact results in unknown ways: it is likely that different tendon types have different inflammatory environments. Additionally, data collected on a review's conclusions, discussions, and classification of the 'inflammatory component' in TD were categorized into pre-defined groups. These groups were general enough to provide intuitive data at the cost of precision: we did not collect specific data on the exact immune cells and/or mediators discussed. Due to this our statistical model was more generalizable and representative of contemporary opinions in the medical literature but was less specific due to the use of categorized groups.

There is the potential for misclassification bias in our inclusion and exclusion criteria. It was difficult to categorize a review as narrative vs. critical if it was not a systematic review. This potential bias was minimized by the piloting all our data extractions to assess reproducibility and validity. Additionally, we tried to reduce this bias by having an independent panel categorize data that was deemed not sufficiently reproducible. Failure to minimize this bias would reduce our model's ability to detect associations between our predictors and conclusions about the role of inflammation in TD (i.e. if hybrid reviews of high quality were more likely to be classified as basic than clinical and quality predicts conclusion, systematic bias could be introduced).

2.5 Discussion

This systematic study of published mechanistic reviews between 1970 and 2015 documented growing support for an inflammatory paradigm in the literature, particularly during

the last 3-6 years. Additionally, there has been increasing interest in monocytes, macrophages, lymphocytes, signalling molecules, and growth factors in TD since 2004. The adoption and/or rejection of a causal theory between inflammation and TD was significantly associated with quality indicators of the review and characterizations of the 'inflammatory component' in TD. Reviews were more likely to conclude inflammation is important in TD if they were of higher quality score or they included critical analyses of relevant primary literature. Additionally, reviews that only discussed acute inflammatory cells, PMNs and/or neutrophils, were significantly more likely to refute an inflammatory component to TD.

The role of inflammation in the pathogenesis of TD has been a topic of debate since the mid-late 1900s. During the past 15 years, TD has been predominately characterized as a degenerative condition devoid of inflammation [Astrom 1995, Movin 1997, Hashimoto 2003, Rees 2006, Rees 2014]. Some reviews have suggested that the current 'degenerative' disease paradigm overlooks inflammation's complex role in TD by precluding all inflammatory pathways and cells on the pretext that an 'inflammatory infiltrate' is absent [Rees 2014, Dean 2012, Dean 2015]. The transition away from 'tendinitis' to 'tendinosis' has been described as an oversimplification of inflammation's complex role in TD, which potentially acts through a variety of immune cell types, activation pathways, cytokines, and growth factors [Rees 2014, Dean 2012, Dean 2015]. Our results support these notions: prior to 2011, the majority of reviews refuted an inflammatory component to TD and did so by citing an absence of neutrophils in diseased tendon tissues. Reviews have only recently begun to include a greater variety of immune cells and signalling molecules into their discussions on the inflammatory component to TD. This increased interrogation of leukocytes and cytokines in TD has led to a majority of reviews concluding that inflammation has a key role in its pathogenesis.

Chapter 3: The Role of Alarmins and HIF-1α in the Pathogenesis of Tendon Disease and Resolution of Pain after Treatment.

3.1 Overview

Evaluation of the literature in Chapter 2 highlighted growing support for the theory that inflammatory cells and mediators play key roles in the in the initiation and propagation of tendon disease (TD). Recent studies have demonstrated inflammation activation pathways in early to intermediate TD: initiation and propagation of inflammation may cause diseased tenocytes and macrophages to upregulate fibrotic/degenerative mechanisms discussed in section 1.4 [Dakin 2015, Goodier 2016]. However, the nature of the cytokine and growth factor milieu that facilitates a pro-inflammatory phenotype have yet to be elucidated.

Alarmins, endogenous molecules released upon tissue damage, stress, and infection, are key effectors in the activation of the immune system that may play important roles the pathogenesis of TD [Millar 2013]. HIF-1, an oxygen-dependent heterodimeric transcription factor, which has been implicated in pro-inflammatory and pro-fibrotic diseases may play similar roles in TD [Semenza 2009, Rosmorduc 2010]. In this study we investigated tissue alarmins (S100A9, IL-33, & HMGB1) and HIF-1α protein expression in healthy, painful diseased, and pain-free treated human supraspinatus tendon tissue to evaluate if expression profiles differ at the onset of disease and after successful treatment as measured by resolution of pain. Changes in the level and location of expression were evaluated to identify potential roles these factors in the pathogenesis and resolution of pain in TD.

3.1.1 Background

The pathological paradigm of tendon disease is shifting away from a purely degenerative theory as evidence mounts that fibrotic mechanisms are closely integrated with inflammatory stimuli and cascades [Goodier 2016]. Recent work from Dakin *et al* has highlighted the plasticity

of inflammation activation signatures in early, intermediate, and advanced-stage tendon disease [Dakin 2015]. These studies show that tendon derived stromal cells adopt a pro-inflammatory phenotype after exposure to inflammation and may be implicated in sustaining chronic inflammation [Dakin 2015]. However, little is known about what initiates and propagates these changes in diseased human tendons. Up regulation of alarmins has been implicated in the pathogenesis of chronic inflammatory disorders: it is possible shared mechanisms exist in TD [Chan 2012].

3.1.2 Purpose

The purpose of this study was to investigate the role of alarmins and HIF-1α in the initiation and propagation of TD using a human supraspinatus patient cohort.

3.1.3 Objectives

Our primary objective was to quantitatively investigate expression of S100A9, HMGB1, IL-33, and HIF-1α among 1) healthy 'control' supraspinatus tendon tissue 2) chronically painful, diseased supraspinatus tendon tissue (not torn) and 3) supraspinatus tendon tissue from patients whose pain remained resolved one to three years after subacromial decompression (SAD).

A secondary objective was to evaluate differences in alarmin and HIF-1α localization (nuclear/cytoplasmic/extracellular) among groups.

A tertiary objective was to determine if there was co-localizations of alarmins/HIF-1α in resident tendon cells and macrophages using immunofluorescent staining of selected tendon tissue samples.

3.1.4 Hypotheses

HIF-1α, HMGB1, and S100A9 up regulation has been implicated with arthritic, autoimmune, and inflammatory pathologies [Chan 2012, Goebeler 1994, Roth 1992, Brun 1994,

Millar 2013]. Therefore, we hypothesized that HIF-1α, HMGB1, and S100A9 would be up regulated in diseased tissues compared to healthy and reduced in pain-free treated tissues.

IL-33 has been shown to have pathogenic, protective, or regenerative roles in musculoskeletal tissues depending on the location and timing of expression (e.g. nuclear IL-33 expression protects against NF-κβ activation while extracellular IL-33 is pro-inflammatory) [Schmitz 2005, Funakoshi-Tago 2008, Hong 2011, Cayrol 2009, Ali 2010, Lüthi 2009, Saluja 2015, Martin 2016]. Therefore, it was hypothesized that increased extracellular IL-33 protein would be found in diseased tissue.

3.1.5 Rationale/novelty

There is a paucity of research on alarmins in tendon disease. The research that exists on alarmins is limited to assessments of differences in alarmin expression between ruptured supraspinatus tendons, representing 'late-stage' disease, and matched subscapularis tendons representing 'early-stage' disease [Millar 2012, Millar 2013]. To our knowledge, this work will be first to 1) measure HMGB1 and S100A9 expression in tendon tissue 2) assess the level of expression of alarmins/HIF-1α in non-ruptured late-stage pathological tendons compared to healthy and pain-free treated tissue of the same tendon (supraspinatus) and 3) examine if changes in expression of alarmins HMGB1/S100A9/IL-33 and HIF-1α correlate with resolution of pain post-treatment.

3.1.6 Potential significance

There is increasing evidence that activation of the immune system after tissue trauma, stress, or injury is, in large part, due to the interactions of alarmins released from necrotic cells with their associated receptors, upregulating several signalling pathways and inflammatory mediators (NF-kβ, IFNs, PGE_1, PGE_2) [Harris 2006, Yoshioka 2012]. Dysregulation of alarmins

and HIF-1α in tendon tissue may therefore implicate these molecules as drivers of inflammation in TD. Further understanding the role of alarmins and HIF-1α in tendon disease may bridge the gap between inflammatory and degenerative theories and provide insight into novel therapeutic treatments.

3.2 Materials/Methods

Tissue was obtained from three unmatched patient groups: 1) healthy supraspinatus tendon 2) diseased supraspinatus tendon from patients with chronic pain shoulder pain and 3) pain-free supraspinatus tendon from patients who previously had sub-acromial decompression surgery for rotator cuff disease (tissue collected 1 to 2 years post-SAD). Samples were collected by a consultant orthopaedic surgeon during surgery (healthy) or by biopsy needle (diseased/treated). All patients included in the study had completed the Oxford Shoulder Score (OSS), a validated and widely used clinical outcome measure scoring from 0 (severe pathology) to 48 (normal function) prior to tissue collection and/or surgery [Dawson 1996]. The tissue samples used in this study had previously been collected and stored by approved ethical guidelines/protocols (section 3.2.2). Full informed consent according to the Declaration of Helsinki was obtained from all patients. OSS scores at the time of tissue collection, age, and other patient information is available in table 3.1.

3.2.1 Patient cohort and tissue collection

Healthy supraspinatus tissue (n = 6, male = 4, female = 2) was collected from patients that were recruited to the Nuffield Orthopaedic Centre (NOC) from orthopedic referral clinics for having shoulder instability. These patients underwent shoulder instability surgery and excess supraspinatus tendon tissue was collected and stored for future use. The status of the patients supraspinatus tendon was assessed via ultrasound and during surgery and were then classified as

intact, small rupture, medium rupture, large rupture, or massive rupture depending on the damage to the tissue. Only tendons classified as intact were used for this thesis.

Diseased supraspinatus tendon tissue (n = 13, male = 6, female = 7) was obtained from patients recruited from orthopedic referral clinics for having chronically painful shoulders. Patients presenting to the shoulder clinic had failed non-operative treatments and had experienced pain for a minimum of 6 months. A shoulder specialist diagnosed the patients' conditions and confirmed the structural integrity of the supraspinatus tendon via ultrasound. Painful arc tests and external rotation resistance tests were positive in all patients. The tendon tissue was collected via percutaneous ultrasound-guided biopsy under local anesthesia by a senior consultant shoulder surgeon. The biopsy was taken using a trucut needle 5 to 10 mm posterior to the anterior edge of the supraspinatus tendon. This validated biopsy technique is described in detail elsewhere [Murphy 2015].

Post-treatment pain-free supraspinatus tissue (n = 5, male = 2, female = 3) was obtained from patients who underwent the referral process for chronic shoulder pain/supraspinatus tendinopathy, chose to have sub-acromial decompression surgery (SAD), and who were clinically asymptomatic one year after SAD. Patients in this pain-free group had significant pain before SAD that resolved post-treatment, evidenced by a median OSS of 48 (range, 45 to 48) post-SAD. These samples were collected 1-3 years after treatment using the same percutaneous ultrasound-guided biopsy technique. Samples were not matched pre- and post-treatment.

Exclusion criteria for any patient included significant problems in the other shoulder, significant neck problems, rheumatoid arthritis, systemic inflammatory disease, osteoarthritis, previous shoulder surgery, and dual shoulder pathological lesions. OSS scores at the time of tissue collection, age, and other patient cohorts information is in table 3.1.

Tissue Group	n	Median Age	History	Prior Treatment	Median OSS
Healthy	6	24 (range: 19-26)	Shoulder Instability	N/A	33 (range: 11-44)
Diseased	13	45 (range: 36-62)	Painful Tendinopathy	N/A	30 (range: 16-36)
Pain-free post-treatment	5	57 (range: 43-72)	Painful Tendinopathy	SAD	48 (range: 45-48)

Table 3.1 Patient cohort details for tendon tissue samples

3.2.2 Study approval and ethics

Healthy tissue were obtained 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). Patients in the UKUFF trial were recruited for control, small, medium, large, and massive rupture supraspinatus tendon samples. A multicenter research committee and a local ethics committee approved the study (REC reference 10/H0402/24). Diseased tissues were obtained from the Serial Biopsy Study (REC reference 09/H0605/111). Patients in the Serial Biopsy Study were recruited for having shoulder impingement or partial thickness rotator cuff tear and planned on having glucocorticoid treatment or SAD. All post-treatment pain-free tissue samples were from the Why Are Shoulders Painful Trial (WASP), funded by the NIHR Oxford Biomedical Research Unit (REC reference 14/SC/0222). Patients in WASP were recruited for having long-term painful shoulder tendinopathy, had previously tried other treatment methods (most commonly glucocorticoids) that did not help the pain, and now wanted to try SAD surgery.

3.2.3 Tissue processing

Adapted from Dakin *et al* 2015. Samples of tendons were immersed in 10% buffered formalin for 0.5 mm/hour. After formalin fixation, tendon samples were processed in a Leica ASP300S tissue processor and subsequently embedded in paraffin wax. 4mm tissue sections

were cut using a RM2135 microtome (Leica Microsystems Ltd.) and baked onto adhesive glass slides at 60°C for 30 min and 37°C for 60 min.

3.2.4 Immunohistochemistry protocol

Adapted from Dakin *et al* 2015. Prior to antibody staining and antigen retrieval, slides were baked at 60°C for 60 min. Deparaffinization and target retrieval was performed using an automated PT link (Dako, heat-mediated antigen retrieval at high pH). An EnVision FLEX visualization system with an Autostainer Link 48 (Dako) was used to perform antibody staining. Using protocols provided by the manufacturer, antibody binding was visualized using a FLEX 3,3′-diaminobenzidine (DAB) substrate working solution and hematoxylin counterstain (Dako). After staining, slides were taken through graded industrial methylated spirit and xylene and mounted in Pertex mounting medium (Histolab). Details of antibodies and their working dilutions are shown in (Table 3.2). Negative controls were completed to confirm staining seen in tendon tissues was specific: the primary antibody was substituted for universal isotype control antibodies available from the manufacture (Dako). The universal isotype control for mice was a cocktail of mouse immunoglobulin G IgG1, IgG2a, IgG2b, IgG3, and IgM (Dako IR750). The universal isotype control for rabbits was an immunoglobulin fraction of serum from non-immunized rabbits, solid-phase absorbed (Dako IR600). Isotype control images for immunohistochemistry and fluorescent imaging are shown in Figure 3.3. Controls were performed at the outset of the experiment rather than every time staining was conducted. We did not adjust the values found in experimental results based on any background staining.

Protein	Clone	Antibody Info	Dilution	Species	Isotype
HIF-1α	H1alpha67	LSBio; LS-B110/26323	1:150	Mouse	IgG_{2b}
S100A9	EPR3555	ABcam; ab92507	1:200	Rabbit	IgG
IL-33		Biorbyt; orb74856	1:150	Rabbit	IgG Polyclonal
HMGB1	1F3	ABcam; ab190377	1:200	Mouse	IgG_{2b}
CD68	KP1	Dako; M0814	1:100	Mouse	IgG_1
POPO-1		Life Technologies			

Table 3.2 Primary antibodies used for immunohistochemistry and immunofluorescence. POPO-1 is a nuclear counterstain used in immunofluorescence.

