Age-Related Changes in Structure and Biomechanics of Human Sartorius Tendon Collagen

by

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Neizmjerno sam zahvalna.

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Abstract

Injuries to soft tissues such as tendons affect millions of people annually. Injuries produced by *in vitro* mechanical overload result in damage to constituent collagen. Using bovine models, it has been found that overload results in serial kink formation within collagen fibrils in low-load tendons – a mechanism called discrete plasticity.

Despite the prevalence of injury and our aging population, the exact mechanism behind the failure of collagen in aging human tendons has not been investigated until now. In this study, fresh contralateral human sartorius tendons from donors aged 20 to 60 were used to assess potential age-related changes in failure mechanics. Thermal stability of tendon collagen was examined and was expected to increase with age due to increased crosslinking. Damage motifs were investigated following tendon rupture using scanning electron microscopy. It was thought that discrete plasticity kinks would form following rupture in younger samples, but that the mechanism would dissipate with age.

The thermal stability results suggest that there is a high density of mature crosslinks present. The exact relationship between crosslinking and age remains inconclusive. Despite these structural changes, the mechanical properties did not change with age. Discrete plasticity was not found in any tendon sample, likely due to heavy crosslinking. Individual fibrils displayed sites of local damage with exposed substructure, and kinks/turns that propagated across fibrils. These failure motifs along with the thermal stability test results support the notion that discrete plasticity is a feature of tendons that are sparsely crosslinked.

This study was the first to examine how the nanoscaled, structuro-mechanical features of overload failure in human tendons varies with age. As we increase our understanding of the effect of tendon type and age on damage motifs, we will also better understand how injury occurs on the nanoscale and how healing is mediated in the body.

List of Abbreviations and Symbols Used

AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
AGE	Advanced Glycation End-Product
BTT	Bovine Tail Tendon
CDE	Common Digital Extensor
CHP	Collagen Hybridizing Peptide
CSA	Cross-Sectional Area
deH-HLNL	Dehydro-hydroxylysinonorleucine
deH-LNL	Dehydro-lysinonorleucine
DSC	Differential Scanning Calorimetry
E	Tissue Modulus
ECM	Extracellular Matrix
FR_1	Fractional Rise in Load 1
FR_2	Fractional Rise in Load 2
FWHM	Full Width at Half Maximum
GAG	Glycosaminoglycan

HIT	Hydrothermal Isometric Tension
HHL	Hydroxylysinonorleucine
HLKNL	Hydroxylysino-ketonorleucine
Hyl-Pyr	Hydroxylysino-pyridinoline
Hyp	Hydroxyproline
k	Relaxation constant
k_B	Boltzmann's Constant
L_{max}	Maximum Load
l_o	Intergrip Length
$L(t)$	Load at Given Time
$Load_{RT}$	Load at Room Temperature
$Load_{T_d}$	Load at Denaturation Temperature
MGO	Methylglyoxal
mRNA	Messenger Ribonucleic Acid
MT	Mechanical Testing
MTJ	Myotendinous Junction
$NaBH_4$	Sodium Borohydride
OTJ	Osteotendinous Junction
PBS	Phosphate Buffered Saline
PG	Proteoglycan
Pro	Proline
RTT	Rat Tail Tendon
SAXS	Small-Angle X-Ray Scattering
SD	Standard Deviation
SDF	Superficial Digital Flexor
SEM	Scanning Electron Microscopy

$t_{1/2}$	Half-Time of Load Decay
T_d	Denaturation Temperature
$T_{F_{max}}$	Temperature at Maximum Force
TEM	Transmission Electron Microscopy
T_{dev}	Deviation Temperature
T_{onset}	Onset Temperature
T_{peak}	Peak Temperature
UTS	Ultimate Tensile Strength
W	Number of Molecular Conformations
ΔG	Gibbs Free Energy
ΔH	Enthalpy
Δl	Displacement
ΔS	Entropy
ϵ	Strain
σ	Stress

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Chapter 1

Introduction

1.1 Tendon Structure

Tendons are soft-tissues that connect muscle to bone, transferring forces uniaxially between muscle and bone[1]. The primary component of tendons is collagen, which is the most abundant mammalian protein[2]. Many different tendons are present within the human body and they can be classified into two major categories – positional and energy-storing tendons.

1.1.1 Hierarchy & Assembly

The hierarchical structure within tendons is shown in Figure 1.1. Tendons are made up of fascicles (50-300 μm) that are composed of many fibres. These fibres are made up of fibrils (50-500 nm) which are composed of subfibrils, structures that are made up of collagen molecules[3]. Collagen crimp is a waveform pattern visible at the level of the fascicle. The fascicles of a tendon are surrounded by connective tissue known as endotenon, while the whole tendon is enveloped by peritenon[4]. The matrix materials that hold together fibres/fibrils have a high proteoglycan content, which influence the mechanical properties of the soft tissues.

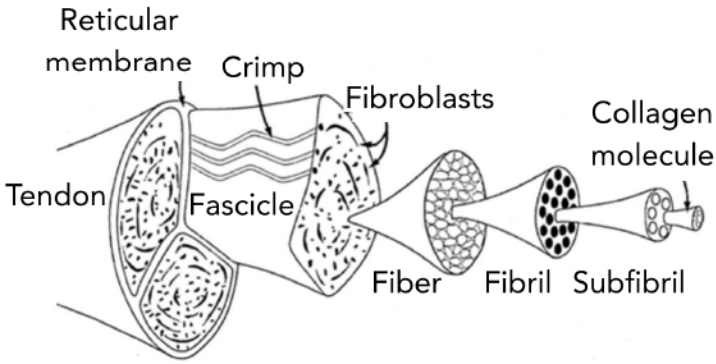

Figure 1.1: Schematic of the various components that make up tendons. Adapted with permission from[3].

Tendons insert into bones at the enthesis, or osteotendinous junction, and it is incredibly important that the collagen is continuous along the length of a tendon[5]. The enthesis is crucial in ensuring proper load transmission from musculature to bones. The insertion of the tendon into the muscle occurs at an interface known as the myotendinous junction (MTJ). This region is also incredibly important in force transmission, resisting muscle forces between 1.8 to $3.5 \times 10^4\,\mathrm{N\,m^{-2}}$[6]. The structure of the MTJ contains many protein complexes, actin filaments, transmembrane protein complexes and proteins that link the extracellular matrix to the basement membrane[6]. The exact spatial configuration of these components along with the constituents present changes with exercise[7] and aging[8], something that may have an implication on tendon biomechanics *in vivo*.

1.1.2 Components

Besides type I collagen which makes up about 60-85% of the dry mass[9], tendons also contain small amount of cells, other collagen types (III, V, etc.), fibrillin, elastin, and proteoglycans[10]. Proteoglycans retain large amounts of water due to the presence of negatively charged sulfated glycosaminoglycan chains. The exact amount of proteoglycans depends on the type of tendon, anatomical location within tissues, and

the age of the tissue. This will be expanded on further in Section 1.9. While tendons are largely acellular, tenocytes, which are specialized fibroblasts, make up the largest proportion of cells present[10]. These cells are able to sense changes in mechanics within their environments through mechanotransduction and therefore are able to repair tendon and maintain homeostasis[9].

1.2 Tendon Injury

Musculoskeletal injuries such as injuries to tendons and ligaments affect over 10 million people in the United States annually[11]. Ligaments are similar in structure to tendons, but instead connect bone-to-bone and will not be dicussed herein. Tendons undergo many loading cycles per year and about 30% of visits to the family doctor for musculoskeletal pain are the result of tendinopathy[12]. Injuries can be chronic or acute, and in some cases, injury can result in complete tendon rupture. While all tendons are susceptible to injury, the major tendons, which experience greater *in vivo* mechanical loading, are most frequently affected (e.g. Achilles tendon, rotator cuff, patellar tendon)[13–18]. There are many different mechanisms of injury that lead to tendon ruptures or developement of tendinopathy[19]. Despite their prevalence, the mechanisms that guide healing in tendon injury have not been completely determined.

Tendinopathy is an umbrella term that refers to any type of overuse tendon disorder[13,20–23]. Usually degenerative in nature, it often results in pain, tenderness, decreased strength, and limited mobility[13,15,22]. It can be characterized histologically by collagen fibril disorganization, increased cellularity and increased proteoglycan/glycosaminoglycan content[13,22,24–26]. The exact relationship between tendinopathy and complete rupture is unknown but the two are definitely related. Acute tendon injury usually implies partial or complete rupture of tendon and is usually debilitating. Both acute and chronic injuries can be caused by excessive, abnormal or repetitive loading in combination with extrinsic and intrinsic factors[22]. Intrinsic factors include sex, age, diabetes, and obesity[13,27,28]. The primary extrinsic factor is abnormal or repetitive mechanical loading, which can result from sport, exercise, or work envi-

ronment[21,29–31]. Overloading of tendons may trigger inflammatory responses, tendon degeneration, or both[32], although this has been a subject of disagreement within the literature[33]. These structures are actively remodelled and repaired; otherwise the tendon would eventually rupture[21,22].

Current treatment options such as topical and/or systemic anti-inflammatory drugs act to decrease discomfort for patients experiencing tendinopathy. Surgery is also a treatment option in the event of a partial or complete tear. Some studies have shown that reinjuring the affected tendon is common, because the scar tissue that forms weakens the structure, resulting in a tensile strength that is one third of what it was prior to injury[13,34–36]. The above-mentioned treatments are currently the most effective since we do not fully understand the mechanism involved in the healing and injury of tendons. The exact role of inflammatory cells such as macrophages, mast cells, T cells, and neutrophils, in the aetiology of tendinopathy remains unclear[19,23,27,33]. It is known that following injury, cell proliferation, cell migration, and remodelling occur[13,32,34,35]. The processes occur in large part due to the release of growth factors and cytokines following injury. These include interleukins, tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), TGF-alpha, connective tissue growth factor (CTGF), epidermal growth factors (EGF) and insulin-like growth factor (IGF)-1[13,37] (Figure 1.2). There is also an increase in mRNAs that code for matrix metalloproteases and collagen in human tendinopathic tendons[26]. Additional changes that occur within tendinopathic tendons are losses in collagen organization and glycosaminoglycan deposition[33].

1.3 Tendon Collagen

1.3.1 Collagen Structure & Assembly

The primary component of tendons is collagen, the most abundant mammalian protein. If the molecules are arranged uniaxially, as they are in tendon, the collagen functions as the primary load bearing element along its axis. Collagen molecules

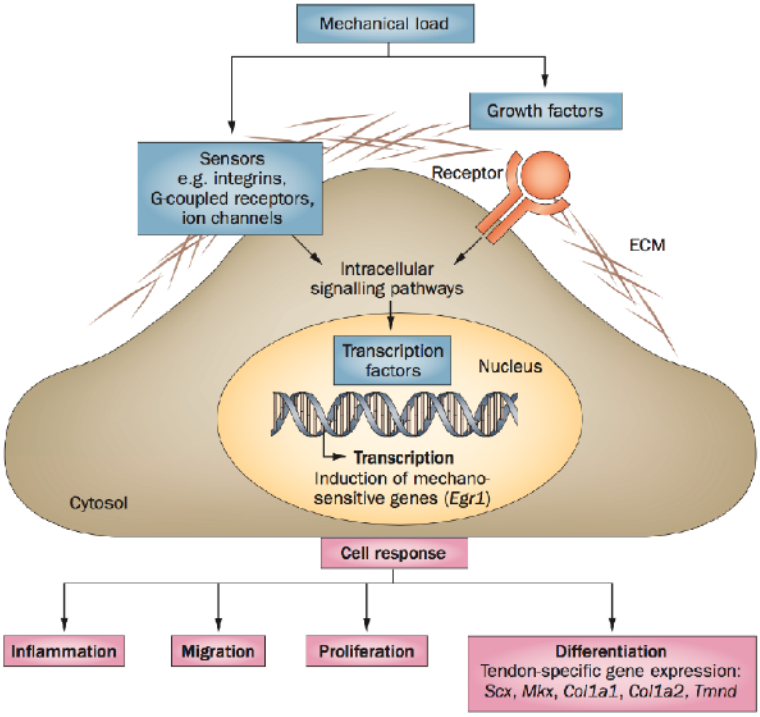

Figure 1.2: Mechanotransduction in tenocytes. Reprinted with permission[13].

themselves are triple helices, that are made up of individual polyproline-II-like helices called alpha-chains[38]. These polypeptides (α-chains) have a primary amino acid sequence of Glycine-X-Y, where X and Y can be any amino acid but are most commonly proline and hydroxyproline[38,39]. Glycine is the smallest amino acid and reduces steric hindrance within the peptide chains, to permit helix formation. The hydroxylation of the proline residues to form hydroxyproline is important to maintain stability of the helical structure through hydrogen bond formation[39]. Lysine residues that are present within the primary structure are also hydroxylated, which has later implications in terms of crosslinking[40]. These post-translational modifications are important and the degree of modification depends on enzyme concentration, and rates of collagen synthesis and turnover, among many other things[41,42]. While there are more than 20 different types of collagen, type I is the most common and makes up the majority of the soft connective tissues in our body[39]. Type I collagen is a heterotrimeric helix that

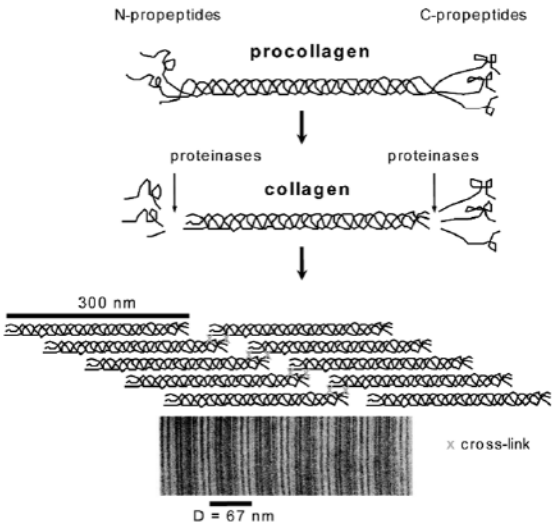

Figure 1.3: Collagen is synthesized as a proprotein, and the telopeptide ends are cleaved off by proteinases. The newly synthesized collagen triple helix self-assembles into a quarter-stagger structure to form fibrils, that are characterized by 67 nm striations called D-bands. Reprinted with modifications with permission[29].

is made up of three polypeptide chains: two $\alpha 1$ chains and one $\alpha 2$ chain[39,43,44]. Once the individual α chains are synthesized, a procollagen molecule self-assembles and is excreted into the extra-cellular matrix. This collagen precursor has a triple helical structure, with non-helical telopeptide ends. Each alpha-chain is 1300-1700 amino acids in length, with about 1000 of these within the triple helical region of the procollagen molecule[45]. Following assembly and excretion from the cell, the carboxy- and amino-termini (i.e. the telopeptides) are enzymatically cleaved off by peptidases[29].

Collagen structure is stabilized by a wide range of covalent and non-covalent interactions such as: hydrogen bonding, hydrophobic interaction, van der Waal's forces, electrostatic interactions, and crosslinking[46]. The collagen molecules are 300 nm in length, 1.5 nm in diameter, and self-assemble to form collagen fibrils[45]. This enthalpy-driven process results in the tight packing of collagen molecules with a quarter stagger, creating 67.0 nm long striations in the structure, referred to as the D-period, which

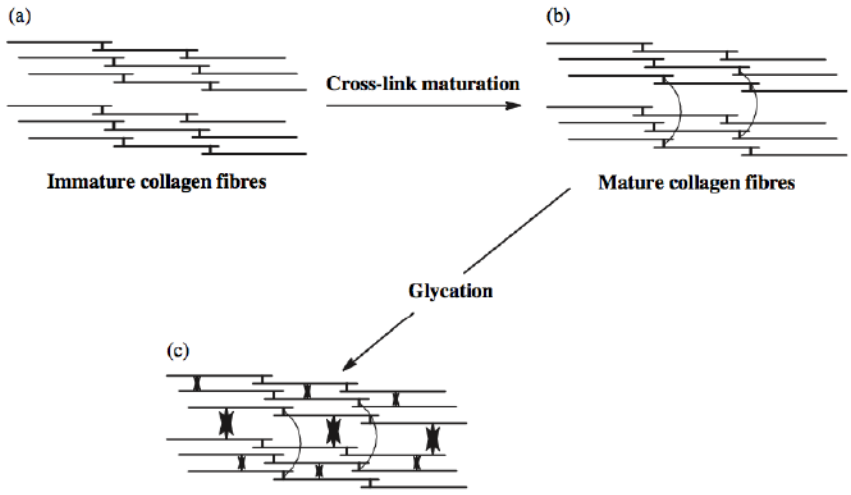

Figure 1.4: Location of collagen crosslinks present in fibres, showing that immature crosslinks are divalent, and mature ones are trivalent, while glycation-derived crosslinks do not occur at the ends but rather within the helical portions of collagen molecules[29,50]. Reprinted with permission[29].

is visible by electron microscopy or atomic force microscopy at the fibril level (Figure 1.3)[47]. Collagen fibrils are thought to be continuous along the length of a tissue[5] and self-assemble to form other hierarchical structures such as fibres[3]. While the longitudinal arrangement of collagen molecules is well understood and widely accepted, there is some debate as to what the lateral molecular arrangement within fibrils looks like[10,39,48].

1.3.2 Crosslinking

The crosslinking of collagen molecules is critical and contributes to the mechanical strength of tendons by preventing the slippage of collagen fibres during loading[49]. Both the number and type of inter- and intramolecular crosslinks present between fibrils determines the stability of the structure and is determined by age and loading history. There are two mechanisms of crosslink formation (Figure 1.4)[50,51]. The first is driven by the enzyme lysyl oxidase and occurs during development and matura-

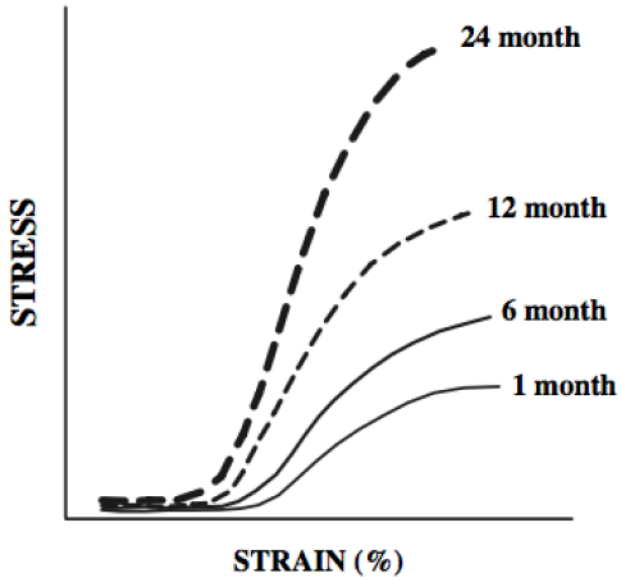

Figure 1.5: Stress-strain curve of aging RTT. In this particular animal model, maturity is reached by about 6 months of age, at which point there would be a plateau in the amount of mature crosslinking present. Any addition increase in crosslinking would be due to glycation (dotted line), and is responsible for any further increase in mechanical strength. Reprinted with permission[51].

tion[40,49]. Eventually, there is some density of enzymatic crosslinks that is achieved in a given mature tissue, and any additional crosslinking is due to glycation. The second occurs non-enzymatically and adventitously between collagen molecules and reducing sugars[50]. Studies have shown that rat tail tendon (RTT) fibres increase in mechanical strength with age, these changes attributed to increases in enzymatic and non-enzymatic crosslinking (Figure 1.5)[51].

Enzymatic Crosslinking

Enzymatic crosslinking occurs following post-translational hydroxylation of lysine. The types and amount of crosslinks present within tendons depends on the relative loading experienced, age, and rate of tissue turnover. This enzyme is responsible for the conversion of lysine and hydroxylysine residues within the C- and N-terminal ends

of procollagen to the corresponding peptidyl aldehyde[50,52]. These products can react with other peptidyl aldehydes to produce the initial divalent inter- and intramolecular crosslinks[50,53]. The exact type of crosslink that is formed is defined by the state of hydroxylation of telopeptide lysines and by the type of tendon[51]. The rate of crosslinking within tissues is regulated by the concentration of the enzyme[50]. Intermolecular crosslinks, such as those produced by the reactions of lysine aldehydes, decrease the slippage between collagen molecules[50].

Immature tissues have low lysine hydroxylation within the telopeptide residues and therefore the primary crosslink present is dehydro-hydroxylysinonorleucine (deH-HLNL) formed from the reaction of allysine with hydroxyallysine[54,55]. Other immature divalent crosslinks present in tendons result from the interaction of two allysine residues to form dehydro-lysinonorleucine (deH-LNL) or the reaction of hydroxylysine with hydroxyallysine to form hydroxylysino-ketonorleucine (HLKNL)[54]. Both deH-HLNL and deH-LNL contain acid- and heat-labile Schiff base double bonds and are found primarily in tendons and skin[54]. By contrast, HLKNL is stable to both acid and heat, and is primarily found in bone and cartilage where hydroxylation of lysine residues occurs more readily[49]. The crosslink deH-HLNL can react spontaneously with histidine residues in the collagen molecule to form a mature crosslink called histidino-hydroxyllysinonorleucine (HHL) (Figure 1.6A)[56]. Moreover, the immature crosslink HLKNL can react with either an allysine or a hydroxyallysine forming either pyrrole or hydroxylysino-pyridinoline (Hyl-Pyr), respectively (Figure 1.6B)[29,57]. These trivalent, "mature" crosslinks increase the mechanical strength of tendons and their concentration increases with age[52]. Low-load tendons contain fewer stable mature crosslinks than tendons experiencing high in vivo loads[52].

Reducing agents such as sodium borohydride can be used *in vitro* to stabilize deH-LNL and deH-HLNL to form LNL and HLNL, respectively, which contain a stable single bond rather than the Schiff-base double bond[49,53,58]. The products of these reactions are not the same as those that occur normally, but the reaction may be used semi-quantitatively identify crosslinks present within tissues. Through borohydride reduction it is possible to compare the stable and total crosslink population within

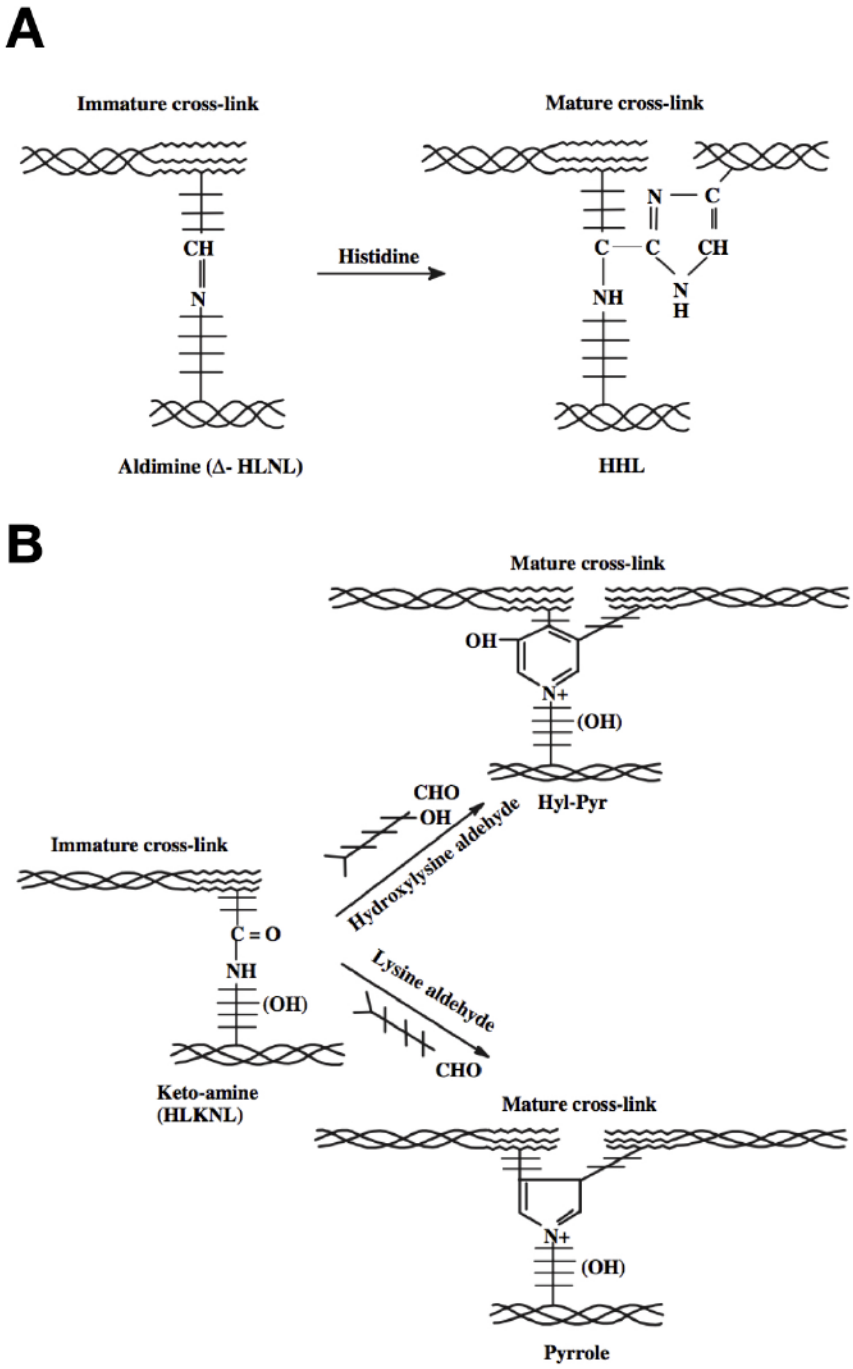

Figure 1.6: The reaction of HLNL, an immature crosslink, with histidine results in the formation of HHL, a mature, trivalent crosslink[29]. The reaction of HLKNL, an immature crosslink, with a hydroxylysine residues results in the formation of Hyl-Pyr while a reaction with an allysine yields a pyrrole. Both pyrrole and Hyl-Pyr are mature trivalent, heat-stable crosslinks[29]. Reprinted with modifications with permission[29].

samples (in control and $NaBH_4$ treated tissues, respectively).