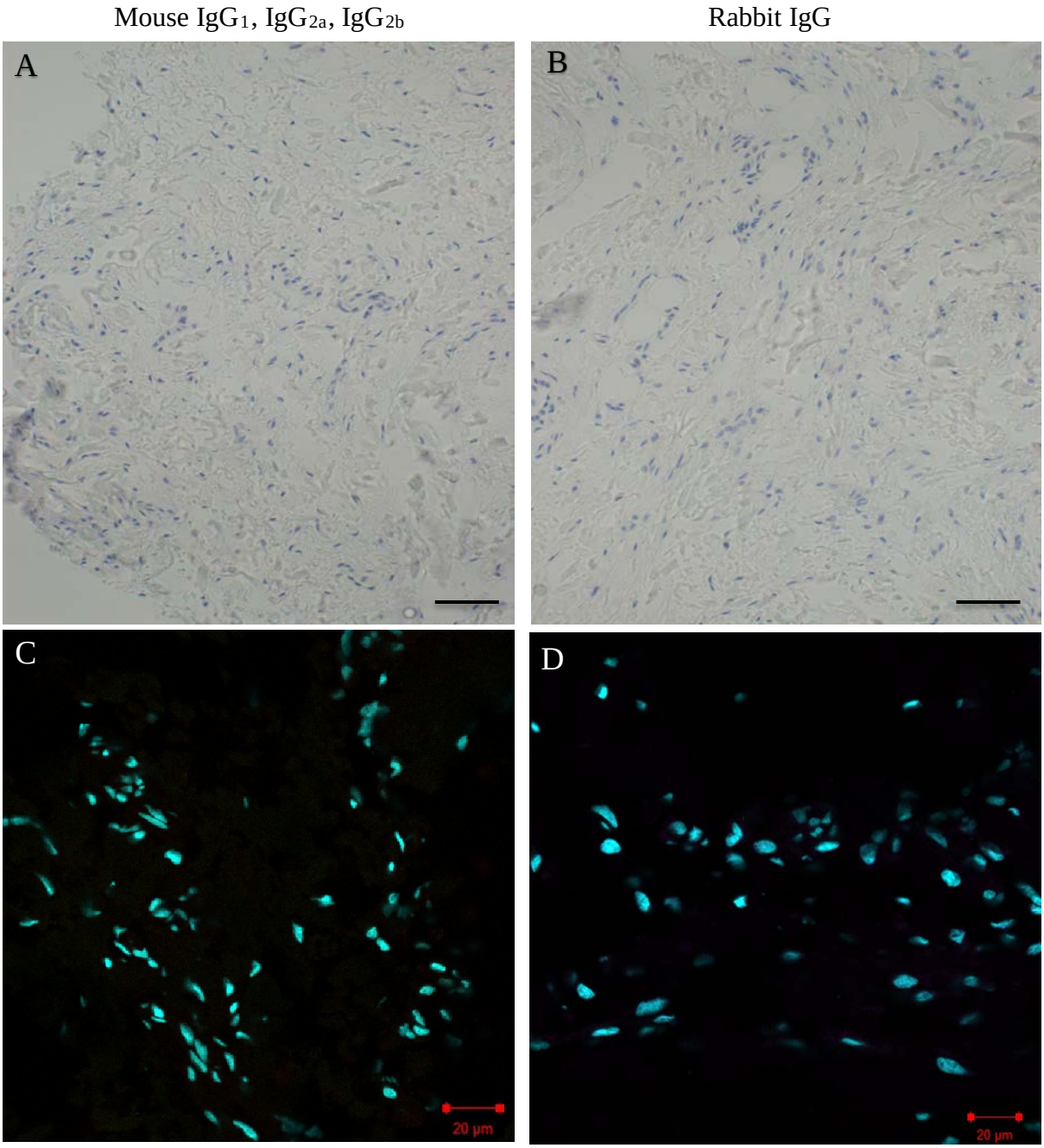


Figure 3.3 Isotype control staining of diseased human supraspinatus tendons. (A, B) Panel shows representative images of pathological tendon sections stained with isotype control antibodies for mouse IgG_1, IgG_{2a}, IgG_{2b}, and rabbit IgG fractions. Nuclear counterstain is hematoxylin. Scale bar = 100µm. (C, D) Representative confocal immunofluorescence images showing image of diseased tendon sections stained with isotype control antibodies for mouse IgG_1, IgG_{2a}, IgG_{2b}, and rabbit IgG fractions. Cyan represents POPO-1 nuclear counterstain. Scale bar = 20µm.

3.2.5 Image acquisition and analysis for quantitative immunohistochemistry

All images were acquired on an inverted microscope using Axiovision software (Zeiss). Twenty images of each stained section were acquired in a systematic manner at ×100 magnification with oil immersion by a single blinded investigator. If the tissue section was not large enough to capture 20 images, images were taken until the available tissue was exhausted. The smallest number of images acquired for a given sample in this study was 9 images; therefore statistical adjustments were not made. ImageJ (National Institutes of Health, Bethesda, Maryland, USA) was used to analyze the acquired images. Previously validated algorithms that quantify DAB staining by a colour deconvolution methods were used in analysis [Ruifrok 2001, Rizzardi 2012]. For each antibody, the colour deconvolution threshold was manually adjusted to best represent positive staining while ignoring background noise. Staining and quantification protocol was piloted on positive control tissues (**Appendix F**). Depending on expression pattern (cytoplasmic/nuclear or extracellular), specific algorithms were used to quantify staining.

For antibodies that showed nuclear and/or cytoplasmic expression, a cell ratio was calculated: positively stained cells/total cells. Cells were considered positive if they had nuclear or cytoplasmic staining. Once positive cells and total cells were counted for each image, the results were summed for each sample to give: total positive cells/total cells. To confirm accuracy of the final positively-stained cell ratio, the positively cells and total nuclei were also counted manually by a single blinded observer using ImageJ's 'cell counter' function: cells were manually clicked on to keep track of positive and non-positively stained nuclei. This was performed for all images acquired, producing a second quantification of the cell ratio. This manually counted ratio was then compared with the algorithms' outcome, showing sufficient

accuracy in measurements. To increase precision and accuracy of measures, these separate measurements of the cell ratio were then averaged to produce the final outcome ratio used for statistical analysis.

For antibodies that showed significant extracellular expression, a ratio of the stained area was calculated: stained area ratio = total positively stained pixels in the image/ total pixels of the image. Once (+) pixels and total pixels were quantified for each image, the results were summed to give the stained area ratio (total number of stained pixels / total pixels of all images).

3.3.6 Immunofluorescence

Triple antibody labelling (Table 3.4) was performed on formalin fixed paraffin embedded sections of diseased supraspinatus tissue to identify whether macrophages (CD68+ cells) and/or non-macrophages (CD68- cells) were expressing/co-localizing HIF-1α, S100A9, IL-33, and HMGB1.

Sample	Channel 1 (Cyan)	Channel 2 (Green)	Channel 3 (Red)	Channel 4 (Purple)
Negative control, mouse	POPO-1	IgG_1	$IgG_{2a/b}$	n/a
Negative control, rabbit	POPO-1	n/a	n/a	IgG
Diseased sample #1	POPO-1	CD68	HIF-1α	IL-33
Diseased sample #2	POPO-1	CD68	HMGB1	S100A9

Table 3.4 Immunofluorescence staining sample channels for negative controls and diseased samples: fluorescent image colors representing given genes/cell markers for each sample.

Slides were stained using a protocol adapted from Dakin *et al* 2015. Prior to antibody staining and antigen retrieval, slides were baked at 60°C for 60 minutes. Slides were taken through deparaffinisation and target retrieval steps (high pH heat mediated antigen retrieval) using an automated PT Link FLEX TRS system (Dako). To prevent drying prior to staining, samples were incubated in PBS tween (PBST) containing 998 mL of PBS and 2mL Tween 20

(Sigma P1379) after target retrieval steps. Slides were placed in a humid chamber throughout staining to prevent drying.

An ImmEdge pen (Vectashield) was used to create a hydrophobic barrier around the tissue section. Prior to antibody staining, tissues were block in 5% normal goat serum (Sigma G9023) in PBS for 60mins at room temperature. This helped to prevent non-specific antibody binding. Slides were then incubated with the primary antibody combination (Table 3.4) and diluted in 5% normal goat serum in PBS at room temperature for 2 hours. Negative controls were completed to confirm staining seen in tendon tissues was specific; the primary antibody was substituted for universal isotype control antibodies available from the manufacture (Dako). The universal isotype control for mice was a cocktail of mouse immunoglobulin G IgG1, IgG2a, IgG2b, IgG3, and IgM (Dako IR750). The universal isotype control for rabbits was an immunoglobulin fraction of serum from non-immunized rabbits, solid-phase absorbed (Dako IR600).

After primary antibody staining, slides were washed 3 times in PBST for 5 minutes each time. Slides were then incubated with three secondary antibodies: 1) goat anti-mouse IgG_1 (Southern Biotech) 2) Alexa Fluor 568 goat anti-mouse IgG_{2a} and 3) Alexa Fluor 633 anti-rabbit IgG (Life Technologies). Secondary antibodies were incubated at 1:200 dilution in PBS containing 5% normal equine serum (Sigma) for 2 hours at room temperature. During this time, the tissue sections were shielded from light and remained in the humid chamber. After secondary antibody staining, slides were washed 3 times in PBST for 5 mins each time. POPO-1 (Life Technologies), the nuclear counterstain, was then applied to all slides diluted in PBS containing 5% Saponin (Sigma) for 20 minutes at room temperature. Slides were then washed 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. After staining was complete, slides were mounted in VectaShield fluorescent mounting medium (H1000) and a coverslip secured with nail varnish. After mounting, slides were stored in a humid box at 4°C until imaging.

3.3.7 Immunofluorescence image acquisition and analysis

Immunofluorescence images were acquired on a Zeiss LSM 710 confocal microscope using a ×40 oil immersion objective following a previously described protocol [Dakin 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. All channels were acquired sequentially to reduce bleed-through. Averaging was set to 2 and the pinhole was set to approximately 1 Airy unit. Two-dimensional reconstructions were created using ZEN 2009 (Zeiss). Analysis of immunofluorescence was qualitative: co-localization of macrophage cells (CD68+) and non-macrophage cells (CD68-) with the alarmin staining is represented by a white colored cell in the composite 2-D image. Representative confocal immunofluorescence images of isotype control sections for mouse IgG1, IgG2a, IgG2b, and rabbit IgG fractions can be found in figure 3.3.

3.3.8 Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software). Kruskal Wallis with Pairwise post-hoc Mann-Whitney U tests were used to test for differences in protein level between: healthy, diseased, and post-treatment pain-free sample groups. Normality was tested using X^2 tests of normality. These tests were performed for DAB stained immunohistochemical images. Statistical significance was set at $p < 0.05$.

3.3 Results

3.3.1 Histological assessment of diseased supraspinatus tendons

Histological assessment of tendon tissues from this study cohort was completed in a previously published study [Dakin 2015]. Tendon tissue structure was evaluated using the Bonar scoring system (0-12) on samples stained with Hematoxylin and Eosin. Healthy supraspinatus tendons exhibited normal tissue architecture, represented by a median Bonar score of 2 and an interquartile range of 1-2. Diseased supraspinatus tendons showed increased vascularity and marked disorganization of the ECM, represented by a median Bonar score of 7 and an interquartile range of 6-8.

3.3.2 HIF-1α and S100A9 proteins increase in diseased tendons

Expression of the HIF-1 protein subunit HIF-1α was investigated in healthy, painful diseased, and pain-free post-subacromial decompression (SAD) supraspinatus tendons (Figures 3.5 & 3.6). Quantitative analysis of immunopositive staining showed increased HIF-1α staining in diseased tissue compared to healthy ($p=0.0002$, 6.2 fold increase); HIF-1α staining decreased post-SAD treatment in patients whose pain resolved compared to diseased tissue ($p=0.03$, 3.2 fold decrease). There was no significant difference of HIF-1α staining between healthy and treated tissue ($p=0.97$).

Qualitative analysis of HIF-1α expression shows that healthy tendon tissue has moderate HIF-1α expression in the cytoplasm of positive cells and that there is consistent low-level extracellular HIF-1α (Figure 3.5, A). Based on visual appearance, diseased tissues showed increased HIF-1α expression in both the cytoplasm and extracellular matrix (Figure 3.5, B). Some nuclear staining was present in these diseased tissues, however, the majority of HIF-1α

expression was cytoplasmic or extracellular. Pain-free treated tissues showed a consistent increased in nuclear staining compared to healthy and diseased supraspinatus tissues (Figure 3.5, C). Cytoplasmic and extracellular staining was still present in treated tissues, but was decreased compared to diseased ones.

Expression of S100A9 was also investigated in healthy, painful diseased, and pain-free post-SAD supraspinatus tendons (Figures 3.7 & 3.8). Quantitative analysis of immunopositive staining showed increased S100A9 staining in diseased tissue compared to healthy ($p=0.0002$, 14.2 fold increase); S100A9 staining decreased post-SAD treatment in patients whose pain resolved compared to diseased tissue ($p=0.03$, 4.5 fold decrease). There was no significant difference between healthy and treated tissue ($p=0.97$).

Qualitative analysis of S100A9 staining showed consistent low-level expression among resident tissue cells in healthy samples (Figure 3.7, A). Where S100A9 expression was present in resident cells of healthy tissue, it was primarily cytoplasmic. The intensity of expression in healthy tissue was highest around blood vessels and adjacent cells, where some extracellular staining was seen. Diseased tissues showed significant up regulation of S100A9 in the cytoplasm and ECM in all locations compared to healthy samples (Figure 3.7, B). Treated tissues showed similar characteristics to healthy samples: low-level nuclear and cytoplasmic expression among resident tissue cells with higher intensity in blood vessels (Figure 3.7, C).

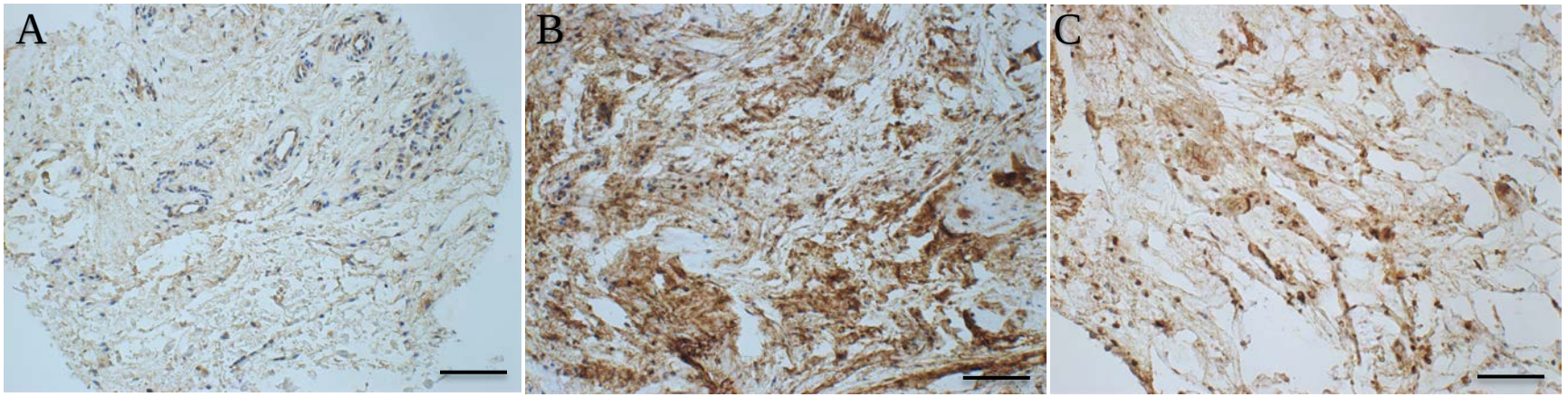


Figure 3.5 Photomicrographs of immunohistochemistry of (A) healthy control, (B) diseased, and (C) pain-free post-treatment supraspinatus tendon samples. Stained with anti-HIF-1α antibody; DAB staining is brown. Nuclear counterstain is hematoxylin (blue). Scale bar = 100μm

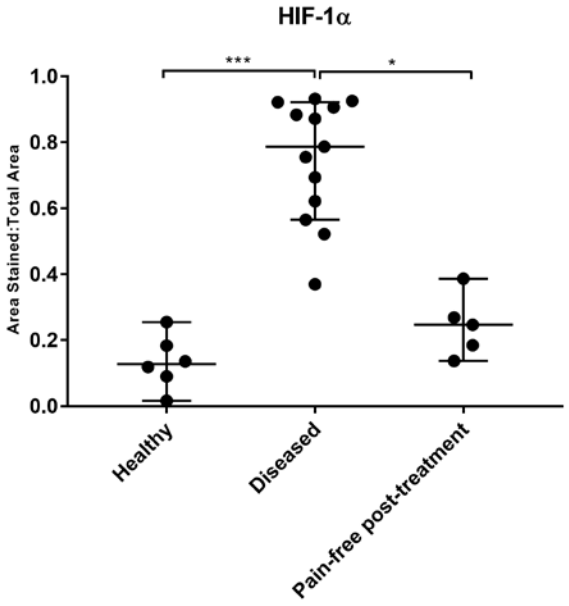


Figure 3.6 Quantitative analysis of immunopositive staining for HIF-1α in healthy, diseased, and post-treatment pain-free tendons. Outcome measure is the percentage area stained = total number of positively stained pixels / the total number of pixels. Healthy (n=6), diseased (n=13), post-treatment pain-free (n=5). Bar represent median value with 95% confidence intervals. Data were analyzed using pairwise Mann-Whitney U tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

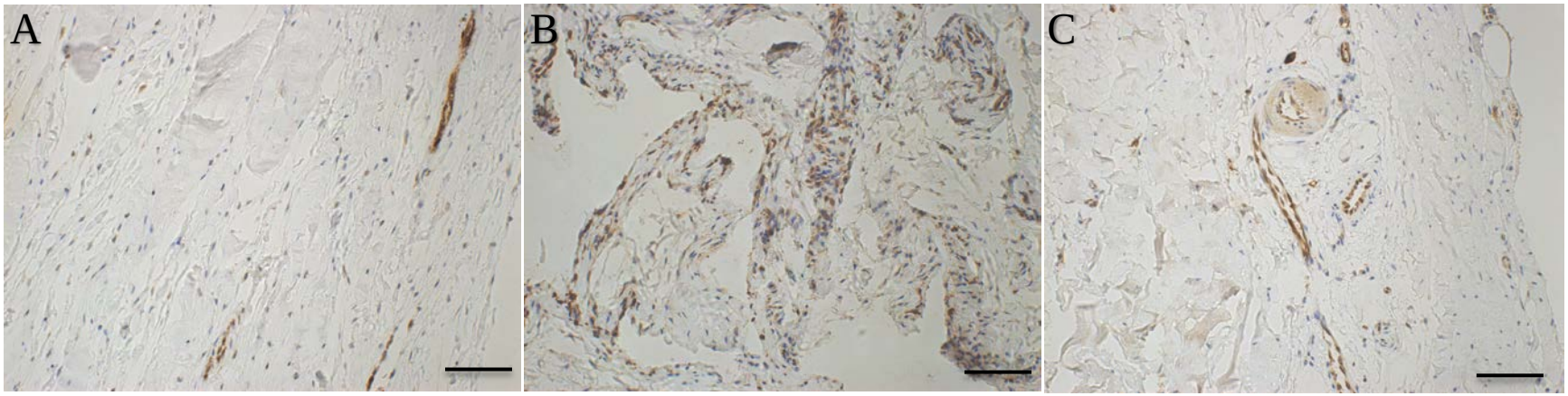


Figure 3.7 Photomicrographs of immunohistochemistry of (A) healthy control, (B) diseased, and (C) post-treatment pain-free supraspinatus tendon samples. Stained with anti-S100A9 antibody; DAB staining is brown. Nuclear counterstain is hematoxylin (blue). Scale bar = 100µm

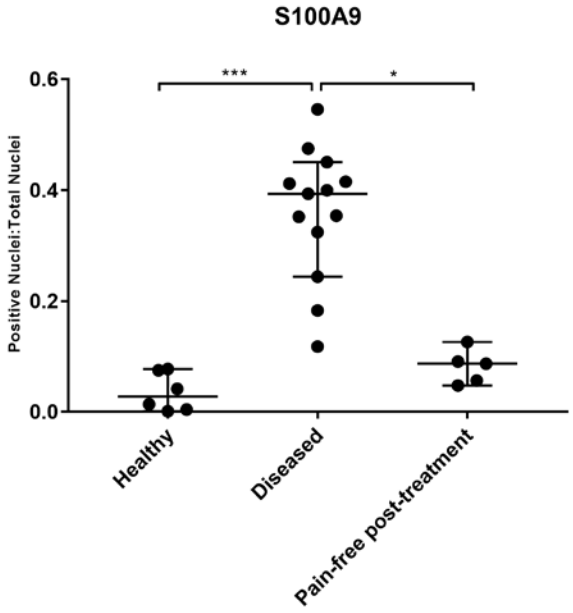


Figure 3.8 Quantitative analysis of immunopositive staining for S100A9 in healthy, diseased, and post-treatment pain-free tendons. Outcome measure is the cell staining ratio = total number of positively stained nuclei / the total number of nuclei. Healthy (n=6), diseased (n=13), post-treatment pain-free (n=5). Bar represent median value with 95% confidence intervals. Data were analyzed using pairwise Mann-Whitney U tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3.3 IL-33 protein expression is reduced in diseased tendons

IL-33 expression in healthy, painful diseased, and pain-free post-SAD supraspinatus tendons is shown in Figures 3.9 and 3.10. Healthy and treated tissue showed virtually identical IL-33 expression ($p > 0.99$). IL-33 expression was significantly higher in healthy tissue compared to diseased ($p = 0.0006$, 2.8 fold increase). The level of IL-33 expression was also significantly higher in post-SAD compared to diseased ($p = 0.01$, 2.6 fold increase).

Based on visual qualitative analysis of IL-33 staining showed consistent high-level expression in healthy and treated tissues (Figure 3.10, A & C). In these tissue groups, expression was primarily nuclear: cytoplasmic expression was common and high in intensity. Some extracellular expression was also present. Diseased tissues showed a reduction in nuclear expression of IL-33 compared to healthy or treated tissue (Figure 3.9 & 3.10, B).

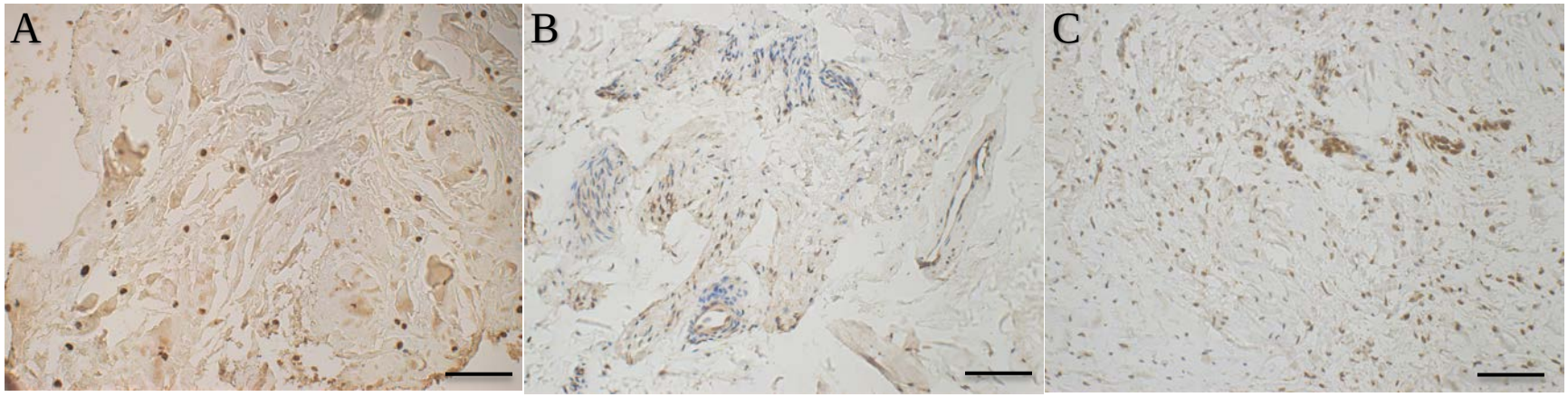


Figure 3.9 Photomicrographs of immunohistochemistry of (A) healthy control, (B) diseased, and (C) post-treatment pain-free supraspinatus tendon samples. Stained with anti- IL-33 antibody; DAB staining is brown. Nuclear counterstain is hematoxylin (blue). Scale bar = 100µm

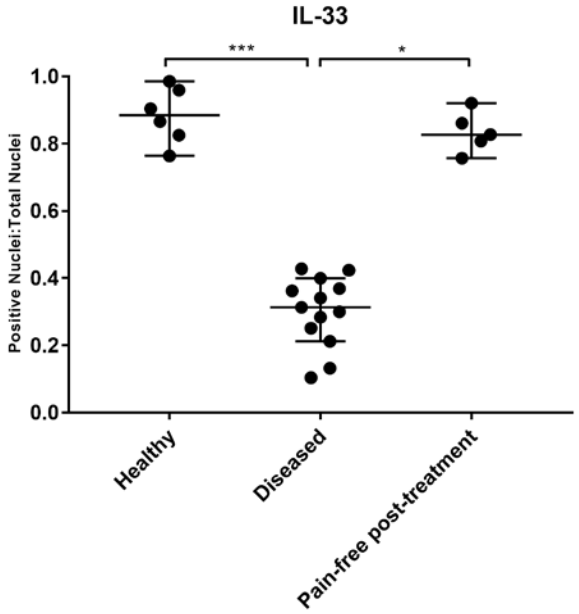


Figure 3.10 Quantitative analysis of immunopositive staining for IL-33 in healthy, diseased, and post-treatment pain-free tendons. Outcome measure is the cell staining ratio = total number of positively stained nuclei / the total number of nuclei. Healthy (n=6), diseased (n=13), post-treatment pain-free (n=5). Bar represent median value with 95% confidence intervals. Data were analyzed using pairwise Mann-Whitney U tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3.4 HMGB1 protein is increased in pain-free post-treatment tendons

HMGB1 expression in healthy, painful diseased, and pain-free post-SAD supraspinatus tendons is shown in Figures 3.11 and 3.12. Quantitative analysis of immunopositive staining showed that HMGB1 expression was primarily up regulated in tissues post-treatment. There was no significant difference in HMGB1 expression between healthy and diseased tendon tissues ($p>0.99$). Nuclear expression of HMGB1 was increased in pain-free post-treatment tissues compared to both healthy and painful diseased tissues ($p=0.006$ for both comparisons, 6.6 fold increase from healthy tissue, 5.3 fold increase from diseased tissue).

Based on visual qualitative analysis of HMGB1 staining showed that its expression is only nuclear in healthy controls and primarily nuclear in diseased tissue with some cytoplasmic expression near blood vessels (Figure 3.11, A & B). Treated tissue showed nuclear and cytoplasmic expression in all tissue locations with some low-level extracellular staining (Figure 3.11 C).

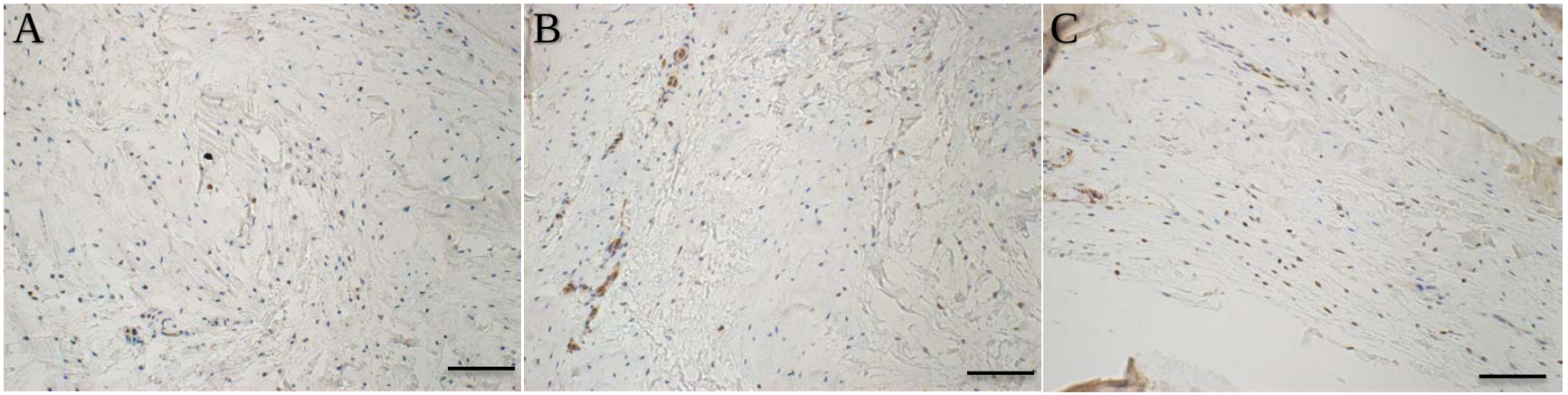


Figure 3.11 Photomicrographs of immunohistochemistry of (A) healthy control, (B) diseased, and (C) post-treatment pain-free supraspinatus tendon samples. Stained with anti-HMGB1 antibody; DAB staining is brown. Nuclear counterstain is hematoxylin (blue). Scale bar = 100µm

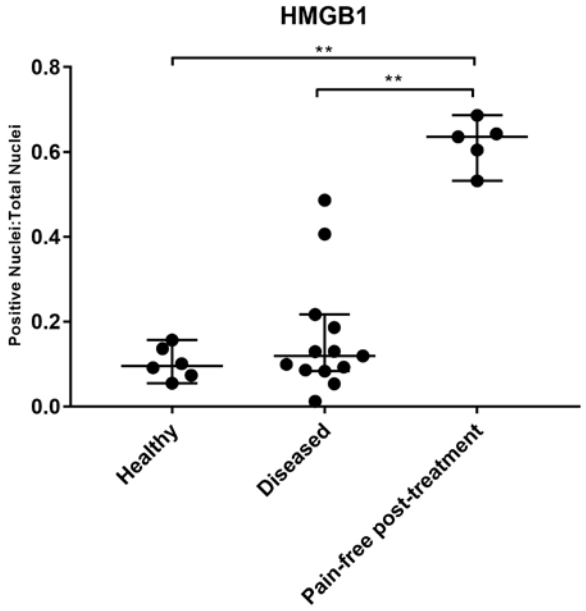


Figure 3.12 Quantitative analysis of immunopositive staining for HMGB1 in healthy, diseased, and post-treatment pain-free tendons. Outcome measure is the cell staining ratio = total number of positively stained nuclei / the total number of nuclei. Healthy (n=6), diseased (n=13), post-treatment pain-free (n=5). Bar represent median value with 95% confidence intervals. Data were analyzed using pairwise Mann-Whitney U tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3.5 Immunofluorescence

Since our IHC staining was not cell-specific, we were interested to see if there was co-localization of alarmins or HIF-1α with a cell marker for macrophages (CD68) in diseased tendon samples. Confocal images illustrating the expression of S100A9, HMGB1, HIF-1α, CD68, and POPO-1, a universal marker for cell nuclei, are shown in figures 3.13 and 3.14. Confocal images show that HIF-1α, S100A9, and IL-33 are expressed by resident cells (CD68- cells: tenocytes, epithelial cells, etc.) and macrophages (CD68+ cells). HMGB1 expression does not show much co-localization with CD68 (Figure 3.14). However, there is notable expression of HMGB1 in CD68- cells. The intensity of HMGB1 expression appears to be highest in epithelial cells and around blood vessels (blood vessels identified are circled in red).