Non-enzymatic Crosslinking

Non-enzymatic crosslinking occurs between collagen molecules adventitiously and is widespread in human tissues, occurring on a long time scale and affecting long lived proteins[59]. These crosslinks are derived from advanced glycation end-products (AGEs), moieties that are formed through the reaction of a reducing open chain sugar with amine groups present on proteins, lipids, and nucleic acids[29,41,42]. AGEs form between collagen molecules and can result in covalent crosslinking between triple helical portions of collagen molecules. Glycation reactions are concentration dependent and are affected by the type of sugar present. Glucose has the slowest glycation rate, while other intracellular sugars such as glucose-6-phosphate and fructose incur faster rates[49,51]. Glycation-derived crosslinks are especially important important to consider in people with type II diabetes, because of their elevated blood glucose levels[60].

The formation of AGEs changes the way that collagenous proteins interact with their surroundings. A family of receptors for AGEs is expressed on smooth muscle cells, monocytes, macrophages, endothelial cells, podocytes, astrocytes, and microglia[59,61]. The binding of AGEs to receptors causes downstream signalling that increases the expression of pro-inflammatory cytokines, along with coagulating and vasoconstrictive factors. This is thought to upregulate the inflammatory response, while slowing down healing. AGE effects at the tissue level include peroxidation of lipids, remodeling, and thrombosis[59,61]. The binding of AGEs to macrophage receptors is thought to be the primary mechanism for AGE elimination, which occurs via the renal system[59].

Examples of AGE crosslinks include pentosidine and glucosepane, which have been shown to increase denaturation temperature[62], decrease solubility[52,63] and increase failure stress and stiffness[50,52,64]. Increased stiffness has been seen in both human diabetic tendons[65], and in RTTs with induced diabetes[64]. Treatment of tendon fascicles with an AGE such as methylglyoxal (MGO), resulted in a loss of stress relaxation response and changes in failure stress and yield behaviour[66,67]. It is possible that

stiffening occurs as a results of AGE formation, and that lowered collagen content would result in the opposite effect[67,68]. AGEs can also alter the charge profile of collagen[50], thus affecting fibril-fibril interactions, which may change the force transmission present between fibrils[49,67].

1.4 Collagen Molecular Stability

1.4.1 Thermal Denaturation and Stability

Native collagen is an ordered triple-helical structure that is stabilized by a variety of interactions, as mentioned above. It is possible to study the structure and stability of collagen through thermal denaturation by taking advantage of changes to the well-known native structure. When collagen is heated in water, there is a structural transition where the triple helical structure begins to unwind and a random coil structure is formed, due to water solvation and the rupture of hydrogen bonds – a process termed denaturation[69,70]. The driving force for this transition derives from the change in Gibbs free energy of the system (ΔG), which is determined by the change in enthalpy (ΔH) and entropy (ΔS).

$$\Delta G = \Delta H - T\Delta S \tag{1.1}$$

Enthalpy is defined as the total heat content within a sample, but more generally is interpreted as the internal energy of a system (i.e. in structural changes, ΔH is a measure of bonding energy changes in the system). Entropy is a measure of the number of molecular configurations within a system and is defined by the Boltzmann equation:

$$S = k_B ln W \tag{1.2}$$

where S is entropy, k_B is Boltzmann's constant ($1.381 \times 10^{-23}\,\mathrm{kJ\,K^{-1}\,kmol^{-1}}$), and W is the number of possible conformations. Simply put, a system with greater disorder has a higher entropy value, S. The highly-ordered native collagen structure transitions to a random coil upon heating due to the breakage of hydrogen bonds via thermal

gyration and collagen molecules begin to solvate in water. This has an unfavourable ΔH, but is overshadowed by the favourable increase in S, resulting from the structural change from helix to coil. Together, these effects result in a negative free energy change, (ΔG), which is a favourable reaction.

Collagen molecules in solution begin to unfold at body temperature, and therefore stabilization of collagen must occur, otherwise newly synthesized molecules would denature and be removed from the body. The stabilization of collagen molecules is increased due to a loss in entropy resulting from the embedding of collagen molecules within fibres. The thermal stability of collagen molecules largely depends on the degree of hydrogen bonding present within the sample, which is related to the amount of hydroxyproline residues present[71]. The amount of proline hydroxylation in turn depends on two prolyl-hydroxlyase enzymes. Within collagen, there are regions along the length of the molecule that do not contain the usual Gly-Pro-Hyp sequence but rather are devoid of hydroxyproline and are called thermally labile domains[72]. The largest of these regions is found near the C-terminus of the molecule[72] while two others are found at the N-terminal end of the molecule and are 26 residues in length[73]. All of the thermally labile domains are found within the gap-region of the collagen molecules, which is thought to create space for steric changes (i.e. increase its instability)[72–74].

Hydrothermal denaturation is thought to initiate in the 65-residue hydroxyproline-free sequence at the C-terminus[70,72,73]. Hydrogen bonds begin to rupture, and the native triple helical structure of collagen begins to transition into an amorphous random coil structure. Once initial uncoiling begins within the thermally labile domain, the entire structure becomes less stable and begins to unzip[73]. Collagen denaturation is affected by lateral spacing of molecules within the fibre. Denaturation temperature will be increased by anything that reduces the free volume available for thermal gyration, because this will limit the configurational entropy of the thermally labile domains[72].

The polymer-in-a-box model is commonly used to describe the denaturation process of collagen[72,75]. In this model, the collagen molecules are seen as polymers that are

constrained in a box created by their neighbouring molecules. Collagen molecules packed in a fibre (i.e. lattice structure) are more thermally stable than those found individually in solution, through reduction of molecular motion[72,76]. Anything that affects the confinement of collagen molecules to a lattice-like structure (i.e. a box) will affect the configurational entropy and as such affect the activation of the thermally labile domain[72,77]. Endogenous crosslinking, dehydration, and swelling of collagen have been shown to have an effect on the thermal stability of collagen[43]. With respect to crosslinking, it was initially thought that the crosslinking itself increases the thermal stability, but in fact, it causes dehydration which decreases the size of the 'box' and a subsequent reduction in the configurational entropy. Overall, this mechanism serves to stabilize the collagen molecule[43,70,72–74,77–80].

1.5 Assessing Structure & Stability of Collagen

1.5.1 Hydrothermal Isometric Tension (HIT) Testing

Hydrothermal isometric tension (HIT) testing is a technique that is able to assess thermally-induced structural changes in collagenous tissues. Specifically it is able to capture the mechanical consequences resulting from thermal denaturation, breakage of thermally unstable crosslinks, and hydrolysis of the the peptide backbone within collagen. This technique involves mounting a sample between two grips and constraining the length under isometric tension (Figure 1.7). One end is fixed, while the other is attached to a load cell, which allows for measurement of tension development in a sample[81]. Following mounting, the samples are submerged in a water bath (Figure 1.7). The temperature of the bath is increased at a rate of approximately 4°C/min from room temperature to 90°C (Figure 1.8)[81–83]. After reaching 90°C, the sample is maintained at this temperature for 5 hours during the isothermal portion (Figure 1.8). This allows for the assessment of the crosslink profile present within the sample.

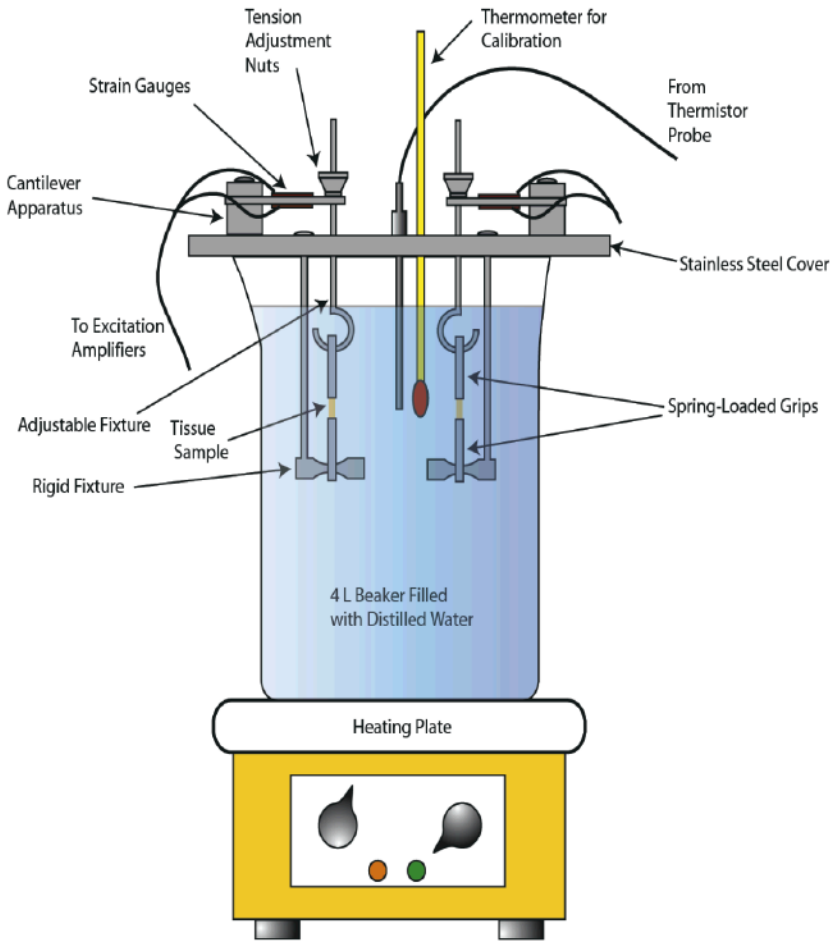

Figure 1.7: Schematic of the custom-built testing apparatus used for hydrothermal isometric tension (HIT) testing. Figure by Peter Massaro with permission.

Denaturation Temperature

The HIT data captures denaturation, assess the scission of thermally labile immature crosslinks, and the hydrolysis of the individual chains within the collagen molecule[81]. The main parameters that are determined from the resulting data are the denaturation temperature (T_d), the temperature at maximum force (T_{Fmax}) and half-time of load decay, $t_{1/2}$ (which will be discussed in the following section) (Figure 1.8).

The temperature at which there is a marked increase in force during heating is the denaturation temperature. It is important to note that with HIT, the T_d is a measure of the thermal stability of the *load-bearing* collagen within the tissue sample[84]. As the collagen triple helix denatures, the helicity decreases and the protein structure energetically favours transition to a random coil[58,81,85,86]. Because the sample is constrained isometrically, this denaturation event produces a marked increase in tension[81] (Figure 1.8). The exact value of the loads produced from this rise in tension is not informative, as they are dependent on the amount of collagen/tissue present between the grips[84,87]. After the denaturation temperature is reached, thermally labile crosslinks begin to break[84,88]. In some tendons, the tension will reach a maximum as hydrothermally unstable crosslinks break, and the temperature at which this happens is known as the $T_{F_{max}}$[84,88]. The $T_{F_{max}}$ can be used a proxy for the number of thermally stable crosslinks present in the tissue[84,88].

Half-time of Load Decay

Following the isothermal portion of HIT, it is possible to obtain the half-time of load relaxation, which is dependent on the amount of thermally stable crosslinks present in the sample[83,89]. During the isothermal portion of the HIT test, the load decays as a result of the hydrolytic scission of backbone peptide bonds within the collagen molecules (Figure 1.8). The rate of isothermal load decay can be measured by calculating the half-time of load decay, $t_{1/2}$ which is defined as the time it takes for the load to reach half of the maximum load value. This relaxation is an exponential decay similar to that of the Maxwell model of stress relaxation, we can write that the load decay will be given by:

$$\frac{L(t)}{L_{max}} = e^{-kt} \tag{1.3}$$

where $L(t)$ is the load at any given time, L_{max} is the maximum load reached in the isothermal portion, k is the constant of relaxation, and t is time[90]. When the load is

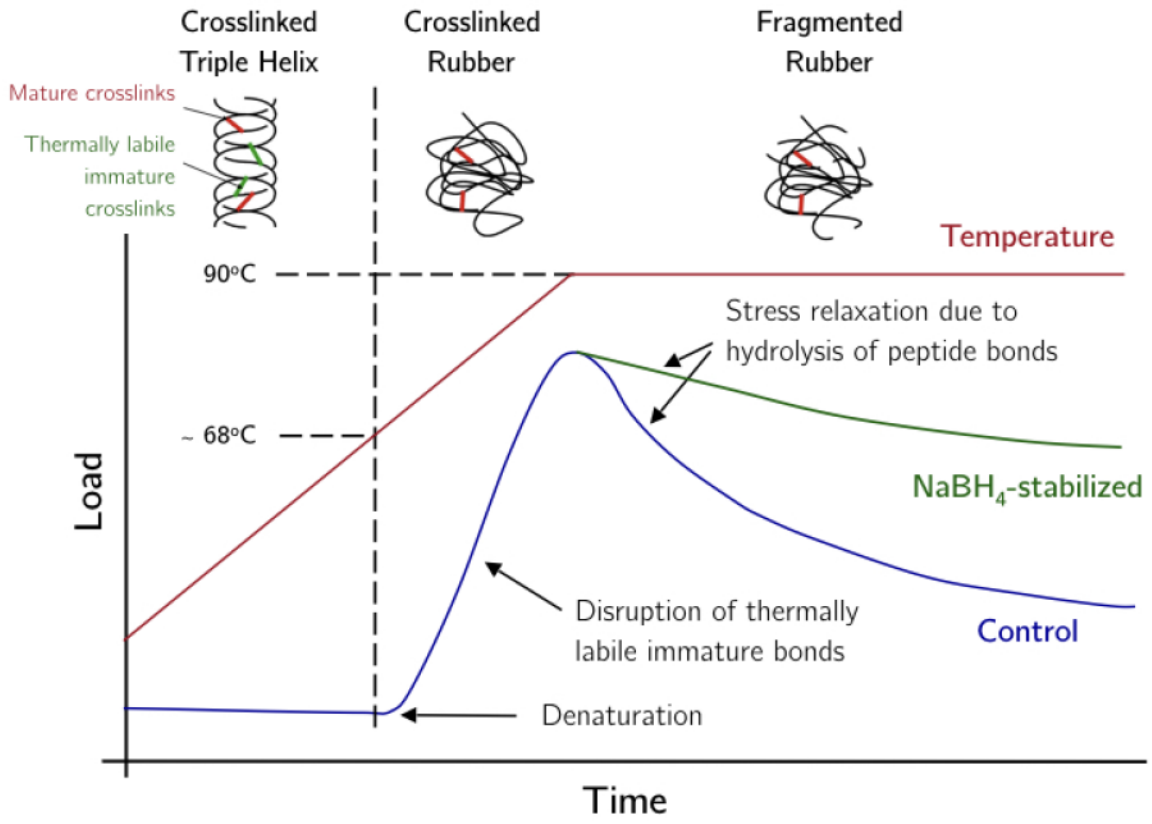

Figure 1.8: Schematic explaining hydrothermal isometric tension (HIT) testing. The temperature (red line) is gradually increased over time until it reaches 90°C, at which point it is held constant for an extended period of time. This portion of the test is called the isotherm. At a certain temperature during the ramp to 90°C, the structure of the collagen triple helix begins to transition to a random coil, due to the breakage of hydrogen bonds and chain solvation (i.e. denaturation). The temperature at which this transition begins to happen is called the denaturation temperature, and because the sample is being held at a fixed length, this transition does not occur but it is registered as an increase in load (blue line). Following denaturation, but prior to the isotherm, thermally labile crosslinks will begin to break which contributes to pre-isothermal contraction. At 90°C, stress relaxation due to the hydrolysis of peptide bonds will begin. Sodium borohydride ($NaBH_4$) will convert thermally labile crosslinks into their thermally stable counterparts, thereby decreasing the amount of load decay that is happening (green line) as compared to the control, untreated samples (blue line). Adapted with permission from J. Michael Lee.

equal to half of the maximum load, the equation becomes:

$$\frac{1}{2} = e^{-kt_{1/2}} \tag{1.4}$$

where k can be determined from the slope of a $ln(\frac{L(t)}{L_{max}})$ versus time plot[90]. This relationship was first described by Le Lous and Allain, and they stated that the constant of relaxation, $-k$, is proportional to the rate of peptide bond scission and is inversely related to the number of chains per unit volume that continue to sustain load[90]. As mentioned above, the $t_{1/2}$ is also related to the amount of thermally stable crosslinks present within the sample. While the peptide bonds are being hydrolyzed during the isotherm, tissue integrity can be maintained by the presence of heat-stable crosslinks. By combining HIT with $NaBH_4$ reduction of tissues, which stabilizes thermally labile crosslinks, it is possible to look at the total crosslinking in a sample by comparing values of $t_{1/2}$. Both inter- and intramolecular crosslinks increase $t_{1/2}$ during the isothermal portion of HIT, but intermolecular crosslinks leave the T_d unaffected[83,89]. Intramolecular crosslinks, however, increase the T_d[89]. This model of load decay works well for tissues that are largely collagenous, and that are decreasing in load over time during the isotherm. For tissues that contain elastin[83] or are heavily crosslinked, the load will continue to be supported and may not decay. In these samples, such as glutaraldehyde crosslinked heart valve tissue[91], it might be prudent to consider other models or to use the presence of load decay as a useful piece of information in itself.

1.5.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a another tool used for analyzing the thermal stability of collagen[86,92]. This method is used to evaluate enthalpic energy changes that occur within thermal transitions. In this experiment, a sample is placed in a sealed aluminum DSC pan and heated alongside an (often empty) reference pan. The heat flow required to keep both samples at the same temperature during a ramp increase is measured, and the differential heat flow is recorded. The DSC heat flow

signal ($\frac{dH}{dt}$) is measured in $\frac{J}{g \cdot s}$ and is determined by the heat capacity ($\frac{dH}{dT}$, in $\frac{J}{K \cdot g}$) and the scanning rate ($\frac{dT}{dt}$ in $\frac{K}{s}$)[93].

$$\frac{dH}{dt} = \frac{dH}{dT} \times \frac{dT}{dt} \tag{1.5}$$

At low temperatures, the rate of denaturation is slow, so DSC does not register the small changes in heat capacity. Once enough thermal energy is applied, denaturation occurs at a faster rate, which is now able to be registered by the DSC since there is a larger increase in heat capacity. Several characteristic features regarding phase transitions during denaturation can be determined from the resulting endotherm curve (Figure 1.9) since denaturation affects the $\frac{dH}{dt}$[73]. These parameters are affected by structural features within collagen, such as molecular packing, crosslinking, and water content. Because thermal transitions involve an increase in heat absorption and capacity, a peak (usually shown inverted as a valley) on the DSC endotherm plot can be seen. The onset temperature (T_{onset}) is the temperature at which the least thermally stable collagen molecules begin to unravel and denature due do the breaking of hydrogen bonds and chain solvation[92,94]. During the DSC experiment, the lower energy bonds within each sample rupture earlier than higher energy ones, and therefore the DSC endotherm can be thought of as a spectra showing the range of thermal stabilities present within the sample. The peak temperature (T_{peak}) is the point at which there is maximum heat flow[92,94]. The specific enthalpy of denaturation (Δh) is defined as the area under the endotherm (1.9) and indicates the amount of energy needed to denature the molecules capable of this transition[92,94].

The parameters that are extracted from the DSC endotherms are important, but the shape of the curve can also provide some information[73]. The full width at half maximum (FWHM) is a measure of the range of thermal stabilities present within the sample (1.9)[94]. While DSC is not able to identify which bonds are broken specifically[95], a broader peak resulting from denaturation would imply that there is a more heterogeneous bond population within that sample than that of a narrow one[96].

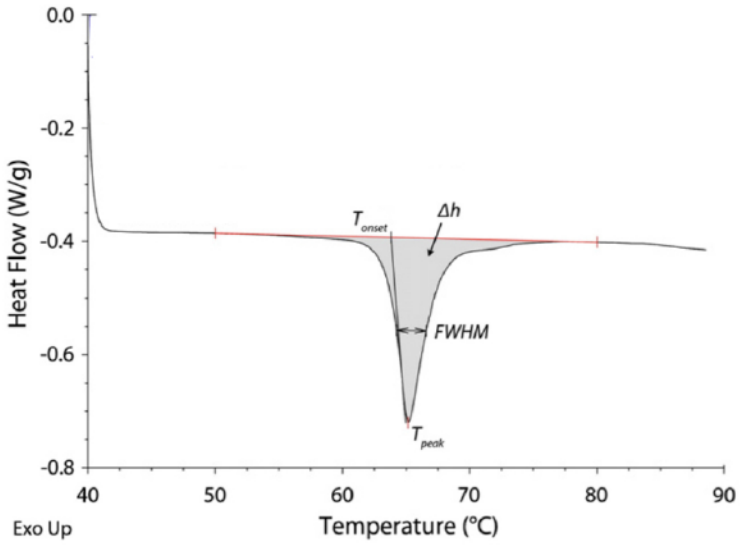

Figure 1.9: Representative DSC endotherm of collagen denaturation with the parameters labelled. Adapted with permission[85].

1.5.3 Thermal Studies on Tendon Collagen

There have been many studies on the thermal stability of collagen, dating back to the early 70s[97]. DSC, in particular, has been used to study the denaturation process in skin and tendon collagen and how this changes with glycation[98], age[96,98], damage[99], or disease[99,100]. It is known that with age and increased glycation, there is an increase in the denaturation temperature in RTT collagen as measured by DSC[64,98]. Miles et al. showed that there is an increase in thermal stability of collagen that is induced by dehydration, which occurs as a result of increased crosslinking[43,72].

Young and old bovine tail tendons (BTTs) were used to study age-related changes in DSC and HIT parameters within tendon collagen. The T_{onset}, T_{peak}, and T_d did not change between the two age groups, while the $T_{F_{max}}$ increased significantly with

age[86]. A previous study demonstrated that young BTTs contained primarily HLNL and HHL crosslinks, and that the amount of thermally stable crosslinking was low, as none of the young samples survived to 90°C in HIT[101]. It has been shown using DSC and HIT that the density and type of crosslinking present within anatomically proximate but functionally distinct tendons is different, with common digital extensor (CDE) tendons having fewer thermally stable crosslinks and a lower total crosslink density than superficial digital flexor (SDF) tendons[102].

Other studies have shown that damage to tendon is also detectable by examining the thermal stability of the constituent collagen. It has demonstrated that damaged BTTs have reduced T_{onset} and T_{peak} values and an increase in FWHM as compared to undamaged samples[94]. This damage-induced decrease in denaturation temperature was also seen in HIT (as measured by T_d)[86]. This decrease in thermal stability with mechanical damage was explained by an increase in entropy leading to an increase in freedom of the thermally labile domain (Figure 1.10)[94].

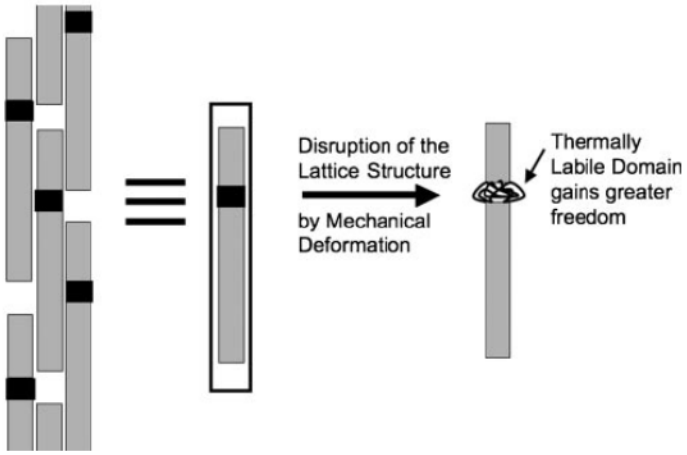

Figure 1.10: Schematic of the Effect of Disruption of Lattice Structure on the Thermally Labile Domain. Modified and reprinted with permission[94].

Thermal Stability of Human Tendon Collagen

While there have been many animal studies on the changes in thermal stabilities of tendon collagen following mechanical overload, there have not been as many calorimetric studies on undamaged or damaged human tendon, or systematic aging studies. Not et al. showed that there is a difference in the structure of collagen in healthy and diseased human tendons, studying a variety of pathologies such as carpal tunnel syndrome, De Quervain tenosynovitis, and Dupuytren disease[100]. Pathologic tendons did not show any changes in denaturation temperature but did show significant decreases in the Δh compared to native intact tendons[100].

Another study looked at the effect of damage on the thermal stability of human tendon. Ruptured Achilles, quadriceps, and patellar tendon had increases in denaturation temperature and FWHM, as compared to healthy control samples, while Δh decreased[99]. These studies, while somewhat insightful, are limited because the control samples and ruptured samples did not come from the same patients. Control tissues came from cadavers who were under 55 years of age at death, while the ruptured and pathological samples came from patients undergoing surgery with no mention of patient age[99,100]. Aging has been shown to affect collagen structure, so it is possible that the differences cited in these studies were age-related or related to the variability in patient population (i.e. a problem of sample size). Additionally, none of the studies used the same tendon or the same methodology, so it is difficult to compare the results to one another and draw a conclusion about the effect of damage or disease on human tendon collagen structure.