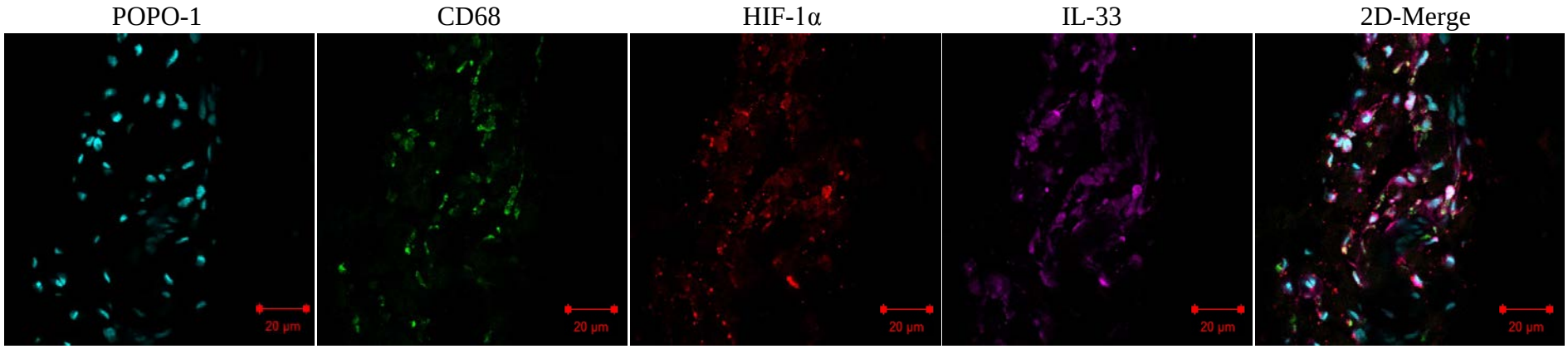


Figure 3.13 Representative confocal images shows markers for resident macrophages (CD68, Green), HIF-1α expression (Red), and IL-33 expression (Purple) in diseased tissue. Nuclear counterstain was POPO-1. Scale bar = 20µm

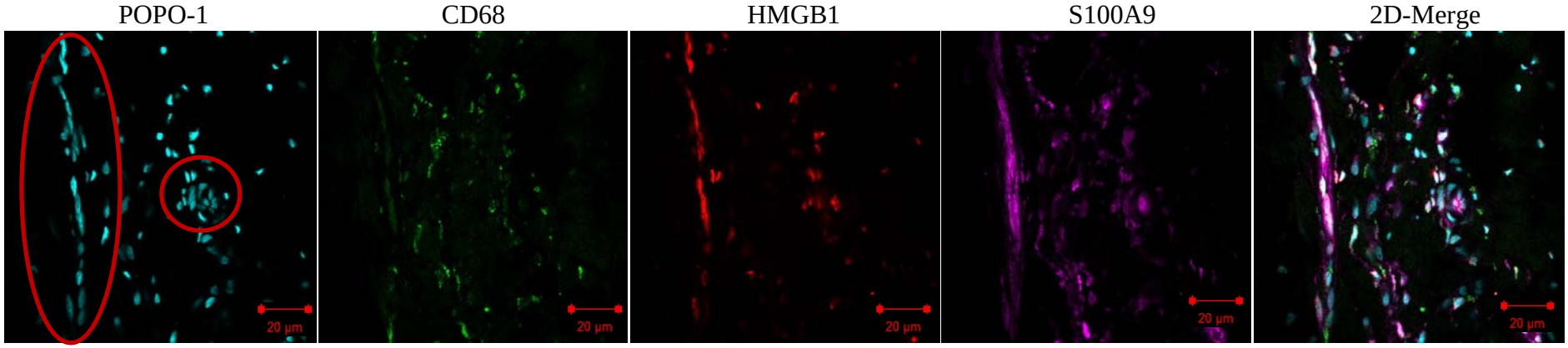


Figure 3.14 Representative confocal images shows markers for resident macrophages (CD68, Green), HMGB1 expression (Red), and S100A9 expression (Purple) in diseased tissue. Nuclear counterstain was POPO-1 and a blood vessels are circled. Scale bar = 20µm

3.4 Limitations

A limitation of this study was the relatively small number of patients studied in healthy and post-treatment groups. Additionally, the diseased and treated samples did not come from the same patient: before and after treatment. Matching pre- and post- treatment samples would provide more robust measurements and allow for more refined conclusions. Age related changes may contribute to differences in expression between healthy and diseased tissues. Our healthy samples came from patients undergoing shoulder stabilization surgery and therefore may not be truly normal controls. Despite the limitations, these were the best available healthy tendons samples, reflected by a Bonar score of 1. The Oxford Shoulder Score (OSS), like all patient-reported outcomes, is an imperfect measurement of function and pain, and is prone to bias. Post-treatment samples were in patients whose pain resolved, but no information is gather regarding differences in function and/or strength after treatment. It is difficult to differentiate between functional and pain outcomes without assessing mechanical properties.

3.5 Discussion

The emerging hypothesis that tendon disease results from abnormal inflammatory signalling is supported by data generated from this work. Atypical HIF-1α, S100A9, IL-33, and HMGB1 immunostaining have been implicated in the initiation and propagation of various chronic inflammatory and autoimmune disorders [Chan 2012]: documenting their immunostaining profiles in tendon tissue highlights potentially shared mechanisms. This study highlights potential pathogenic roles for HIF-1α and S100A9 up regulation in TD. Nuclear expression of IL-33 may have a protective or homeostatic role in tendons that is lost with the onset of disease. HMGB1 was up regulated in post-treatment pain-free tendon tissue, suggesting it may have regenerative potential through HMGB1-stimulated transit of progenitor cells across

the endothelial layer, similar to what has been found in cardiac disease [Mitola 2006, De Mori 2007, Sorci 2004, Kitahara 2008, Oozawa 2008].

Previous work on musculoskeletal disorders has shown that inflammatory resolution can become dysregulated over time and that this failure can result in fibrosis and hyperplasia [Akbar 1997]. Fibroblast and macrophage plasticity is often the result of excessive exposure to pro-inflammatory cytokines/pathways, primarily PG-E2, NF-kβ, Cox-2, IL-1α/β and TGF-β [Buckley 2001, Smith 1997, Zhang 1998, Zhou 1999]. There is increasing evidence that activation of the immune system after tissue trauma, stress, or injury is in large part due to the interactions of alarmins released from necrotic cells with their associated receptors (TLRs) [Yoshioka 2012]. Alarmins have known intra- and extracellular roles in the activation of and/or protection from several inflammatory signalling pathways and have been implicated in cascading effects on tissue morphology and resident cell phenotype of various musculoskeletal disorders [Chan 2012]. Recent studies have shown that diseased tendon cells are primed to inflammatory mediators [Dakin 2015]. However, it is not known what factors or environments contribute to the increased pro-inflammatory activity of tendon cells.

In this study we documented that the immunostaining patterns of HIF-1α, S100A9 and IL-33 are similar in healthy and treated post-treatment pain-free supraspinatus tissue; these patterns are significantly altered in diseased supraspinatus tendon. Our results are consistent with the expression patterns shown in inflammatory disorders, including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis, providing support for the concept that dysregulation of these factors may prime tissues to increasingly react to future inflammatory stimuli [Konisti 2012, Karhausen 2004, Rosenberger 2007, Distler 2004, Yao 2008].

HIF-1α immunostaining was significantly increased in diseased tissue compared to healthy and treated tissue. Healthy and treated tissue showed similar low-level immunostaining patterns, although treated tissue had consistently higher levels than healthy. In diseased tissue, HIF-1α was significantly up regulated in the ECM, the cytoplasm, and nuclei. These results are consistent with previous findings of HIF-1α in chronic inflammatory/autoimmune disorders and previous studies in tendon tissue [Deng 2016, Barreto 2003, Millar 2012]. Stabilization of HIF-1α with subsequent up regulation of HIF-1 is known to stimulate the release of alarmins S100A9, HMGB1, and IL-33 in inflammatory disorders [Vallabhapurpu 2009]. Up regulation of HIF-1 is also known to stimulate expression of VEGF, which has shown to form a positive feedback loop by further stabilizing HIF-1α in all cells. Extracellular HIF-1α has various known pro-inflammatory effects in addition to facilitating alarmin secretion: it protects myeloid cells against apoptosis and it drives macrophages towards an M1 phenotype [Cramer 2003, Sitkovsky 2004, Vanderkooi 1991, Roos 1973]. HIF-1 activation also leads to increased expression of pro-fibrotic mediators [Cannito 2014]. HIF-1α up regulation likely plays similar roles in tendon tissue: our results show that S100A9 and IL-33 dysregulation occurred alongside HIF-1α upregulation.

S100A9 immunostaining was also significantly increased in diseased tissue compared to healthy and post-treatment pain-free tissue. Healthy and post-treatment pain-free tissue showed almost identical immunostaining patterns: primarily low-level cytoplasmic staining. S100A9 has various regulatory roles in the cytoplasm of all cells, including Ca^{2+} regulation, protein phosphorylation, enzyme activity, transcription factor activity, and cellular proliferation and differentiation [Donato 2001]. Our results suggest that low-level cytoplasmic expression of S100A9 is typical of normal function: S100A9 primary function in healthy tissues is cytoplasmic Ca^{2+} regulation. In our diseased supraspinatus samples, S100A9 was significantly up regulated in

the cytoplasm and the ECM. Previous studied have shown that extracellular S100A9 induces pro-inflammatory responses, including facilitating cell adhesion of infiltrating cells and promoting monocyte differentiation [Foell 2004, Roth 2003]. Monocytes stimulated by S100A9 undergo phagocytosis, secrete proteolytic enzymes, PGE1, and PGE_2 [Zwadlo 1988]. S100A9 has implicated in upregulating NF-κβ expression, resulting in increased secretion of TNF-α and IL-1 [Vogl 2007, Roth 2003].

S100A9 overexpression in stressed tissues has been shown to result in a positive feedback mechanism if not properly resolved: the acute inflammatory responses activate downstream expression of TNF-α, a potent stimulator of S100A9 expression [Roth 2003, Gebhardt 2006]. Even if resolution eventually occurs, continued insults to tissues can cause cell phenotype changes over time and predispose tissues to failed healing: S100A9 from myeloid cells promotes further leukocyte recruitment, priming myeloid cells for more intense inflammatory responses following injuries [Roth 2003, Gebhardt 2006]. S100A9 has not previously been looked at in tendon tissues. The up regulation of S100A9 is analogous to chronic inflammatory and autoimmune disorders. These data potentially implicate S100A9 as an important initiator and propagator of chronic inflammation in tendon diseases.

IL-33 immunostaining was significantly decreased in diseased tissue compared to healthy and treated tissue. Healthy and treated tissue showed similar immunostaining patterns: consistent nuclear and cytoplasmic expression with treated tissues showing some extracellular immunostaining. Diseased tissue samples showed almost a substantial loss in nuclear/cytoplasmic IL-33 expression. These results are consistent with the known protective role of nuclear IL-33 against pro-inflammatory stimuli. Physical interaction of intracellular IL-33 with NF-κβ sequesters NF-κβ and curbs any incoming pro-inflammatory or fibrotic signals from

other receptors [Ali 2011]. IL-33 is considered an alarmin because resident tissue cells release it upon tissue damage, stress or infection [Saluja 2015, Martin 2016]. Extracellular IL-33 can stimulate INFγ production and lead to up regulation of NF-κβ signalling pathways in infiltrating cells [Cascao 2010, Fournier 2012**]**. However, the pro-inflammatory 'alarmin' role of extracellular IL-33 does not appear to be mutually exclusive with its protective nuclear function in resident cells [Ali 2011]. Our results suggest that the protective nuclear IL-33 is decreased in the resident cells of diseased tissue and may in part explain how inflammatory resolution fails over time. Previous studies have concluded that this loss of protective IL-33 is likely to be dependent on unexplained environmental conditions [Martin 2016].

HMGB1 has become categorized as an alarmin due to its involvement in mobilization and activation of immune cells; its overexpression has been implicated in chronic inflammatory disorders [Klune 2008, Chan 2012]. Stimuli for the secretion of HMGB1 from monocytes, macrophages, and dendritic cells include cellular stress, PAMPs, and cytokines, including TNF-α, IL-1, and INFγ [Wang 1999, Youn 2006, Jiang 2007, Jiang 2006, Wang 2006]. The primary receptors for extracellular HMGB1 are RAGE, TLR2, and TLR4 [Hori 1995, Chan 2012]. Activation of RAGE by HMGB1 promotes chemotaxis and cytokine production through the up regulation NF-κβ pathways [Palumbo 2007, Park 2003].

HMGB1 has also been suggested to have regenerative potential. HMGB1 can induce migration of stem cells towards inflamed regions, promoting repair and regeneration [Palumbo 2004]. Myoblasts migrating to stressed tissues to stimulate repair have been shown to be more effective through HMGB1-stimulated transit of these progenitors across the endothelial layer [Mitola 2006, De Mori 2007, Sorci 2004]. HMGB1 may also be important in protecting tissues from damage by inducing proliferation and positive cellular architecture changes [Kitahara 2008,

Oozawa 2008, [Degryse 2001, Porto 2006]. The restorative and pathogenic effects of HMGB1 appear to be mediated through the same receptors, RAGE, TLR2 and TLR4, although the mechanisms differentiating regeneration and pathogenesis are unknown [Mitola 2006, De Mori 2007, Sorci 2004, Degryse 2001, Chavakis 2007].

HMGB1 has not previously been studied in tendon tissues. We hypothesized that HMGB1 expression would be up regulated in diseased tissue due to its pro-inflammatory and pathogenic role in some musculoskeletal disorders. However, our results do not show HMGB1 dysregulation between healthy and diseased tendons. HMGB1 immunostaining was up regulated in post-treatment pain-free tendon tissue, suggesting it may have regenerative potential, similar to what has been found in cardiac disease, or yet unknown protective roles [Kitahara 2008, Oozawa 2008].