1.6 Models Used to Mimic Tendon Injury

Modelling tendon injury using animal models has been done for decades and allows for the examination of tissues during all stages of tendon injury. Furthermore, mimicked injuries are repeatable and reproducible whereas clinical human tendons have often been studied during chronic, end-stage tendinopathy. Frequently-used animal models

include the murine, rabbit, ovine, canine, bovine, and primate models. The most studied tendons within these models are the tail, patellar, Achilles, and forelimb tendons. Many of these are limited in that lab animals are quadrupeds and the load distribution is different due to differing anatomy and kinematics than that of humans, but these shortcomings are overshadowed by their ease of extraction and availability[103].

1.7 Visualizing & Assessing Damage in Tendons

Tendons undergo uniaxial tensile forces *in vivo*, and it is thought that various features of tendon collagen help to support these loads. The waveform crimp helps with extensibility at low loads[104,105], while crosslinking between collagen molecules supports increased loads at high extensibilities[51]. Thankfully, extended fibrils do not undergo brittle fracture but rather absorb strain energy. Because of this, strains and sprains are more prevalent than complete rupture.

Damage to tendon is damage to the components within tendons. Initially, it was unknown which structural features within the tendon hierarchy were affected by mechanical overload. While optical microscopy can be used to visualize whole tendons, collagen crimp, and fascicular structures, finer elements such as fibrils, and subfibrils can only be seen under higher magnifications. Light microscopy is limited by the optical resolution, r, which is defined by:

$$r = \frac{\lambda}{2 \times NA} \tag{1.6}$$

where λ is the wavelength of visible light, and NA is the numerical aperture of the lens. This limited resolution led to the development of electron microscopy, where the wavelength of electrons is significantly smaller, allowing for finer details to be distinguishable.

Early studies showed that mechanical rupture or cyclic overload of wet RTTs resulted in the dissociation of collagen fibrils into components that were on the nanoscale[106,107].

In one particular study the D-banding, which is characteristic of properly assembled collagen fibrils, was absent following tendon fascicle rupture, suggesting that there was slippage of subfibrillar components (Figure 1.11)[107].

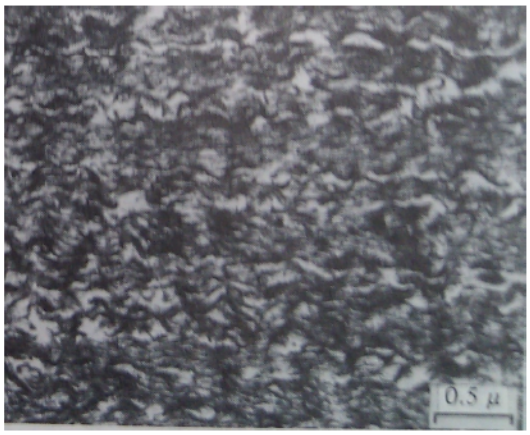

Figure 1.11: The shredding of the collagen fibril structure was seen in RTT fascicles following rupture, resulting in a loss of D-banding which suggests that there is subfibrillar slippage occurring. Reprinted with permission[107].

Many of the initial studies on tendon damage used an RTT model and found that the constituent collagen fibrils dissociated into their subfibrillar components following rupture under transmission electron microscopy (TEM)[106–111]. A study conducted by Nemetschek et al. found that overload of RTTs resulted in the formation of kinked structures, or the dissociation of collagen fibrils into their subfibrillar components (Figure 1.12)[108]. This longitudinal disintegration of RTT collagen fibrils was later confirmed by Knorzer et al., who saw similar damage motifs (Figure 1.13)[109].

In addition to transmission electron microscopy (TEM), X-ray diffraction has been used to examine fibrils, subfibrils, and individual collagen molecules[107,109]. Using this technique, one study discovered that damaged RTTs did not incur damage on the fibril level due to interfibrillar sliding, as molecular level damage was found prior to the occurrence of this sliding[109]. More recent studies have used atomic force microscopy (AFM) to examine collagen fibrils with the added advantage of being able to obtain mechanical data from the scanned structures[112–116].

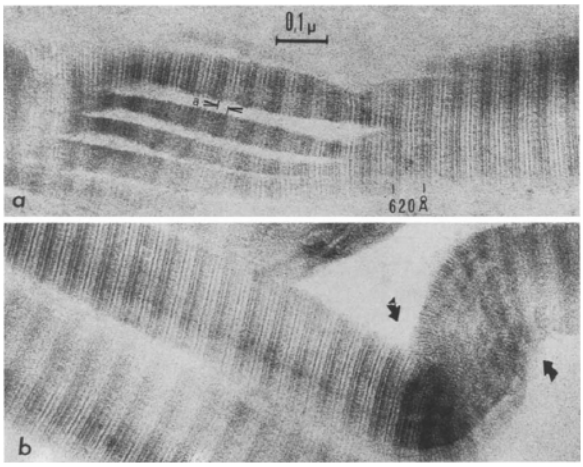

Figure 1.12: Transmission electron micrograph of overloaded RTT reveals the exposure of subfibrillar structure and kink formation on the fibril level. Reprinted with permission[108].

1.7.1 Discrete Plasticity

Veres et al. confirmed that tendon damage does indeed occur at the level of the collagen fibrils on a nanoscale[58,85,102,117–119]. The initial study was done using a BTT model and involved rupturing tendons and imaging them using scanning electron microscopy (SEM)[94,117].The overloaded tendon samples were compared with control tendons, which remained homogeneous and organized. The loaded tendons showed kinks along the length of the fibril at discrete sites, leading to the conclusion that collagen structure may not actually be completely uniform and homogenous (Figure 1.14)[117]. This kinking mechanism was termed 'discrete plasticity' and is thought to contribute to the toughness of collagen[58,85,117–120].

It was discovered that this motif was present after overload even without rupture. One subrupture overload cycle refers to loading tendon until the slope of the stress-strain curve reaches zero, at which point the sample is unloaded. This process can be repeated for a number of cycles. BTTs were cyclically loaded to subrupture overload and it was found that the number of kinks increased with the number of overload cycles, showing an accumulation of damage (Figure 1.15)[85].

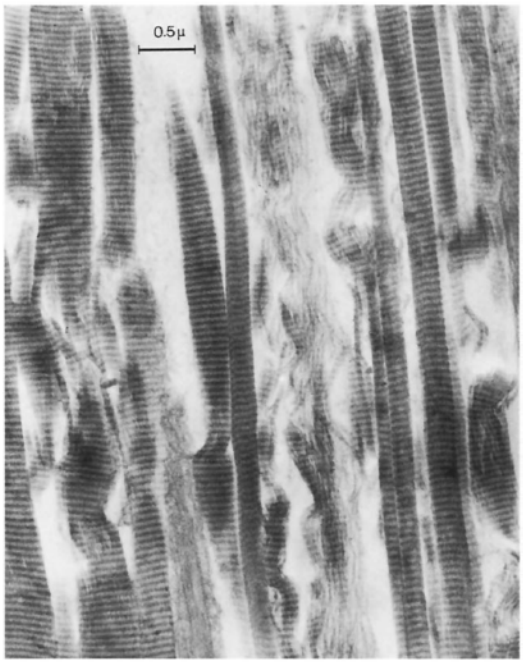

Figure 1.13: Transmission electron micrograph of overloaded RTT shows the longitudinal disintegration of fibrils into smaller components. Reprinted with permission[109].

Trypsin, a serine protease that selectively cleaves non-helical portions of collagen, was used to determine the amount of denatured collagen present[94,117]. The enzymatic digestion, followed by SEM, showed that there was a layer of denatured collagen present on the surface of the fibrils following overload cycles (Figure 1.16).

Along with contributing to collagen toughness and prevention of brittle fracture, it was thought that these discrete kink sites could serve as recognition sites to promote healing following injury. Macrophage-like (U937) cells, which are capable of releasing enzymes that selectively degrade denatured collagen, were cultured on the surface of damaged collagen. Following seeding, the cells showed an increased number of filapodia, which is associated with phagocytosis of damaged tissues, and it appeared as though a layer of material was removed (Figure 1.17)[85,119]. These findings suggest that discrete plasticity may not only serve to toughen collagen, but to help with the body's recognition of damage[119].

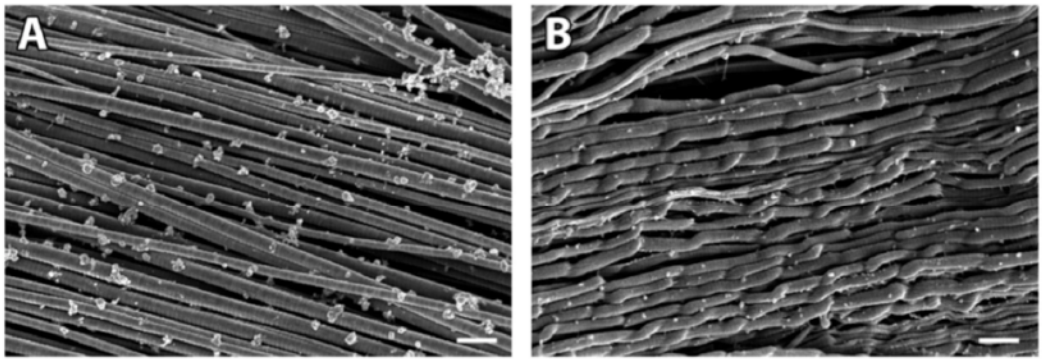

Figure 1.14: Micrographs of undamaged control (A) and ruptured (B) tendon samples. The ruptured tendons show longitudinal kinking on the fibril level, while D-banding remains intact between kink sites. Scale bars are 500 nm (20,000X). Reprinted with modifications with permission[117].

Another model used to investigate this phenomenon is the bovine forelimb model, which contains structurally proximate but functionally distinct tendons. The superficial digital flexors (SDF) and common digital extensors (CDE) are energy-storing and positional tendons, respectively. Mechanical overload and subsequent SEM of the two revealed that CDEs were stronger and tougher than SDFs, and showed higher levels of plastic damage on the nanoscale following rupture (Figure 1.18)[102]. This differential damage mechanism between these two tendons was confirmed using AFM and second harmonic generation, where it was also found that individual flexor tendon collagen fibrils did not undergo extensive molecular damage while the matching extensor tendon collagen fibrils did[121]. These studies suggest that the formation of discrete plasticity kink sites may be dependent on the amount of crosslinking present in tendon collagen, as flexor tendons are more heavily crosslinked than their extensor tendon counterparts[102].

1.8 Biomechanical Properties of Tendons

In order to transmit forces that allow for locomotion and movement, tendons must be able to transfer energy from musculature to the skeletal system across joints in the

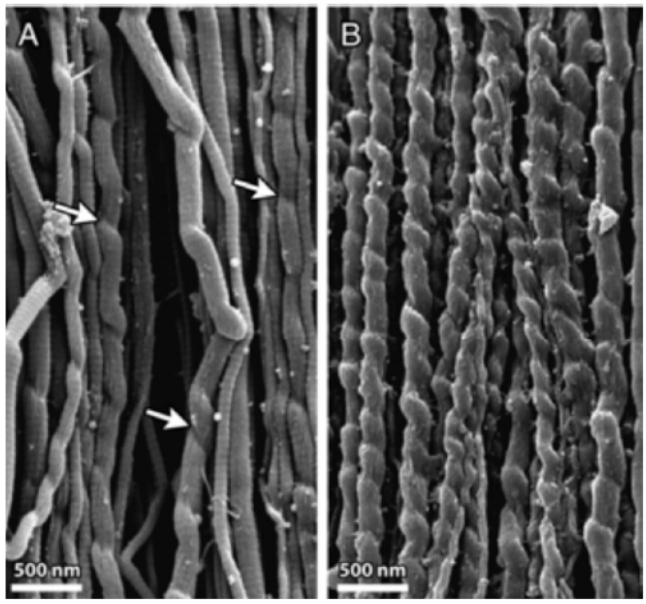

Figure 1.15: SEM images of ruptured and subrupture overloaded BTT samples (A and B, respectively). The linear kink density increased with increasing subrupture overload cycles. Scale bars are 500 nm (20,000X). Reprinted with modifications with permission[85].

body. During gait, potential energy is stored in tendons as strain energy. The recoil of tendons following extension results in the conversion of this potential strain energy into kinetic energy[29]. With aging and disease, the ability to convert between strain energy and kinetic energy decreases, and with this it is possible to infer changes in the structure of the ECM in tendon[29,48].