Chapter 4: Re-assessing the Pathological Paradigm of Tendon Disease: A New Era for Inflammation?

4.1 Discussion

The role of inflammation in the pathogenesis of tendon disease (TD) is a topic of ongoing debate. Prior to the turn of the century, inflammation was believed to contribute to the pain and pathology associated with non-acute, non-ruptured diseased tendons [Khan 1998, Khan 1999, Khan 2002]. During this time TD was often referred to as ‘tendinitis’ [Weiler 1992]. However, after multiple histological studies showed an absence of neutrophils in diseased tendons, the term ‘tendinitis’ was replaced with ‘tendinosis,’ indicating that inflammation was not central to tendon pathology [Puddu 1976, Astrom 1995, Jozsa 1997, Khan 1999, Khan 2002]. Subsequently, TD was reframed as primarily a multifactorial ‘degenerative’ disorder [Astrom 1995, Movin 1997, Hashimoto 2003, Rees 2006, Lewis 2008, Rees 2009, Rees 2014]. More recently, it has been suggested that this disease paradigm may have overlooked inflammation’s complex role in TD by precluding all inflammatory pathways, cells, and mediators on the pretext that an ‘inflammatory infiltrate’ is absent [Rees 2014, Dean 2012, Dean 2015]. These debates, coupled with an increased understanding of inflammation’s cellular and molecular roles in other musculoskeletal diseases, have led to a re-emergence of interest in the potential for inflammation to initiate and propagate TD. [Gilmore 2006, Gilmore 1999, Monaco 2004, De Andrea 2002, Cohen 2001, Chan 2012, Buckley 2001, Rees 2014].

Recent investigations about the role of immune cells and mediators in TD have provided substantial evidence for an inflammatory component in TD [Millar 2012a, Millar 2012b, Dean 2012, Dean 2013b, Dean 2015, Dakin 2014, Dakin 2015, Goodier 2016]. It has been suggested that alarmins, endogenous molecules released upon tissue damage, stress, or infection could play integral roles in TD [Millar 2013]. There have been a paucity of studies related to alarmins in

TD: understanding more about these inflammatory effectors in tendons may illuminate mechanisms analogous to musculoskeletal disorders whose inflammatory components have been more thoroughly examined [Chan 2012].

The overarching goal of this thesis was to examine the role of inflammation in the initiation and propagation of TD through two specific objectives: first to examine paradigm shifts and potential explanations for scientific equipoise regarding the role of inflammation in TD through a quantitative analysis of published mechanistic reviews and, second, to determine the protein expression of a set of alarmins (S100A9, HMGB1, & IL-33) and HIF-1α in three different groups of human supraspinatus tissue including healthy, persistently painful diseased, and treated pain-free tendons.

Results from the two scientific aims are synthesized to help elucidate the role of inflammation, with particular focus on alarmins, in the pathogenesis of TD. The findings may help to explain dissonance in the scientific and clinical community, and may provide future direction for therapeutic targets for the prevention and treatment of TD. If inflammatory cells and signals are further implicated in TD through an alarmin-mediated mechanism, the ongoing debate about the role of inflammation in TD could potentially be resolved.

The notion that inflammation plays a key role in TD and that the reframing of ‘tendinitis’ as ‘tendinosis’ was an oversimplification of a disease paradigm is supported by the data in this body of work. The resurgence of interest in the role of inflammation in TD reflects recent advances in cellular and molecular medicine as well as a growing appreciation for immune cells, mediators, and effectors as constituents of an ‘inflammatory component’ in the context of TD. An increased understanding of inflammation’s role in other musculoskeletal diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, sepsis, asthma, and

atherosclerosis helped to reignite this interest, sparking more thorough investigations of inflammation in TD [Gilmore 2006, Gilmore 1999, Monaco 2004, De Andrea 2002, Cohen 2001, Chan 2012, Buckley 2001, Rees 2014]. Alarmins and HIF-1α have been suggested as potential mediators of TD for similar reasons: investigations of these effectors in inflammatory and autoimmune disorders have implicated them in chronic inflammation and fibrosis [Millar 2013, Lawrence 2007, Buckley 2001, Buckley 2007, Cannito 2014, Chan 2012, Deng 2016]. Documenting expression profiles of specific alarmins and HIF-1α in healthy, diseased, and pain-free post-treatment human supraspinatus tendon tissues highlights potentially analogous mechanisms.

This work supports pro-inflammatory roles for HIF-1α and S100A9 in TD. Additionally, IL-33 appears to have protective and/or homeostatic roles in TD that are lost upon the onset of disease. The findings in this body of work with recent novel inflammatory discoveries in TD fit into known mechanisms of chronic inflammation and fibrosis of other musculoskeletal diseases. A novel finding from this research is that the immunostaining of HIF-1α, S100A9, and IL-33 are analogous to immunostaining in autoimmune disorders with established mechanisms of chronic inflammation, failure of resolution, and fibrosis [Lawrence 2007, Cannito 2014, Chan 2012, Deng 2016]. Of particular interest is HIF-1's role in promoting chronic inflammation: HIF-1 activity has been suggested to play a role in the switch from acute resolving to chronic inflammation by allowing immune cells to survive in a hypoxic environment [Deng 2016, Cramer 2003]. Ongoing immune responses have been associated with increased oxygen consumption and subsequent anaerobic metabolism of cells within the inflammatory lesion [Kominsky 2010, Eltzschig 2011]. A HIF-1-mediated metabolic shift has been proposed to explain these findings in autoimmune disease:

> "Recent studies have demonstrated that ongoing inflammatory and immune responses are associated with increased oxygen consumption, a process resulting in localized tissue hypoxia within inflammatory lesions (''inflammatory hypoxia"), in which hypoxia-inducible factor 1 (HIF-1), an oxygen-sensitive transcription factor that allows adaptation to hypoxia environments, has been shown to play an important function" [Deng 2016].

HIF-1 activity induces expression of VEGF, its associated receptors, and pro-fibrotic mediators, including PDGF, bFGF, and PAI-1 [Peyssonnaux 2005, Rosmorduc 2010]. Up regulation of HIF-1α in myeloid cells protects them from apoptosis [Cramer 2003]. Hypoxia causes tendon cell necrosis, release of alarmins, particularly S100A9 and IL-33, with downstream up regulation of pro-inflammatory cytokines [Vallabhapurpu 2009]. Hypoxia also drives macrophages to a M1 phenotype, priming them to release pro-inflammatory cytokines, including IL-1β and TNF-α [Sitkovsky 2004, Vanderkooi 1991, Roos 1973, Hume 1978, Greiner 1994]. HIF-1 activity is up regulated physiologically through the stabilization of HIF-1α in low-oxygen environments and pathologically by induction of pro-inflammatory cytokines [Konisti 2012, Karhausen 2004, Rosenberger 2007, Distler 2004, Yao 2008, Deng 2016]. Pro-inflammatory cytokines, including IL-1β and TNF-α, act on cell-surface receptors and intracellularly through NF-κβ to reduce HIF-1α degradation, thus increasing HIF-1 activity.

These mechanisms form a feedback loop: the increased oxygen consumption from infiltrating immune cells causes up regulation of HIF-1 activity via physiological hypoxia, which has downstream effects on alarmin secretion, immune cell survival, stromal cell phenotype, and further HIF-1 activity via pathological induction of HIF-1α stabilization [Deng 2016, Peyssonnaux 2005, Rosmorduc 2010]. A failure of acute inflammation to resolve has been shown to be, in large part, due to pathological fibroblast activation and subsequent dysregulation of inflammatory cytokines, chemokines, and prostaglandins [Buckley 2001]. These findings

from autoimmune and arthritic diseases suggest that a state of chronic inflammation can develop over time through pathological activation of resident tissue cells towards a pro-inflammatory phenotype. Once a threshold of resident cell activity is reached, inflammation fails to resolve, resulting in chronic inflammation and fibrosis [Buckley 2001, Lawrence 2007, Cannito 2014, Chan 2012, Deng 2016]. Chronic inflammation and fibrosis in TD may also be partially driven by a HIF-1 mediated metabolic shift: our data shows that HIF-1α and S100A9 are up regulated in TD and that IL-33 loses its protective/homeostatic roles with the onset of disease.

The totality of evidence suggests that pathological activation of resident tendon cells in TD through 'inflammatory hypoxia' [Lawrence 2007, Buckley 2001, Buckley 2007, Chan 2012, Deng 2016] can transform an acute resolving immune response into chronic inflammation with subsequent fibrosis [Millar 2012, Millar 2013, Dean 2012, Dean 2015, Dakin 2014, Dakin 2015]. Proposed mechanisms for the initiation and propagation of chronic inflammation and fibrosis in TD via HIF-1 and alarmins are shown in figures 4.1 and 4.2. These diagrams integrate the findings in this body of work on HIF-1, S100A9, IL-33, and HMGB1 with 1) proposed mechanisms of alarmin activity and inflammation from various musculoskeletal diseases 2) proposed mechanisms of fibroblast activation in sustaining chronic inflammation 3) recent findings that immune cells and inflammatory/fibrotic mediators are present in TD and 4) recent findings that resident tendon tissue macrophages are primed to release these mediators.

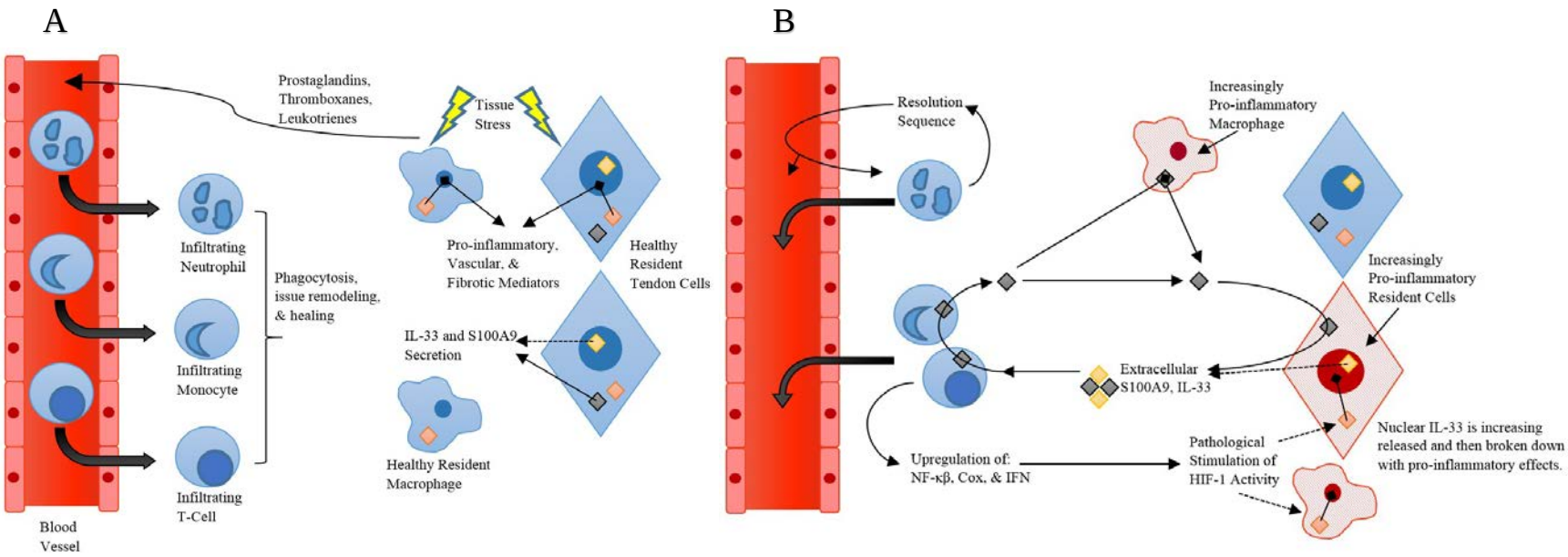


Figure 4.1 Development of chronic inflammation in tendon tissues: (A) Tissue stress, acute injury, and/or vessel damage occurs in healthy tendon tissue, resulting in temporary physiological hypoxia. HIF-1α stabilizes and translocates to the nucleus of resident tendon cells and macrophages, activating the HIF-1 transcription factor. Activated HIF-1 upregulates expression/secretion of vascular and fibrotic mediators in resident cells. At the same time, stressed cells release S100A9 and pro-inflammatory cytokines. IL-33 is secreted in small amounts, but is largely sequestered in tenocyte nuclei, protecting the cell against excessive up regulation of pro-inflammatory cytokines via the protein complex NF-κβ. Platelets/neutrophils arrive at injury tissue, releasing additional cytokines/factors to recruit/stimulate monocytes to differentiate, remove necrotic materials, induce fibroblast proliferation, and extracellular matrix remodeling. (B) During tissue remodeling, pro-resolving mediators are released, initiating the termination sequence. Additionally, several feedback loops form between resident and infiltrating cells, resulting in lasting effects on tissue environment and resident tendon cell phenotype. S100A9 secreted from tenocytes stimulates immune cells to upregulate NF-κβ and Cox activation pathways in addition to stimulating S100A9 secretion. S100A9 then stimulates resident cells to upregulate NF-κβ, Cox, and IFN pathways in addition to further S100A9. Up regulation of pro-inflammatory pathways leads to increased concentrations of cytokines/fibrotic mediators/proteolytic enzymes: many of which are pathological stimuli of HIF-1α stabilization/secretion from all cell types. HIF-1α and VEGF also form a positive feedback loop, resulting in increased vascularity and fibrosis. As the initial inflammatory episode resolves, resident macrophages/tenocytes become primed to future inflammatory and hypoxic stimuli, driving them towards a pro-inflammatory phenotype.