Many tissues in our bodies are primarily made up of collagen yet are viscoelastic. This means that these materials can store some energy elastically while dissipating some energy to heat. These properties can be attributed in part to the large water content within tendons. The collagen within tendons is largely arranged in parallel, and therefore tendons are anisotropic materials where the mechanical properties are greater along their length than their width. As muscles transmit force through tendons, extension occurs which results in the storage of energy within the tendon. This is not a 100% transmission of energy, as 10-25% of the energy is dissipated due to sliding of collagen fibrils[29]. The origins of energy storage within tendons can be

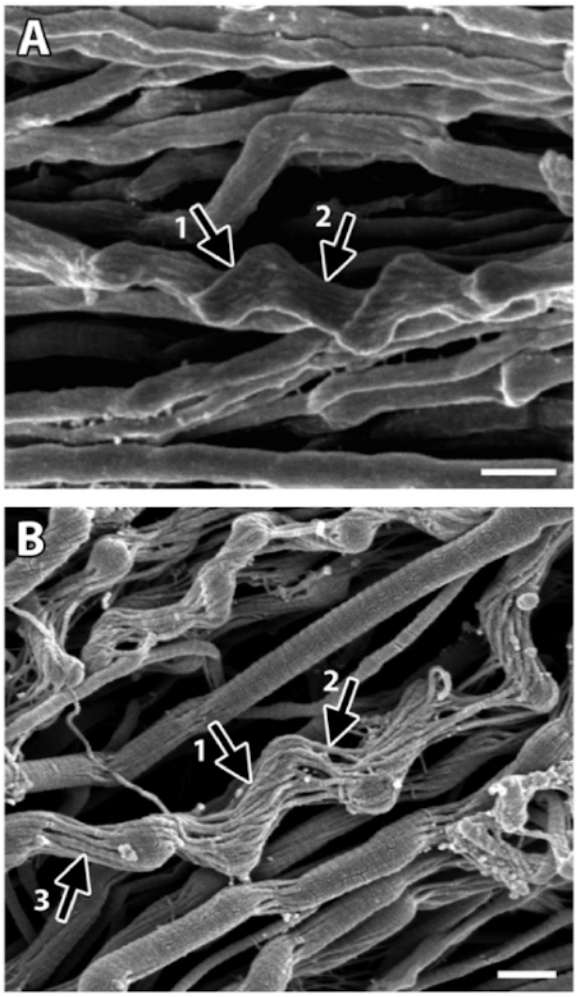

Figure 1.16: SEM images of damaged BTT samples (A) and damaged samples treated with the protease, trypsin (B). Following enzymatic digestion, some material is missing around the kink sites, confirming that denatured collagen was removed. Scale bars are 300 nm. Reprinted with permission[117].

attributed to changes of the triple-helical structure of collagen, stretching of fibre bundles, and straightening of collagen crimp. Because they are viscoelastic, tendons display hysteresis[29]. The mechanical properties of tendons are dependent on strain rate, where higher strain rates yield higher moduli and slow strain rates result in an apparent decrease in stiffness. Collagenous tissues also have a high modulus, E, and

remarkable toughness and strength[14,29,48].

The exact shape of stress-strain curves of tendons depends on the nature of the sample, but all share similar features. The non-linear stress-strain curves of collagenous tissues have characteristic regions (Figure 1.19). The initial portion is called the toe region, in which the collagen crimp is straightened out due by low forces[21]. It is followed by the linear region, in which the collagen molecules are aligned, elongated, and are able to slip past each other. The slope of the linear portion of the stress-strain curve is referred to as the elastic or tissue modulus, the magnitude of which depends on the tendon in question and the exact nature of measurement. Once collagen fibrils begin to fail, there is deviation from linearity and the point at which this happens is known as the yield point. The portion of the graph after the yield point is known as the yield region in which the collagen continues to fail until the failure point, which is typically characterized as a sharp drop in load on the stress-strain curve[21]. The stress and strain at which failure occurs are known as the ultimate tensile strength and failure strain of the tendon, respectively. Similar to the modulus, these parameters are also affects by the strain rate.

Specifically, the yield region of the curve can plateau for an extended period of time before failure denoting that there is significant plastic damage being incurred. Other tendons may have a shorter yield region prior to the failure point, suggesting that the sample is more brittle in nature. The shape of the stress-strain curve can therefore be indicative of the mechanism of failure. For example, a tendon that is comprised of many individual fascicles or fibres can show numerous small fractures before the entire structure fails as a whole, which may indicate that some fibres are susceptible to failure prior to other ones. Conversely, other tendons may not show individual fractures, but rather fail as one whole unit, denoted by the presence of a single failure point on the stress-strain curve.

The general class of tendon is important to consider when comparing mechanical parameters of tendons. While all tendons are viscoelastic, not all tendons are equal in terms of their mechanical properties[122]. Positional tendons such as extensor tendons in the hands and feet, generally require more speed and precision than energy-storing

tendons such as the Achilles or patellar tendons, and therefore these 'low-energy' tendons are generally stiffer and less extensible[122,123].

1.8.1 Measuring Mechanical Properties

Typically, the mechanical properties of tendons are obtained *in vitro* by gripping isolated samples in a tensile materials testing system, and measuring the resulting load and deformation from which resulting stress (σ) and strain (ϵ) can be calculated. Ensuring that the sample is properly mounted within the system is important, as any slippage will result in inaccurate measures of deformation. It is possible to test whole tendons, however is it also possible to break the tissues down into smaller subsamples (such as individual fascicles) to combat slippage issues. Along with traditional tensile tests performed in materials testing systems, AFM can be used to measure mechanical properties of individual collagen fibrils (lateral indentation stiffness)[112,114,116].

More recent studies of the mechanical properties of tendons have been using *in vivo* methods, where an imaging modality (e.g. X-ray or ultrasound) is combined with voluntary muscle contraction and joint moments are calculated to determine the applied load[124]. The forces are calculated from external moments, and it may be difficult to determine which moment arms are acting on the tendon[125] since it is possible that a moment is resulting from the action of more than one tendon or muscle group[126]. Ultrasound imaging allows for the real-time tracking of deformation, but is limited because it relies heavily on tracking anatomical landmarks such as the myotendinous or osteotendinous junctions, which may be difficult to image simultaneously for long tendons. More recent studies using ultrasound have used speckle tracking as a new method of measuring tendon deformation, which not only bypasses the need for anatomical landmarks but also allows for strain measurements within specific parts of tendons[127,128].

1.9 Age-Related Changes in Structure & Mechanics of Tendon

While age-related changes can pertain to embryonic development, maturation, or puberty, in this thesis aging will be defined as time after maturation, through adulthood, into near-geriatric life. The rate of aging is not equal between species or even within species, as it depends on many factors including lifestyle and genetics. There are many changes that occur slowly over time, and it is important to consider life-span when studying aging in non-human models. Life-span refers to both the relative and absolute age of the species. Compared to most animals, humans have a long life span in terms of absolute age, and as such there are many features that will be more pronounced and evident than in common animal models have shorter absolute life spans (e.g. mice and rats only live a few years)[129,130]. For example, the age-related increase in glycation derived crosslinks is dependent on absolute age, and therefore old humans will have higher amounts of AGE-crosslinks than old rats, dogs, or cattle[129]. This difference in absolute life span, combined with differences in rates of aging[129] along with varying species and tendon types makes it difficult to compare studies, and even more difficult to draw firm conclusions regarding changes that occur within tendons during aging.

1.9.1 Changes in Tendon Structure with Aging

Numerous age-related changes in structure have been reported in the literature, however many of these findings have been contradictory and were found to depend upon the tendon in question. The relationship between tendon cross-sectional area (CSA) and age remains unknown. Some studies have found that the CSA of animal and human tendons increase, decrease, or do not change with age. The collagen content[68,131,132], cellular content and volume density of cells[133] within human and animal tendons decreases with age. The morphology of fibroblasts and tenocytes change, yielding elongated cells with higher nucleus-to-cytoplasm ratios[134]. The rates of protein synthesis within cells decreases, which contributes to the decrease in collagen turnover rates[134]. The amount of water in tendons decreases from about 80% at birth

to 30-70% in older populations[133,134]. It is thought that this dehydration partially stems from an increase in enzymatic and non-enzymatic crosslinking that occurs with age[43,135].

Perhaps the most marked change in the structure of tendons is the presence of non-enzymatically derived AGE-crosslinks, which as previously discussed have been shown to, decrease the solubility of collagen, increase the thermal stability, and may increase mechanical stiffness[50,51]. *In vitro* glycation of animal and human tendon has resulted in increased collagen molecular spacing[62,136,137] further demonstrating that AGEs have an effect on collagen structure. AGEs are related to both diabetes and aging, however, their role on tendon collagen structure *in vivo* remain unclear. In one study on human toe extensor tendons, there was an age-related increase in collagen molecular spacing that was not seen in diabetic tendons[138]. Conversely, James et al. found that human diabetic extensor tendons resulted in increased molecular spacing while age did not play a role[136]. Gautieri et al. found that human semitendinosus and gracilis tendons had age-related increases in molecular spacing as measured by small-angle X-ray scattering[62]. The exact changes that occur to the structure and composition of tendons with aging has not fully been elucidated, however the consensus is that aging results in lower collagen content, decreased cellularity, and increased crosslinking.

1.9.2 Changes in Tendon Mechanics with Aging

The changes in mechanical properties of tendons that occur with aging have been a topic of debate in the literature as they depend on the nature of the experiment. It is difficult to compare mechanical overload experiments that were conducted *in vivo* as compared to *in vitro*. Moreover, it is difficult to compare different tendon types (both within and across species). The structural changes that occur within tendons will certainly have an impact on the resulting tendon, and there are many confounding results seen in the literature. Additionally, age-related mechanical changes are difficult to anticipate because the structural changes may counteract one another. In other words, a lower collagen content and decreased molecular density may result in a decrease in strength and stiffness, but an increase in enzymatic or non-enzymatic crosslinking

(and resulting reduced water content) would have the opposite effect[124].

Animal studies have been performed on a variety of aging tendons and there is no consensus on what changes occur with age. In rodent tendon models, increases[139–141] and decreases[142–144] in the modulus and strength have been found to occur with age, while others have found no age-related mechanical changes[131,145,146]. The animal models are limited with respect to the fact that most animals have a shorter life span than humans and some of the tendons do not accurately represent a load-bearing human tendon model.

Experiments using human tendons are often influenced by a variety of factors that may influence tendon properties. Diet, lifestyle, physical activity, sex, and diabetic status have all been found to affect the mechanical properties of tendons[124]. Furthermore, there is often missing information regarding the injury history or the preceding degenerative changes that may be present[147]. *In vitro* human studies have been performed on fresh-frozen cadaveric patellar tendons and some have found a reduction in modulus with aging[148], while others have found no changes[18,149]. Cadaveric human extensor tendons from the hands and feet also showed that age had no effect on modulus[150].

Similar to the *in vitro* studies, *in vivo* human studies have found inconclusive information about age-related mechanical changes in tendon. Studies have found that the modulus remains unchanged[68,151] or decreases with aging. Within aging patellar tendon, there were no age-related differences found in mechanical properties[68,151], which correlates well with the *in vitro* studies[18,149]. Age-related reductions in modulus have been found in *in vivo* studies of female vastus lateralis tendons[152], male Achilles tendons[153], and both male and female calf muscle tendon complexes[154]. The *in vivo* studies suggest that any mechanical differences in tendon mechanics may be due to changes in the force output from musculature that arise with age[151]. In any case, the variable nature of the experiments in terms of sex and tendon type, makes it difficult to draw any definitive conclusions regarding age-related changes in tendon mechanics.

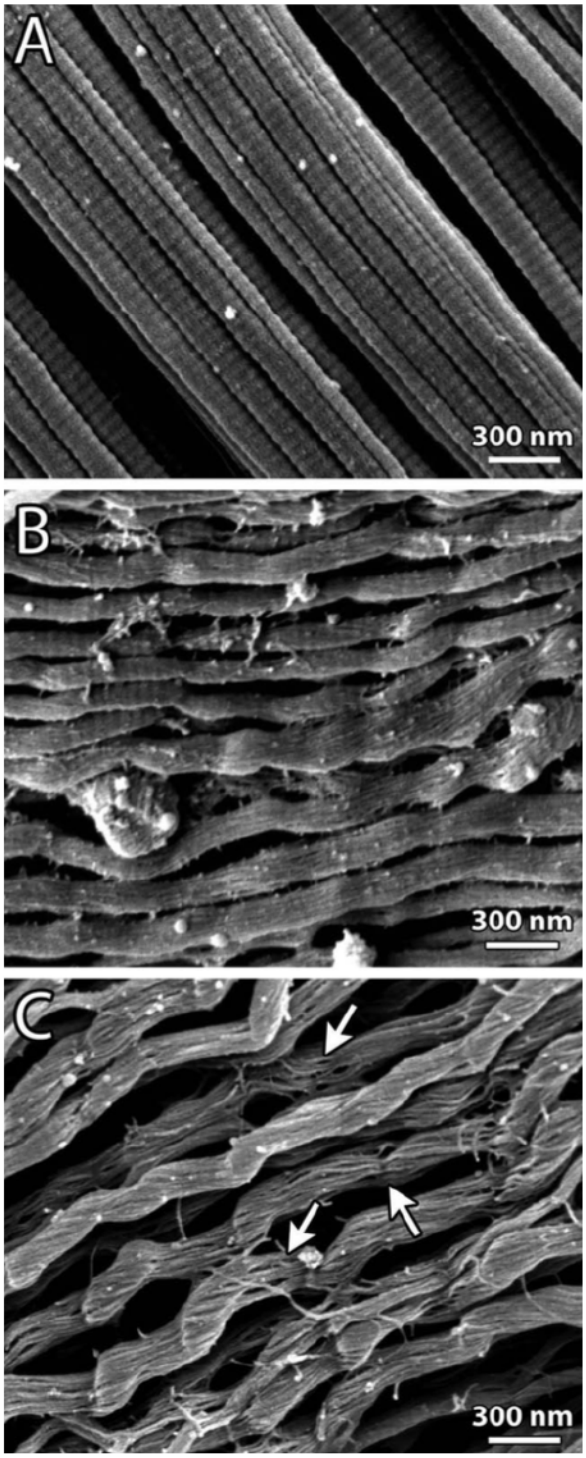

Figure 1.17: SEM images of (A) unloaded, control BTT; (B) damaged BTT following overload without cells; (C) damaged BTT cultured with U937 cells. These macrophage-like cells removed matrix material surrounding damaged fibrils. Reprinted with permission[119].

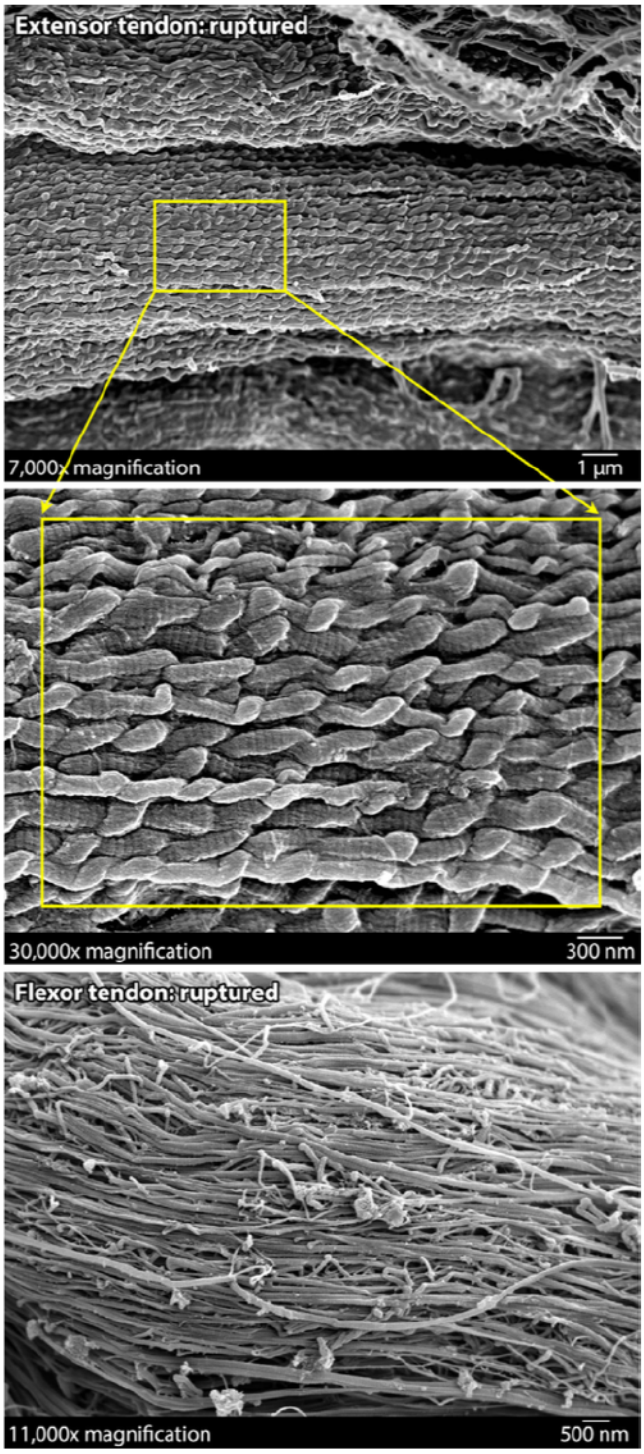

Figure 1.18: SEM images of ruptured bovine forelimb tendons. Extensor tendons show extensive plastic damage in the form of discrete plasticity kink sites, while flexor tendons do not show the same damage motifs. Reprinted with permission[102].

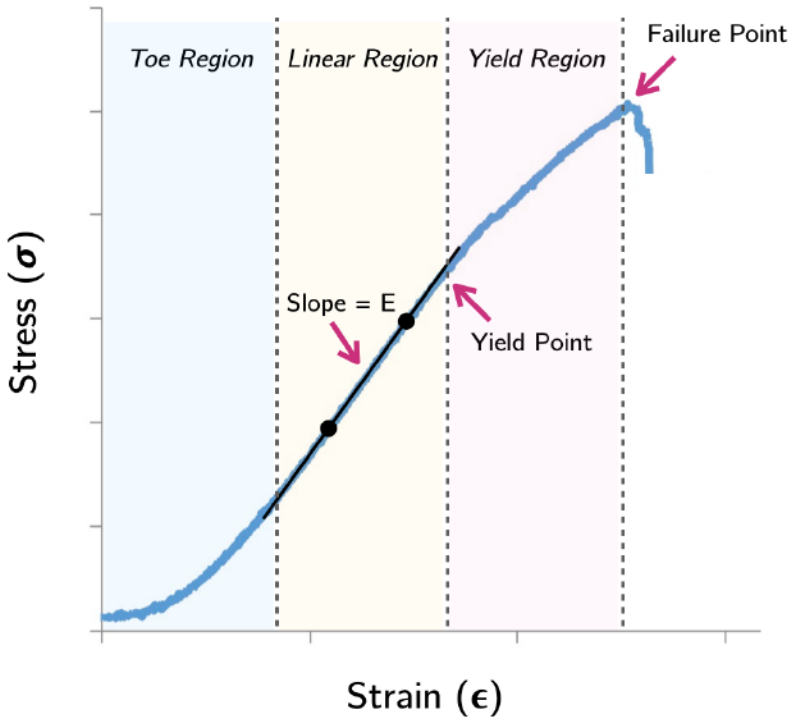

Figure 1.19: Schematic of a typical stress-strain curve of a ruptured tendon, with the characteristic regions labelled accordingly. E: Tissue modulus.

Chapter 2

Thesis Rationale, Research Questions & Objectives

2.1 Overview

Soft-tissue injuries are prevalent in our society, in both young and old populations, yet the underlying failure mechanisms remain elusive. It is known that with age, there is a steady decline in muscle strength and power,[155,156] and in our bone mass[157], all of which impact the ability of elderly people to remain mobile without sustaining injuries[158]. Despite understanding other components of the musculoskeletal system and how their properties change with age, the changes that occur in human tendon structure are not yet fully understood and effects of age on tendon mechanical properties remains inconclusive. While it has been determined that there are changes in crosslinking which sometimes lead to changes in mechanical properties, the relationship between these factors and features of failure has yet to be fully elucidated in an aging human tendon model.

2.2 Experiment I: Thermal Stability Assessment by HIT and DSC

Rationale: Collagen is the main load-bearing component within tendons, and changes in crosslinking have an effect on the thermal stability of the constituent collagen. It is possible that changes in crosslinking will also have an effect on the tendon mechanical properties and damage motifs present. While is is possible to quantify and identify crosslinks present within tendons by chromatography[61,86], it is also possible to assess collagen thermal stability by HIT and DSC, and parameters from theses tests can serve as a proxy for amount and type of crosslinking present.

Objective: To determine the thermal stability of collagen within aging human sartorius tendons and assess the maturity of crosslinking present.

Hypothesis: The level and maturity of crosslinking in the human sartorius tendon will increase with age in male, non-diabetic samples. This would result in an increase in the denaturation temperature and the half-time of load decay during the isothermal portion of HIT. For DSC, it is predicted that older samples will have higher onset and peak temperatures than will younger ones, due to the increased presence of enzymatic and non-enzymatic crosslinks. It is also thought that the FWHM will decrease with age, meaning that the range of molecular stabilities within each sample will narrow due to decreased remodelling in older tissues and increased crosslinking. The enthalpy of denaturation is predicted to increase with age, due to the increase in energy needed to break the thermally stable crosslinks present within the sample.

2.3 Experiment II: Analysis of Nanoscale Damage Motifs

Rationale: It has previously been shown that damage to tendons occurs on the nanoscale[58,117,118], characterized by serial kinks that have a superficial layer of denatured collagen on them[85]. These kink sites are potential recognition sites for wound healing cells[119], and could help with triggering remodelling/repair within the body.

Since discrete plasticity kinks are thought to prevent brittle fracture of tendons it may disappear after reproductive age, as there would no longer an evolutionary benefit to promote survival after genetic material has been passed on. To date, this mechanism has only been looked at in rat and cattle models, but it is unknown whether this mechanism represents what overload damage looks like within human tendons and how this may change with age.

Objective: Determine whether the discrete plasticity mechanism works in humans and if the previous models of tendon injury are predictive of what happens in a maturing human tendon such as the sartorius tendon.

Hypothesis: The failure mechanism in tendon collagen changes with age - discrete plasticity works until reproductive age (<40 years of age) and then disappears.

2.4 Experiment III: Mechanical Characterization

Rationale: The mechanical properties of aging tendons have been studied in a variety of animal and human models, and the results are inconclusive. A systematic study on age-related changes in mechanics within a human tendon model have not previously been investigated. The rupture experiments proposed for this thesis will be performed at 0.25%/s, as per the initial discrete plasticity study in bovine tail tendons[117]. Moreover, the tendons will be ruptured to validate the models that have previously been used. It has been found that the damage motifs present in low or high load tendons depend on the degree of crosslinking, which in turn, depends on the intensity of *in vivo* loading and age[102]. Furthermore, the amount of crosslinking is expected to greatly influence the mechanical properties. It is possible that tough, plastic tendons, with low crosslinking exhibit discrete plasticity damage[102]. Therefore, it is of interest to compare the mechanical properties of the human sartorius tendon to our existing animal models, and to previously established models of human tendon injury (i.e. patellar and Achilles tendon). If the mechanical properties and the crosslinking profile, and thus in turn the damage motifs, are similar to our previous models, it would mean that they are a useful representation of damage in this specific

human tendon. More importantly though, if they are different, this would establish a new model for tendon injury in humans.

Objective: Characterize the mechanical properties of the sartorius tendon and establish a loading protocol for damage simulation.

Hypothesis: The mechanical properties of the human sartorius tendon will change with age, in step with the predicted increase in crosslinking with increasing age. An increased amount of crosslinking will cause stiffening of the fibres, and result in higher strength and stiffness in older populations as compared to younger ones. As compared to other tendons studied in the literature, the sartorius tendon will not be as stiff as higher loaded tendons such as the Achilles and patellar tendon, but will be stronger and stiffer than the well-established bovine tail tendons.

Chapter 3

Materials & Methods

3.1 Tissue Collection

Sartorius tendons were collected from tissue donors at the Capital Health Regional Tissue Bank. The sartorius muscle forms the lateral border of the femoral triangle with the adductor longus and the inguinal ligament (Figure 3.1)[159,160], and is responsible for knee and thigh flexion, and hip abduction. The tendon itself is part of the pes anserinus, a conjoined tendon that is a highly loaded complex made up of the tendons of the sartorius, gracilis, and semitendinosus (Figure 3.2)[161]. The sartorius tendon itself is a flat, ribbon-like structure made up of well-defined fascicles[159]. There is very little information about this tendon reported in the literature.

The tissue harvesting protocol was approved by the Capital Health Research Ethics Board (CDHA-RS/2015-168 and renewals). The age, sex, and diabetic state of each donor were recorded, along with the number of tendons (in some situations, only one tendon from each donor was harvested) (Figure 3.3). Immediately following collection, the tendons were wrapped in gauze soaked in isotonic phosphate buffered saline (PBS), pH 7.4, containing a 1% solution of amphotericin B and a penicillin/streptomycin. No more than 24 hours post-collection, the tendons were stored frozen at -86^{o} C until use.

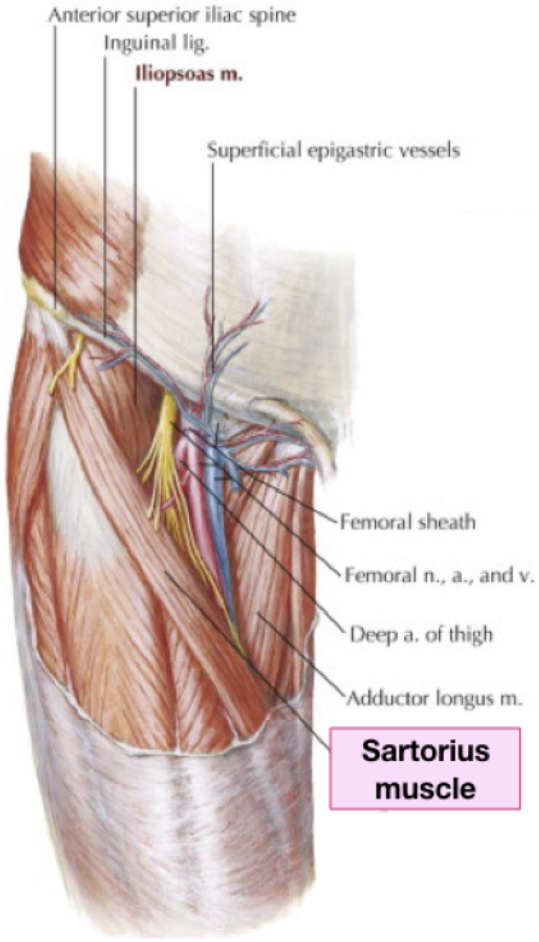

Figure 3.1: Anatomy of Lower Limb from Netter's Clinical Anatomy. Reprinted with modifications with permission[160].