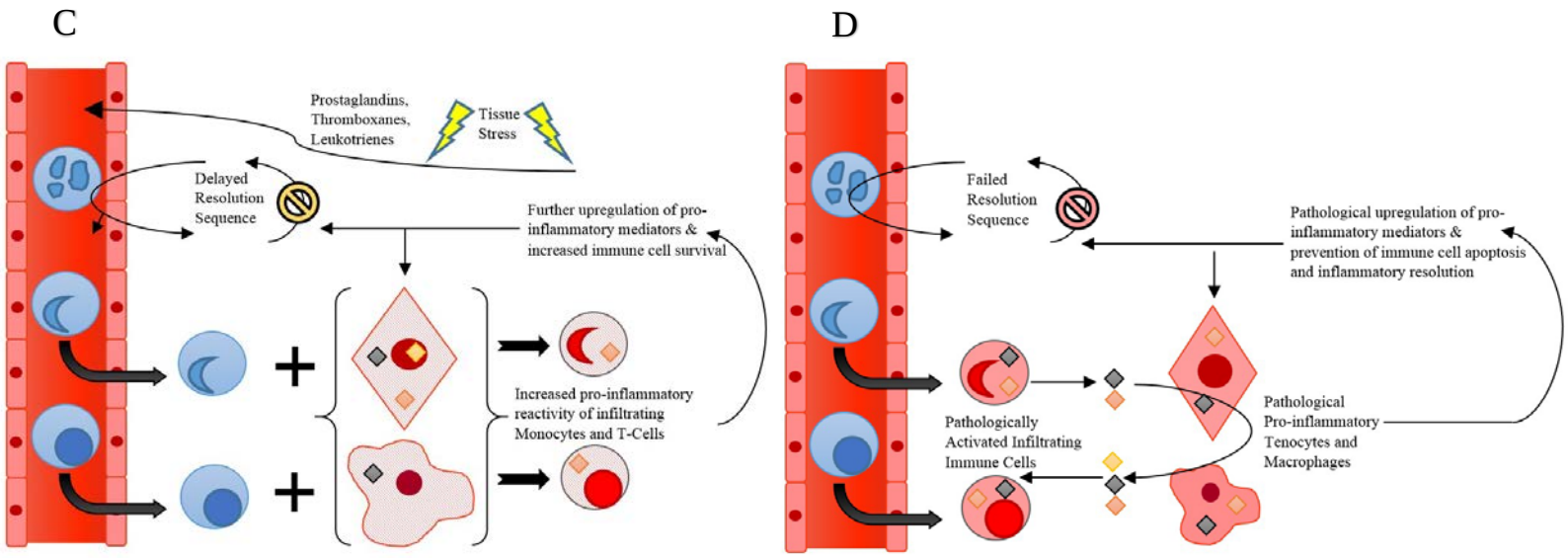


Figure 4.2 Development of chronic inflammation in tendon tissues continued: (C) Subsequent tissue injuries become dysregulated due to increased reactivity of resident cells to inflammatory stimuli. Pro-inflammatory mediators are again released in response to acute injury, with subsequent infiltration of leukocytes via release of alarmins/cytokines from resident cells. The heightened interaction between infiltrating and resident cells increase the overall metabolic activity of the tissue, causing increased stabilization of HIF-1α in all cells and therefore increased immune cell survival. Due to increased reactivity of resident cells, HIF-1 activity is up regulated longer, resulting in oxygen-depletion and glycolytic energy production. Inflammation takes longer to resolve as arachidonic acid-derived prostaglandins – stimulated from the various cascades of HIF-1α stabilization – begin to overtake the effects of pro-resolving mediators. (D) After a certain threshold unknown threshold of HIF-1α stabilization and/or tissue metabolic activity is reached, infiltrating immune cells (primarily monocytes/T-cells) become constitutively activated by resident tenocytes/macrophages. The effects of pro-resolving mediators are completely overwhelmed and/or dysregulated by the up regulation of various pro-inflammatory mechanisms. This state, known as inflammatory hypoxia, results in constant up regulation of inflammatory/vascular/fibrotic/proteolytic factors. The up regulation of these factors has deleterious consequences for tendon tissue structure and composition.

4.2 Summarized Findings

Systematic Review of Mechanistic Literature and Conclusions about the Role of Inflammation in TD

- ❖ There was little support for an inflammatory paradigm in TD until recent published reviews (2012-2015).
- ❖ Prior to 2012, the majority of published mechanistic reviews did not discuss monocytes, macrophages, or lymphocytes in TD, rather they focused on the lack of PMNs, 'inflammatory infiltrate,' or immune cells were not discussed.
- ❖ Prior to 2012, less than half of published mechanistic reviews included discussions of signalling molecules, growth factors, activation pathways, and/or molecular effectors in TD; in contrast to X% included in discussion post-2012.
- ❖ Reviews that concluded there was a link between inflammation and TD had significantly higher mean quality scores, adapted from AMSTAR, and/or were significantly more likely to include critical analyses of relevant primary literature, compared to reviews that refuted a role of inflammation in TD.
- ❖ Reviews were more likely to conclude inflammation is important in TD if they were of higher methodological quality or they included critical analyses of relevant primary literature.
- ❖ Reviews that included monocytes, macrophages, or lymphocytes, in their discussions were 5.2-times more likely to support that inflammation contributes to TD compared to reviews that only discussed PMNs or an 'inflammatory infiltrate.'
- ❖ Reviews that discussed signalling molecules, growth factors, activation pathways, and/or molecular effectors 4.9-times more likely to support that inflammation contributes to TD compared to reviews that did not include a discussion of these factors.
- ❖ These data show there has been growing support for an inflammatory component to TD, particularly among reviews of higher quality and those that utilized more robust definitions of the 'inflammatory component.'

Alarmins and HIF-1α in Healthy, Persistently Painful Diseased, and Post-treatment pain-free Supraspinatus Tendons

- ❖ The immunopositive staining of HIF-1α and S100A9 were significantly higher in persistently painful diseased tissue compared to healthy and post-treatment pain-free tissue.
- ❖ IL-33 immunopositive staining was significantly lower in persistently painful diseased tissue compared to both healthy and post-treatment pain-free tissue.
- ❖ HMGB1 showed lower immunopositive staining in healthy and diseased supraspinatus tendons, compared to post-treatment pain-free tendon tissues.
- ❖ These variable expression patterns implicate alarmins and HIF-1α as potential mediators of TD.

4.3 Conclusion

This body of work supports a role of inflammation in TD and suggests that alarmin-mediated mechanisms may be important in the pathological activation of tendon cells. To the best of our knowledge this research is the first to 1) systematically evaluate published reviews' conclusions about the role of inflammation in TD and analyze potential correlates of those conclusions through standardized data collection and statistical analysis, 2) perform quantitative analysis of HMGB1 and S100A9 immunostaining in healthy and diseased tendon tissue, 3) assess the level of expression of alarmins/HIF-1α in non-ruptured late-stage diseased tendons compared to healthy and pain-free (supraspinatus tendons) and, 4) investigate if changes in expression of alarmins HMGB1/S100A9/ IL-33 and HIF-1α correlate with resolution of pain post-treatment.

The work submitted in this thesis suggest that there is some evidence for an integral role of inflammation in TD and that it may be an alarmin-mediated pathology. Pathological activation of resident tendon cells and macrophages through alarmin involvement may sustain chronic inflammation and lead to fibrosis. This may be of significant clinical relevance as it may lead to novel therapeutic targets and shift management approaches.

4.4 Future Work

Future work in TD should evaluate assumptions in the proposed alarmin-mediated pathological model and address the limitations of the research conducted. Larger studies that include more subjects in different stages of disease (e.g. early, moderate, and advanced-stage TD) may provide more robust conclusions. Before and after treatment samples obtained from the same patient will provide more precision than independent samples from subjects with different

tendon disease profiles as conducted in this research and will provide more definitive conclusions regarding causality.

The research in this body of work suggests that alarmins are up regulated in TD, however, the proposed interactions between alarmins and HIF-1α with infiltrating immune cells are based on research in arthritic and cardiac diseases. An experimental study could lend credibility to analogous alarmin-mediated mechanisms by examining if monocytes, macrophages, and/or lymphocytes are present in diseased tendons and respond to alarmin stimulation. Similarly, interactions between alarmins and other inflammatory stimuli with infiltrating immune cells has been implicated in a failure of inflammation to resolve in autoimmune diseases. To date there are no studies that examine the presence of pro-resolving mediators in TD. A future study could evaluate the presence of pro-resolving mediators in TD in different stages of disease (e.g. early, moderate, and advanced-stage TD). Subsequently, studies could evaluate if the expression of pro-resolving mediators (e.g. lipoxins, resolvins, protectins, and maresins) changes in response to alarmin stimulation.

Understanding how pro-resolving mediators behave in TD and determining if there is a correlation with alarmin secretion could help to confirm or refute assumptions in the proposed pathological paradigm. A more robust understanding regarding how acute inflammation turns into chronic inflammation may provide insight for novel treatment methods in patients with chronic tendon inflammation.

Future studies might also examine how the expression of cytokines and fibrotic mediators are affected in response to various alarmin stimulation. TD has classically been considered ‘degenerative’ but it may be more accurate to describe structural and compositional changes in diseased tendons as a result of fibrosis. It is possible that alarmin up regulation promotes fibrosis

in TD by increasing pro-inflammatory stimuli, similar to what has been found in autoimmune diseases.

Finally, future studies should examine the relationship between HIF-1 activity and anaerobic metabolism bi-products (e.g. lactic acid). The proposed pathological paradigm assumes a pathological state of hypoxia due to ongoing immune responses and anaerobic metabolism of cells within the inflammatory lesion. It has been suggested that a HIF-1-mediated metabolic shift allows for infiltrating immune cells to adapt and increase survivability. This body of work suggests that HIF-1 activity plays a key role in the pathogenesis of TD but it is not known if this affects immune cell survivability or causes any metabolic shifts within resident or infiltrating cells as it does in other chronic inflammatory diseases. Studies have found increased lactic acid levels in diseased tendons, however no studies have examined the interaction of HIF-1, alarmins, and anaerobic metabolism. Such data could advance the knowledge generated from this thesis regarding the role of inflammatory molecules and their interactions in the pathogenesis of TD, and provide potential insight into therapeutic approaches.

Appendix A: Data extraction sheet for mechanistic reviews

For any question – answer "P" if categorization cannot be easily determined, that question will be answered with the input of the expert panel: SD, SS, and AC

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1) What are the review's overall conclusions about the role of inflammation in TD?
    0 = Pathogenesis is not discussed or mentioned briefly as multifactorial
    1 = TD is described as degenerative with an absence of a chronic inflammatory component
    2 = Inflammation is described as a contributing factor in pathogenesis of TD
    3 = Inflammation is discussed but author's conclusion about its importance on pathogenesis is inconclusive or not readily apparent.
    X = Other/not applicable/panel determines answer is unclear
2) What immune cells are discussed during discussions on the role of inflammation in TD
    0 = Immune cells in diseased tendon tissue are not discussed or mentioned
    1 = Inflammatory infiltrate or cells are mentioned but are not defined/outlined
    2 = Neutrophils and/or PMNs the only immune cells including in cellular discussions
    3 = Monocytes, macrophages, or lymphocytes are included in cellular discussions
    X = Other/not applicable/panel determines answer is unclear
3) Are signalling molecules, growth factors included during discussions on the role of inflammation in TD and how are they defined/discussed in relation to 'inflammation' as a whole? (Cytokines, prostaglandins, inflammatory mediators etc.)
    0 = Signalling molecules/effectors are not discussed, defined, or mentioned within the context of TD
    1 = Signalling molecules/effectors' role in the context of TD is discussed but is treated/examined as separate from the umbrella of inflammation
    2 = Signalling molecules/effectors' role in the context of TD is discussed and is treated/examined as an integral part of the umbrella of inflammation
    X = Other/not applicable/panel determines answer is unclear
4) What are the review's conclusions about the role these signalling molecules play in the pathogenesis of TD?
    0 = Review does not mention these signalling molecules/effectors in context of TD
    1 = Review states that molecules/effectors do not appear to play a role in pathogenesis to chronicity
    2 = Review states that molecules/effectors have pathogenic effects primarily during early stages of disease
    3 = Review states that molecules/effectors have sustained pathogenic effects throughout all stages of disease
    X = Other/not applicable/panel determines answer is unclear
5) Is the review a narrative overview or does it have a specific area of focus?
    0 = Narrative
    1 = Specific
    X = Other/not applicable/panel determines answer is unclear

6) What type of journal is the review published in?
    0 = Basic science orthopaedic
    1 = Clinical orthopaedic
    2 = Orthopaedic hybrid
    3 = Non-orthopaedic basic science
    4 = Non-orthopaedic clinical
    5 = Other
7) Journal impact factor (most current available): _____
8) Number of citations: _____
9) Year published: _____

Appendix B: Methodological quality and scientific rigor of included mechanistic reviews

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For any question – answer "P" if categorization cannot be easily determined, that question will be answered with the input of the expert panel: SD, SS, and AC

Search Methods:

1) Does the review clearly state its purpose/objective in the abstract or introduction?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
2) Does the review explicitly describe the outcomes to be documented (if any) in its methods?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
3) Was a systematic literature search performed? (At least two databases)
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
4) Was there duplicate study selection and data extraction?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
5) Was a PRISMA flow diagram included with inclusion/exclusion criteria?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
6) Were the characteristics of the included studies provided in a table?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear

Level of Evidence/Methods of Analysis:

7) What was level of evidence used to discuss/evaluate research question?
    2 = Randomized control trials (RCT) or meta-analysis of RCTs (Level 1); Prospective comparative study or meta-analysis of Level 2 studies or Level 1 studies with inconsistent results (Level 2).
    1 = Retrospective cohort study, Case-control study, Meta-analysis of Level 3 studies
    0 = The review was an expert opinion: it did not critically engage with substantial primary literature on the relevant topic. OR other/not applicable/the panel determined answer is unclear
8) Was the methodological or scientific quality of the review's included studies formally assessed and documented?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
9) Were the scientific quality and/or depth of research of the included studies used appropriately and explicitly stated in formulating final conclusions?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear
10) Does the review pool data from original research for its own analysis (meta-analysis)?
    1 = Yes
    0 = Not met/not applicable/panel determined answer is unclear

11) Were the methods used to combine the findings of studies appropriate? For the pooling of results to be accurate and for the correct method to be used, studies need to be combinable and so their homogeneity needs to be assessed (for example using a statistical test such as the chi-square test for homogeneity or the I2 test for heterogeneity).

1 = Test(s) conducted to assess if studies are combinable

0 = Not met/not applicable/panel determined answer is unclear

12) Was the likelihood of publication bias assessed? An assessment of publication bias should include a combination of graphical aids (e.g., funnel plot, other available tests) and/or statistical tests (e.g., Egger regression test).

1 = Test(s) conducted to account for possible publication bias

0 = Not met/not applicable/panel determined answer is unclear

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Total quality score: _____

This quality score was adapted from AMSTAR (Original questions below):

1. Was an “a priori” design provided?
2. Was there duplicate study selection and data extraction?
3. Was a comprehensive literature search performed?
4. Was the status of publication (i.e. grey literature) used as an inclusion criterion?
5. Was a list of studies (included and excluded) provided?
6. Were the characteristics of the included studies provided?
7. Was the scientific quality of the included studies assessed and documented?
8. Was the scientific quality of the included studies used appropriately in formulating conclusions?
9. Were the methods used to combine the findings of studies appropriate?
10. Was the likelihood of publication bias assessed?
11. Was the conflict of interest stated?

Appendix C: Results of Reproducibility Sample, Kappa Coefficients

Data extractors: Michael Mosca (MM), Stephanie Dakin (SD), & Sarah Snelling (SS)

	MM vs. SD	MM vs. SS	SS vs. SD
Appendix A, Q1	0.828	0.839	0.783
Appendix A, Q2	0.889	0.889	0.706
Appendix A, Q3	0.833	0.690	0.847
Appendix A, Q4	0.722	0.722	1
Appendix A, Q5	1	1	1
Appendix B, Q1	0.800	0.800	1
Appendix B, Q2	1	0.800	0.800
Appendix B, Q3	1	1	1
Appendix B, Q4	0.737	1	1
Appendix B, Q5	1	1	1
Appendix B, Q6	1	0.737	1
Appendix B, Q7	1	0.615	1
Appendix B, Q8	0.737	1	1
Appendix B, Q9	1	1	0.737
Appendix B, Q10	1	1	1
Appendix B, Q11	1	1	1
Appendix B, Q12	0.737	1	1

Appendix D: List of Included Reviews

Authors	Title	Journal	Year
S. C. Chan Tan, O.	Achilles and patellar tendinopathy: Current understanding of pathophysiology and management	Disability and Rehabilitation	2008
I. M. Chazan	Achilles tendinitis part I: Anatomy, histology, classification, etiology, and pathomechanics	Journal of Manual and Manipulative Therapy	1998
M. Kannus Paavola, P. Jarvinen, T. A. Khan, K. Jozsa, L. Jarvinen, M.	Achilles tendinopathy	Journal of Bone & Joint Surgery - American Volume	2002
A. Khan Abbassian, R.	Achilles tendinopathy: Pathology and management strategies	British Journal of Hospital Medicine	2009
T. A. H. Kannus Jarvinen, P. Maffulli, N. Khan, K. M.	Achilles tendon disorders: Etiology and epidemiology	Foot and Ankle Clinics	2005
T. A. H. Kannus Jarvinen, P. Paavola, M. Jarvinen, T. L. N. Jozsa, L. Jarvinen, M.	Achilles tendon injuries	Current Opinion in Rheumatology	2001
M. Kvist	Achilles tendon injuries in athletes	Sports Medicine	1994
J. C. Rich Patterson-Kane, T.	Achilles tendon injuries in elite athletes: lessons in pathophysiology from their equine counterparts	ILAR journal / National Research Council, Institute of Laboratory Animal Resources	2014
M. T. Jokl Galloway, P. Dayton, O. W.	Achilles tendon overuse injuries	Clinics in Sports Medicine	1992
J. E. Cook Gaida, J. L. Bass, S. L.	Adiposity and tendinopathy	Disability & Rehabilitation	2008
N. L. Murrell Millar, G. A. C. McInnes, I. B.	Alarmins in tendinopathy: Unravelling new mechanisms in a common disease	Rheumatology (United Kingdom)	2013
L. Rui Shi, Y. F. Li, G. Wang, C.	Alterations of tendons in diabetes mellitus: what are the current findings?	International Orthopaedics	2015
R. R. Lemos de Oliveira, A. de Castro Silveira, P. V. da Silva, R. J. de Moraes, S. R. A.	Alterations of tendons in patients with diabetes mellitus: A systematic review	Diabetic Medicine	2011
J. T. Russell Laseter, J. A.	Anabolic steroid-induced tendon pathology: A review of the literature	Medicine and Science in Sports and Exercise	1991
S. J. Warden	Animal models for the study of tendinopathy	British journal of sports medicine	2007

A. Andarawis-Puri Neviaser, N. Flatow, E.	Basic mechanisms of tendon fatigue damage	Journal of Shoulder and Elbow Surgery	2012
M. S. Mahmood Zafar, A. Maffulli, N.	Basic science and clinical aspects of Achilles tendinopathy	Sports Medicine and Arthroscopy Review	2009
Y. Murrell Xu, G. A.	The basic science of tendinopathy	Clinical Orthopaedics & Related Research	2008
W. McCluskey Christopher Patton, Iii G. M.	Biceps tendinitis and subluxation	Clinics in Sports Medicine	2001
B. M. Murrell Andres, G. A. C.	The biology of rotator cuff tears	Current Orthopaedic Practice	2008
P. Maffulli Sharma, N.	Biology of tendon injury: Healing, modeling and remodeling	Journal of Musculoskeletal Neuronal Interactions	2006
J. H. C. Iosifidis Wang, M. I. Fu, F. H.	Biomechanical basis for tendinopathy	Clinical Orthopaedics and Related Research	2006
G. Baker Jr Hurt, C. L.	Calcific tendinitis of the shoulder	Orthopedic Clinics of North America	2003
F. Via Oliva, A. G. Maffulli, N.	Calcific tendinopathy of the rotator cuff tendons	Sports Medicine and Arthroscopy Review	2011
N. J. Bureau	Calcific tendinopathy of the shoulder	Seminars in Musculoskeletal Radiology	2013
H. K. Sarkar Uhthoff, K.	Calcifying tendinitis	Bailliere's Clinical Rheumatology	1989
J. Wang Yuan, M. X. Murrell, G. A. C.	Cell death and tendinopathy	Clinics in Sports Medicine	2003
P. D. Strassburg Clegg, S. Smith, R. K.	Cell phenotypic variation in normal and damaged tendons	International Journal of Experimental Pathology	2007
W. B. Leadbetter	Cell-matrix response in tendon injury	Clinics in Sports Medicine	1992
U. Stengaard-Pedersen Fredberg, K.	Chronic tendinopathy tissue pathology, pain mechanisms, and etiology with a special focus on inflammation	Scandinavian journal of medicine & science in sports	2008
H. Ohberg Alfredson, L.	Chronic tendon pain: No tendinitis, but high levels of glutamate and a vasculoneural ingrowth - Implications for a new treatment?	Therapy	2005
G. Riley	Chronic tendon pathology: molecular basis and therapeutic implications	Expert Reviews in Molecular Medicine	2005
L. C. Weinhold Almekinders, P. S.	Compression etiology in tendinopathy	Clinics in Sports Medicine	2003

Maffulli, N.			
I. Uhl Blanco, S. K. Schlienger, R. G.	Corticosteroid-associated tendinopathies: An analysis of the published literature and spontaneous pharmacovigilance data	Drug Safety	2005
A. Maak Bedi, T. Walsh, C. Rodeo, S. A. Grande, D. Dines, D. M. Dines, J. S.	Cytokines in rotator cuff degeneration and repair	Journal of Shoulder and Elbow Surgery	2012
J. D. Keeling Duerden, J. J.	Disorders of the Achilles tendon	Current Orthopaedic Practice	2008
T. Larbi Kirchgesner, A. Omoumi, P. Malghem, J. Zamali, N. Manelfe, J. Lecouvet, F. Vande Berg, B. Djebbar, S. Dallaudiere, B.	Drug-induced tendinopathy: From physiology to clinical applications	Joint Bone Spine	2014
M. A. Sandrey	Effects of acute and chronic pathomechanics on the normal histology and biomechanics of tendons: A review	Journal of Sport Rehabilitation	2000
M. E. Seidenberg Pitzer, P. H. Bader, D. A.	Elbow tendinopathy	Medical Clinics of North America	2014
R. P. Ashman Nirschl, E. S.	Elbow tendinopathy: Tennis elbow	Clinics in Sports Medicine	2003
R. P. Nirschl	Elbow tendinosis/tennis elbow	Clinics in Sports Medicine	1992
R. Moro Papalia, L. Franceschi, F. Albo, E. D'Adamio, S. Di Martino, A. Vadala, G. Faldini, C. Denaro, V.	Endothelial dysfunction and tendinopathy: how far have we come?	Musculoskeletal Surgery	2013
S. Gimbel Mehta, J. A. Soslowsky, L. J.	Etiologic and pathogenetic factors for rotator cuff tendinopathy	Clinics in Sports Medicine	2003
S. A. Hale	Etiology of patellar tendinopathy in athletes	Journal of Sport Rehabilitation	2005
L. C. Temple Almekinders, J. D.	Etiology, diagnosis, and treatment of tendonitis: An analysis of the literature	Medicine and Science in Sports and Exercise	1998
J. M. Wiley Archambault, J. P. Bray, R. C.	Exercise loading of tendons and the development of overuse injuries: A review of current literature	Sports Medicine	1995
J. H. Screen Shepherd, H. R. C.	Fatigue loading of tendon	International Journal of Experimental Pathology	2013

W. C. Yang Tsai, Y. M.	Fluoroquinolone-associated Tendinopathy	Chang Gung Medical Journal	2011
Y. Zhanel Khaliq, G. G.	Fluoroquinolone-associated tendinopathy: A critical review of the literature	Clinical Infectious Diseases	2003
F. Olcay Kaleagasioglu, E.	Fluoroquinolone-induced tendinopathy: etiology and preventive measures	The Tohoku journal of experimental medicine	2012
T. Cook Lewis, J.	Fluoroquinolones and tendinopathy: a guide for athletes and sports clinicians and a systematic review of the literature	Journal of athletic training	2014
A. Melhus	Fluoroquinolones and tendon disorders	Expert Opinion on Drug Safety	2005
D. A. Archambault Hart, J. M. Kydd, A. Reno, C. Frank, C. B. Herzog, W.	Gender and neurogenic variables in tendon biology and repetitive motion disorders	Clinical Orthopaedics & Related Research	1998
M. Maffulli Magra, N.	Genetic aspects of tendinopathy	Journal of Science and Medicine in Sport	2008
N. L. Murrell Millar, G. A.	Heat shock proteins in tendinopathy: novel molecular regulators	Mediators of Inflammation	2012
E. J. Frank O'Brien, C. B. Shrive, N. G. Hallgrimsson, B. Hart, D. A.	Heterotopic mineralization (ossification or calcification) in tendinopathy or following surgical tendon trauma	International Journal of Experimental Pathology	2012
P. P. Lui	Histopathological changes in tendinopathy--potential roles of BMPs?	Rheumatology	2013
M. Jozsa Jarvinen, L. Kannus, P. Jarvinen, T. L. N. Kvist, M. Leadbetter, W.	Histopathological findings in chronic tendon disorders	Scandinavian journal of medicine & science in sports	1997
K. M. Cook Khan, J. L. Bonar, F. Harcourt, P. Astrom, M.	Histopathology of common tendinopathies. Update and implications for clinical management	Sports Medicine	1999
A. Malliaras Tardioli, P. Maffulli, N.	Immediate and short-term effects of exercise on tendon structure: Biochemical, biomechanical and imaging responses	British Medical Bulletin	2012
L. Maffulli Battery, N.	Inflammation in overuse tendon injuries	Sports Medicine and Arthroscopy Review	2011
R. K. Birch Smith, H. L. Goodman, S. Heinegard, D. Goodship, A. E.	The influence of ageing and exercise on tendon growth and degeneration--hypotheses for the initiation and prevention of strain-induced	Comparative Biochemistry & Physiology. Part A, Molecular & Integrative Physiology	2002

	tendinopathies		
M. K. Maffulli Sayana, N.	Insertional achilles tendinopathy	Foot and Ankle Clinics	2005
G. M. Hart Thornton, D. A.	The interface of mechanical loading and biological variables as they pertain to the development of tendinosis	Journal of Musculoskeletal Neuronal Interactions	2011
J. Samiric Parkinson, T. Ilic, M. Z. Cook, J. Handley, C. J.	Involvement of proteoglycans in tendinopathy	Journal of Musculoskeletal Neuronal Interactions	2011
J. E. Ashe Gaida, M. C. Bass, S. L. Cook, J. L.	Is adiposity an under-recognized risk factor for tendinopathy? A systematic review	Arthritis Care and Research	2009
J. L. Purdam Cook, C.	Is compressive load a factor in the development of tendinopathy?	British journal of sports medicine	2012
B. J. Tilley, J.L Cook S.I Docking J. E. Gaida	Is higher serum cholesterol associated with altered tendon structure or tendon pain? A systematic review	British journal of sports medicine	2015
A. Shakibaei Mobasheri, M.	Is tendinitis an inflammatory disease initiated and driven by pro-inflammatory cytokines such as interleukin 1beta?	Histology & Histopathology	2013
J. L. Purdam Cook, C. R.	Is tendon pathology a continuum? A pathology model to explain the clinical presentation of load-induced tendinopathy	British journal of sports medicine	2009
S. Carr Chaudhury, A. J.	Lessons we can learn from gene expression patterns in rotator cuff tears and tendinopathies	Journal of Shoulder and Elbow Surgery	2012
S. J. Strauss Nho, E. J. Lenart, B. A. Provencher, C. M. T. Mazzocca, A. D. Verma, N. N. Romeo, A. A.	Long head of the biceps tendinopathy: Diagnosis and management	Journal of the American Academy of Orthopaedic Surgeons	2010
A. L. McClure Seitz, P. W. Finucane, S. Boardman, Iii N. D. Michener, L. A.	Mechanisms of rotator cuff tendinopathy: Intrinsic, extrinsic, or both?	Clinical Biomechanics	2011
S. Parks Thomopoulos, W. C. Rifkin, D. B. Derwin, K. A.	Mechanisms of tendon injury and repair	Journal of Orthopaedic Research	2015
S. P. Lavagnino Arnoczky, M. Egerbacher, M.	The mechanobiological aetiopathogenesis of tendinopathy: is it the over-stimulation or the under-stimulation of tendon cells?	International Journal of Experimental Pathology	2007

S. G. Dent Rees, C. M. Caterson, B.	Metabolism of proteoglycans in tendon	Scandinavian Journal of Medicine and Science in Sports	2009
A. Oliva Del Buono, F. Longo, U. G. Rodeo, S. A. Orchard, J. Denaro, V. Maffulli, N.	Metalloproteases and rotator cuff disease	Journal of Shoulder and Elbow Surgery	2012
B. Aspenberg Pasternak, P.	Metalloproteinases and their inhibitors-diagnostic and therapeutic opportunities in orthopedics	Acta Orthopaedica	2009
M. Maffulli Magra, N.	Molecular events in tendinopathy: A role for metalloproteases	Foot and Ankle Clinics	2005
N. Cowan Wyndow, S. M. Wrigley, T. V. Crossley, K. M.	Neuromotor control of the lower limb in Achilles tendinopathy: implications for foot orthotic therapy	Sports medicine (Auckland, N.Z.)	2010
P. W. Salo Ackermann, P. T. Hart, D. A.	Neuronal pathways in tendon healing	Frontiers in bioscience : a journal and virtual library	2009
P. W. Franklin Ackermann, S. L. Dean, B. J. Carr, A. J. Salo, P. T. Hart, D. A.	Neuronal pathways in tendon healing and tendinopathy--update	Frontiers in bioscience (Landmark edition)	2014
P. W. Ackermann	Neuronal regulation of tendon homoeostasis	International Journal of Experimental Pathology	2013
A. Bahr Scott, R.	Neuropeptides in tendinopathy	Frontiers in bioscience : a journal and virtual library	2009
B. K. Bisset Coombes, L. Vicenzino, B.	A new integrative model of lateral epicondylalgia	British journal of sports medicine	2009
M. S. Molloy Hennessy, A. P. Sturdee, S. W.	Noninsertional Achilles Tendinopathy	Foot and Ankle Clinics	2007
A. D. Webborn	Novel approaches to tendinopathy	Disability & Rehabilitation	2008
F. Papalia Franceschi, R. Paciotti, M. Franceschetti, E. Martino, A. D. Maffulli, N. Denaro, V.	Obesity as a risk factor for tendinopathy: A systematic review	International Journal of Endocrinology	2014
K. M. Cook Khan, J. L. Taunton, J. E. Bonar, F.	Overuse tendinosis, not tendinitis. Part 1: A new paradigm for a difficult clinical problem	Physician and Sportsmedicine	2000
J. R. Stanish Giffin, W. D.	Overuse tendonitis and rehabilitation	Canadian family physician Medecin de famille canadien	1993
U. G. Olivia Longo, F. Denaro, V. Maffulli, N.	Oxygen species and overuse tendinopathy in athletes	Disability and Rehabilitation	2008

M. Jarvinen Paavola, T. A. H.	Paratendinopathy	Foot and Ankle Clinics	2005
K. M. Maffulli Khan, N. Coleman, B. D. Cook, J. L. Taunton, J. E.	Patellar tendinopathy: some aspects of basic science and clinical management	British journal of sports medicine	1998
B. Purdam Hamilton, C.	Patellar tendinosis as an adaptive process: a new hypothesis	British journal of sports medicine	2004
S. M. Pomeroy Mosier, G. Manoli, A., 2nd	Pathoanatomy and etiology of posterior tibial tendon dysfunction	Clinical Orthopaedics & Related Research	1999
J. C. Firth Patterson-Kane, E. C.	The pathobiology of exercise-induced superficial digital flexor tendon injury in Thoroughbred racehorses	Veterinary Journal	2009
B. Bondi Magnan, M. Pierantoni, S. Samaila, E.	The pathogenesis of Achilles tendinopathy: A systematic review	Foot and Ankle Surgery	2014
M. Gravare Silbernagel Abate, K. Siljeholm, C. Di Iorio, A. De Amicis, D. Salini, V. Werner, S. Paganelli, R.	Pathogenesis of tendinopathies: inflammation or degeneration?	Arthritis research & therapy	2009
S. P. Langberg Magnusson, H. Kjaer, M.	The pathogenesis of tendinopathy: Balancing the response to loading	Nature Reviews Rheumatology	2010
G. Riley	The pathogenesis of tendinopathy. A molecular perspective	Rheumatology	2004
J. C. Becker Patterson-Kane, D. L. Rich, T.	The Pathogenesis of Tendon Microdamage in Athletes: The Horse as a Natural Model for Basic Cellular Research	Journal of Comparative Pathology	2012
W. J. Collins Ribbans, M.	Pathology of the tendo Achillis: do our genes contribute?	Bone & Joint Journal	2013
B. J. F. Franklin Dean, S. L. Carr, A. J.	The peripheral neuronal phenotype is important in the pathogenesis of painful human tendinopathy: A systematic review	Clinical Orthopaedics and Related Research	2013
F. Via Oliva, A. G. Maffulli, N.	Physiopathology of intratendinous calcific deposition	BMC Medicine	2012
M. H. J. Frost Sejersen, P. Hansen, T. B. Deutch, S. R. Svendsen, S. W.	Proteomics perspectives in rotator cuff research a systematic review of gene expression and protein composition in human tendinopathy	PLoS ONE	2015
S. G. Dudhia Dakin, J. Smith, R. K.	Resolving an inflammatory concept: the importance of inflammation and resolution in tendinopathy	Veterinary Immunology and Immunopathology	2014

C. T. Clegg Thorpe, P. D. Birch, H. L.	A review of tendon injury: Why is the equine superficial digital flexor tendon most at risk?	Equine Veterinary Journal	2010
P. Danielson	Reviving the "biochemical" hypothesis for tendinopathy: New findings suggest the involvement of locally produced signal substances	British journal of sports medicine	2009
H. van Ark van der Worp, M. Roerink, S. Pepping, G. J. van den Akker-Scheek, I. Zwerver, J.	Risk factors for patellar tendinopathy: a systematic review of the literature	British journal of sports medicine	2011
R. Cesari Garofalo, E. Vinci, E. Castagna, A.	Role of metalloproteinases in rotator cuff tear	Sports Medicine and Arthroscopy Review	2011
D. Singh Bachasson, A. Shah, S. B. Lane, J. G. Ward, S. R.	The role of the peripheral and central nervous systems in rotator cuff disease	Journal of Shoulder and Elbow Surgery	2015
T. Petersen Pufe, W. J. Mentlein, R. Tillmann, B. N.	The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease	Scandinavian journal of medicine & science in sports	2005
J. S. Lewis	Rotator cuff tendinopathy	British journal of sports medicine	2009
J. S. Lewis	Rotator cuff tendinopathy: a model for the continuum of pathology and related management	British journal of sports medicine	2010
F. H. Harner Fu, C. D. Klein, A. H.	Shoulder impingement syndrome: A critical review	Clinical Orthopaedics and Related Research	1991
Jacob Lambert Jewson, Gavin Storr, Michael Gaida, James	The Sympathetic Nervous System and Tendinopathy: A Systematic Review	Sports Medicine	2015
BJF Dean, SL Franklin, AJ Carr	A systematic review of the histological and molecular changes in rotator cuff disease	Bone Joint Research	2012
L. C. Almekinders	Tendinitis and other chronic tendinopathies	The Journal of the American Academy of Orthopaedic Surgeons	1998
T. L. Wilson Sevier, J. K. Helfst, R. H. Stover, S. A.	Tendinitis: A critical review	Critical Reviews in Physical and Rehabilitation Medicine	2000
J. M. Fedorczyk	Tendinopathies of the elbow, wrist, and hand: Histopathology and clinical considerations	Journal of Hand Therapy	2012
A. Battery Del Buono, L. Denaro, V. Maccauro, G. Maffulli, N.	Tendinopathy and inflammation: some truths	International journal of immunopathology and pharmacology	2011
D. Wang Jiang, J. H. C.	Tendinopathy and its treatment with platelet-rich plasma (PRP)	Histology and Histopathology	2013

G. Riley	Tendinopathy--from basic science to treatment	Nature Clinical Practice Rheumatology	2008
B. S. Nirschl Kraushaar, R. P.	Tendinosis of the elbow (Tennis elbow): Clinical features and findings of histological, immunohistochemical, and electron microscopy studies	Journal of Bone and Joint Surgery - Series A	1999
T. L. Wang Dela Rosa, A. W. Zheng, M. H.	Tendinosis of the rotator cuff: A review	Journal of Musculoskeletal Research	2001
J. A. McAuliffe	Tendon disorders of the hand and wrist	Journal of Hand Surgery - American Volume	2010
N. Flatow Andarawis-Puri, E. L.	Tendon fatigue in response to mechanical loading	Journal of Musculoskeletal Neuronal Interactions	2011
M. Saxby Hope, T. S.	Tendon Healing	Foot and Ankle Clinics	2007
Sebastian Todorov Müller, Atanas Heisterbach, Patricia Martin, Ivan Majewski, Martin	Tendon healing: an overview of physiology, biology, and pathology of tendon healing and systematic review of state of the art in tendon bioengineering	Knee Surgery, Sports Traumatology, Arthroscopy	2015
A. L. Wu Stephenson, W. Cortes, D. Rochon, P. A.	Tendon injury and fluoroquinolone use: A systematic review	Drug Safety	2013
J. D. Stride Rees, M. Scott, A.	Tendons--time to revisit inflammation	British journal of sports medicine	2014
E. J. Cook Hegedus, C. Brennan, M. Wyland, D. Garrison, J. C. Driesner, D.	Vascularity and tendon pathology in the rotator cuff: a review of literature and implications for rehabilitation and surgery	British journal of sports medicine	2010
H. A. Iannotti Chansky, J. P.	The vascularity of the rotator cuff	Clinics in Sports Medicine	1991
S. A. Hazleman Fenwick, B. L. Riley, G. P.	The vasculature and its role in the damaged and healing tendon	Arthritis Research	2002
M. Bayer Kjaer, M. L. Eliasson, P. Heinemeier, K. M.	What is the impact of inflammation on the critical interplay between mechanical signalling and biochemical changes in tendon matrix?	Journal of Applied Physiology	2013
M. Toumi Benjamin, H. Ralphs, J. R. Bydder, G. Best, T. M. Milz, S.	Where tendons and ligaments meet bone: Attachment sites ('entheses') in relation to exercise and/or mechanical load	Journal of Anatomy	2006
A. E. Barbe Barr, M. F. Clark, B. D.	Work-related musculoskeletal disorders of the hand and wrist: epidemiology, pathophysiology, and sensorimotor changes	Journal of Orthopaedic & Sports Physical Therapy	2004

Appendix E: Summary of systematic reviews included in review

Reviews are ordered sequentially from highest quality score to least quality score

Author(s)	Title	Year	Quality Score	Conclusions about inflammation in TD	Immune cells discussed	Mediators discussed
Maria Hee Jung Sejersen *et al*	Proteomics perspectives in rotator cuff research a systematic review of gene expression and protein composition in human tendinopathy	2015	10	Supports inflammatory component	Inflammatory infiltrate mentioned, not defined	Yes
RR de Oliveira *et al*	Alterations of tendons in patients with diabetes mellitus: A systematic review	2011	9	Pathogenesis not discussed	None discussed	None discussed
James E. Gaida *et al*	Is adiposity an under-recognized risk factor for tendinopathy? A systematic review	2009	9	Supports inflammatory component	Immune cells not discussed	Yes
Benjamin John Floyd Dean *et al*	The peripheral neuronal phenotype is important in the pathogenesis of painful human tendinopathy: A systematic review	2013	9	Supports inflammatory component	Monocytes, macrophages, and/or lymphocytes discussed	Yes
Henk van der Worp *et al*	Risk factors for patellar tendinopathy: a systematic review of the literature	2011	9	Pathogenesis not discussed	None discussed	None discussed
Anne L. Stephenson *et al*	Tendon injury and fluoroquinolone use: A systematic review	2013	9	Pathogenesis not discussed	None discussed	None discussed
R. Papalia *et al*	Endothelial dysfunction and tendinopathy: how far have we come?	2013	8	Supports inflammatory component	Monocytes, macrophages, and/or lymphocytes discussed	Yes
Benjamin J Tilley *et al*	Is higher serum cholesterol associated with altered tendon structure or tendon pain? A systematic review	2015	8	Supports inflammatory component	Monocytes, macrophages, and/or lymphocytes discussed	Yes

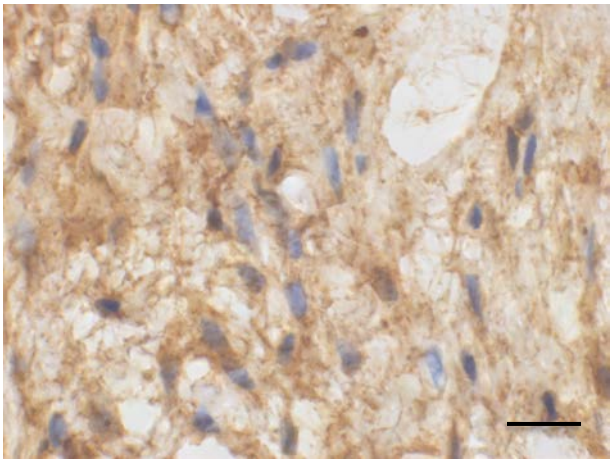
Francesco Franceschi *et al*	Obesity as a risk factor for tendinopathy: A systematic review	2014	8	Inflammation may play a role in TD	Inflammatory infiltrate mentioned, not defined	None discussed
Jacob L. Jewson *et al*	The Sympathetic Nervous System and Tendinopathy: A Systematic Review	2015	8	Inflammation may play a role in TD	Monocytes, macrophages, and/or lymphocytes discussed	Yes
Irene Blanco *et al*	Corticosteroid-associated tendinopathies: An analysis of the published literature and spontaneous pharmacovigilance data	2005	6	Other: Pathogenesis is discussed but inflammation is not mentioned. Topic of discussion is the role of anabolic steroids in TD	None discussed	None discussed
Alex Tardioli *et al*	Immediate and short-term effects of exercise on tendon structure: Biochemical, biomechanical and imaging responses	2012	6	Inflammation may play a role in TD	None discussed	Yes
Benjamin John Floyd Dean *et al*	A systematic review of the histological and molecular changes in rotator cuff disease	2012	6	Supports inflammatory component	Monocytes, macrophages, and/or lymphocytes discussed	Yes
Sebastian A. Muller *et al*	Tendon healing: an overview of physiology, biology, and pathology of tendon healing and systematic review of state of the art in tendon bioengineering	2015	6	Supports inflammatory component	Monocytes, macrophages, and/or lymphocytes discussed	Yes
J. E. Gaida *et al*	Adiposity and tendinopathy	2008	5	Supports inflammatory component	None discussed	Yes
Trevor Lewis	Fluoroquinolones and	2014	3	Other: chronic	None discussed	None

et al	tendinopathy: a guide for athletes and sports clinicians and a systematic review of the literature			tendinitis precedes 50% of tendon ruptures.		discussed
Yinghua Xu *et al*	The basic science of tendinopathy	2008	3	There is no inflammatory component to TD	Inflammatory infiltrate mentioned, not defined	None discussed
Bruno Magnan *et al*	The pathogenesis of Achilles tendinopathy: A systematic review	2014	3	There is no inflammatory component to TD	Inflammatory infiltrate mentioned, not defined	Yes

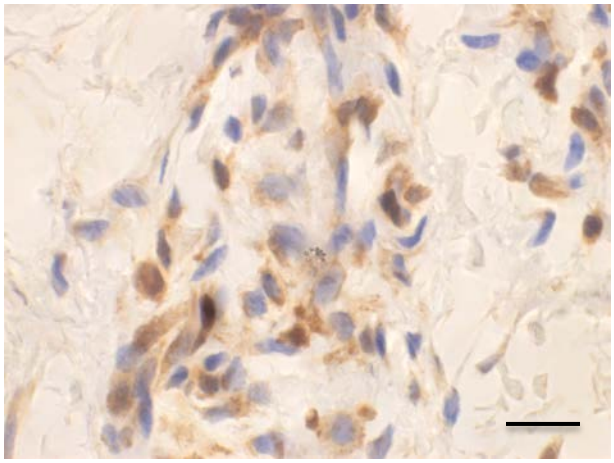
Appendix F: Positive control tissues for immunohistochemistry: representative images

Positive control tissues were diseased human supraspinatus tendon samples. Brown is positive DAB staining for given primary antibody, blue is nuclear counterstain. Scale bar = 20µm

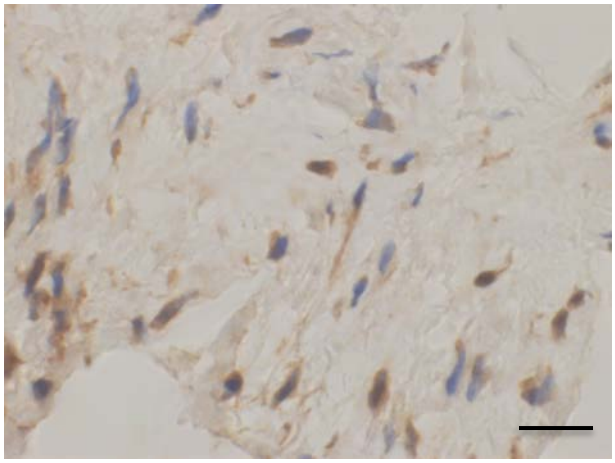
HIF-1α



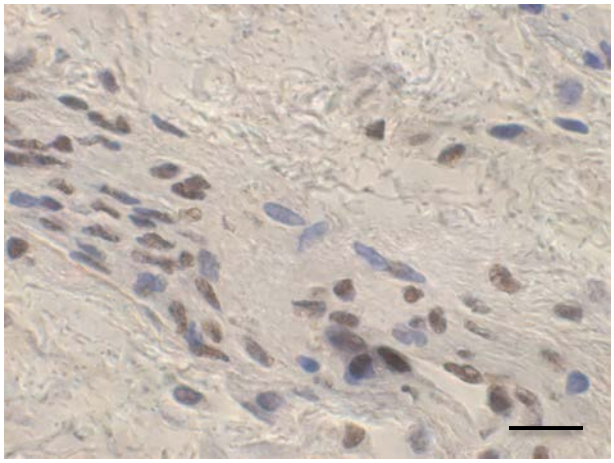
S100A9



IL-33



HMGB1



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