3.2 Tissue Dissection & Handling

For the purpose of this thesis, only male non-diabetic tendons with a matching contralateral pair were used. To study how properties of the sartorius tendon change with age, the samples were subdivided into young (< 40 years of age) and old (≥ 40 years of

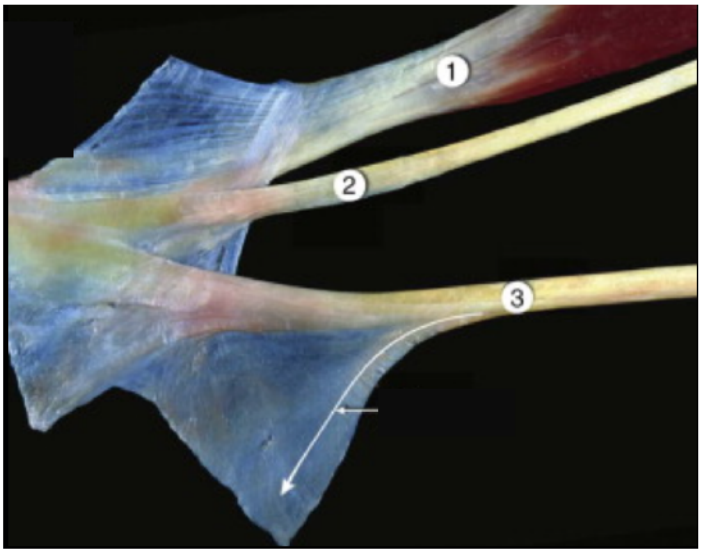

Figure 3.2: Anatomy of human pes anserinus where 1, 2, and 3 are the sartorius, gracilis, and semitendinosus tendons, respectively. Reprinted with modifications with permission[161].

age) age groups. One tendon was used for the thermal analyses while the contralateral one was used for mechanical testing and overload. Each tendon was carefully dissected following thawing at room temperature and subdivided using microsurgical scissors and a scalpel into samples for thermal crosslink analysis by DSC and HIT testing, and for mechanical rupture and structural assessment using SEM.

Aside from variations in tendon morphology due to genetic factors, each tendon varied with respect to its geometry and amount of other tissues present (i.e. fat, muscle, cartilage), resulting in a heterogeneous pool of sartorius samples (Figure 3.4). Additionally, the structure of the sartorius tendon is different than that of most tendons that have been previously used to study mechanics in our group[16,101,102,116,117]. Bovine tail and forelimb tendons are firm and uniform, while the human sartorius tendon is flat, ribbon-like, and subdivided into many small individual fascicles with muscle fibres often extending well into the belly of the tendon (Figure 3.5). After making an incision into the connective tissue layer that surrounds the entire tendon, the whole structure unravelled into smaller components, making it extremely difficult to create uniform tendon subsamples. Troubleshooting was performed on unpaired male sam-

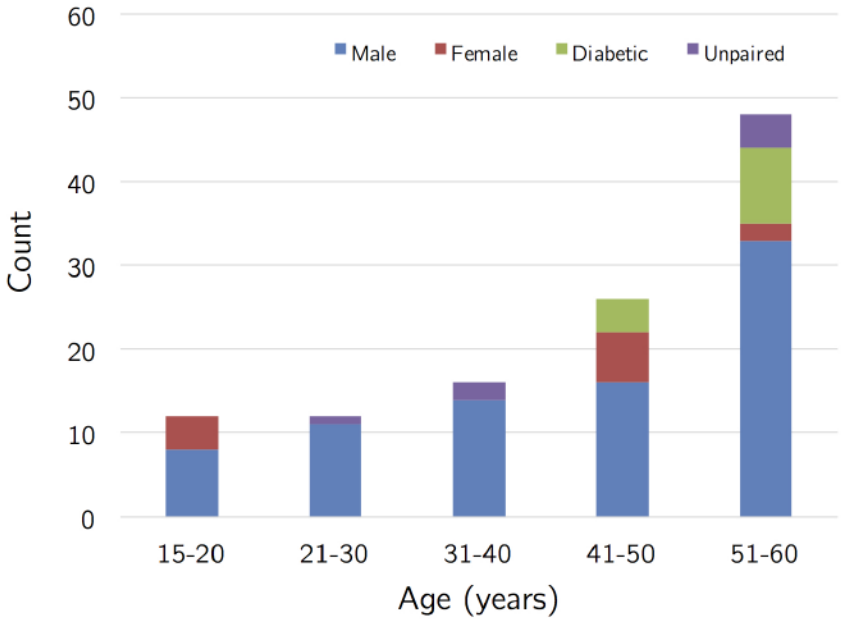

Figure 3.3: Sartorius tendons collected from the Regional Tissue Bank, separated into age groups, paired vs. unpaired tendons, sex, and diabetic state. All of the unpaired and diabetic donors were male, and only one unpaired tendon was from a diabetic donor.

ples from old donors, since there was a larger population of these tissues and younger samples were comparatively hard to come by. Fat and muscle were dissected away to expose the underlying sartorius tendon fascicles, which were then subdivided into sizes appropriate for each experiment (Figure 3.6).

For HIT, the tendon was cut into 6 mm long pieces (6 per tendon), while ensuring that the bone and muscle attachments were not included. Each tendon sample was mapped to keep track of sampling location, as it has been found in the literature that the collagen within the tendon is not the same at the MTJ as it is at the OTJ[13,162,163], so it was thought that the two attachment sites may have varying thermal stabilities. Intermediate thermally labile crosslinks were stabilized in half of the HIT subsamples using sodium borohydride ($NaBH_4$)[40,164–166] (a procedure which will be described below). A small portion from the central portion of the tendon was excised and used to create as many DSC samples (~10 mg each) as allowed by the geometry of the tendon.

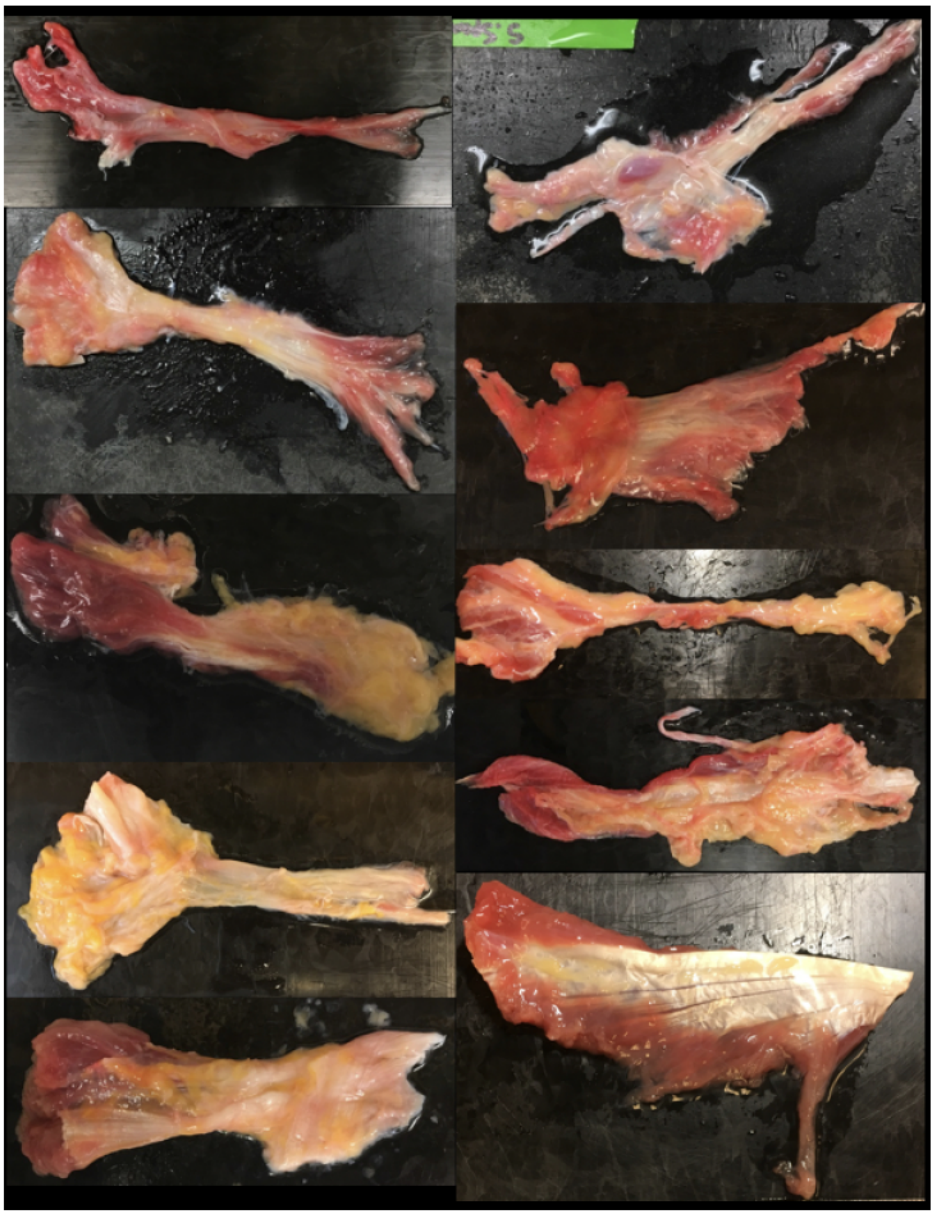

Figure 3.4: Sartorius tendons collected from ten different donors, showing the heterogeneous nature of the population obtained from the Tissue Bank. Some tendons have a large amount of fat and muscle present (e.g. bottom left), while others had portions missing following harvest (e.g. bottom right). The variability in length and width of the tendon is visible even in this small representative subpopulation.

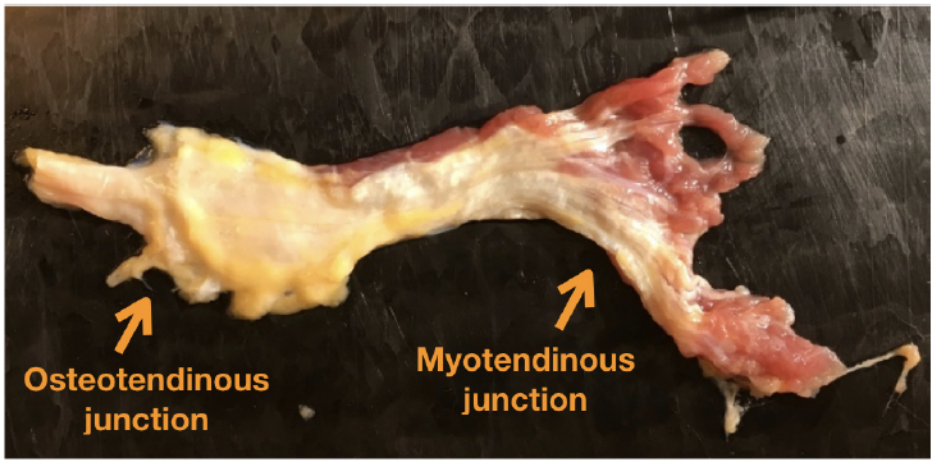

Figure 3.5: Sartorius tendon labelled with its respective attachment sites, prior to removal of fat and muscle tissue.

Tendons that were to be mechanically ruptured were cut into sections that were ~45 mm-long and more or less 1-3 mm in width. The number of subsamples depended on the tendon geometry, however at least five mechanical subsamples were created from each tendon, with a central portion of the tendon being used as a control.

3.2.1 Cross-sectional Area Measurements

Following sectioning into longitudinal subsamples, the samples were suspended in plane with a ruler, and photographs were taken using a camera at 0^o, 90^o, 180^o, and 270^o of rotation (Figure 3.6)[117]. The photographs of each sample were analyzed with ImageJ (Version 1.44; National Institutes of Health, Bethesda, MD) to determine the thinnest diameter of each sample, three times at each rotational angle, and then averaged (d_0, d_{90}, d_{180}, and d_{270}, respectively). Once these were determined, the minimum cross-sectional area (CSA) was estimated by using the formula for the cross-sectional area of an ellipse[117]:

$$CSA = \pi \times \frac{\frac{d_0}{2} + \frac{d_{180}}{2}}{2} \times \frac{\frac{d_{90}}{2} + \frac{d_{270}}{2}}{2} \tag{3.1}$$

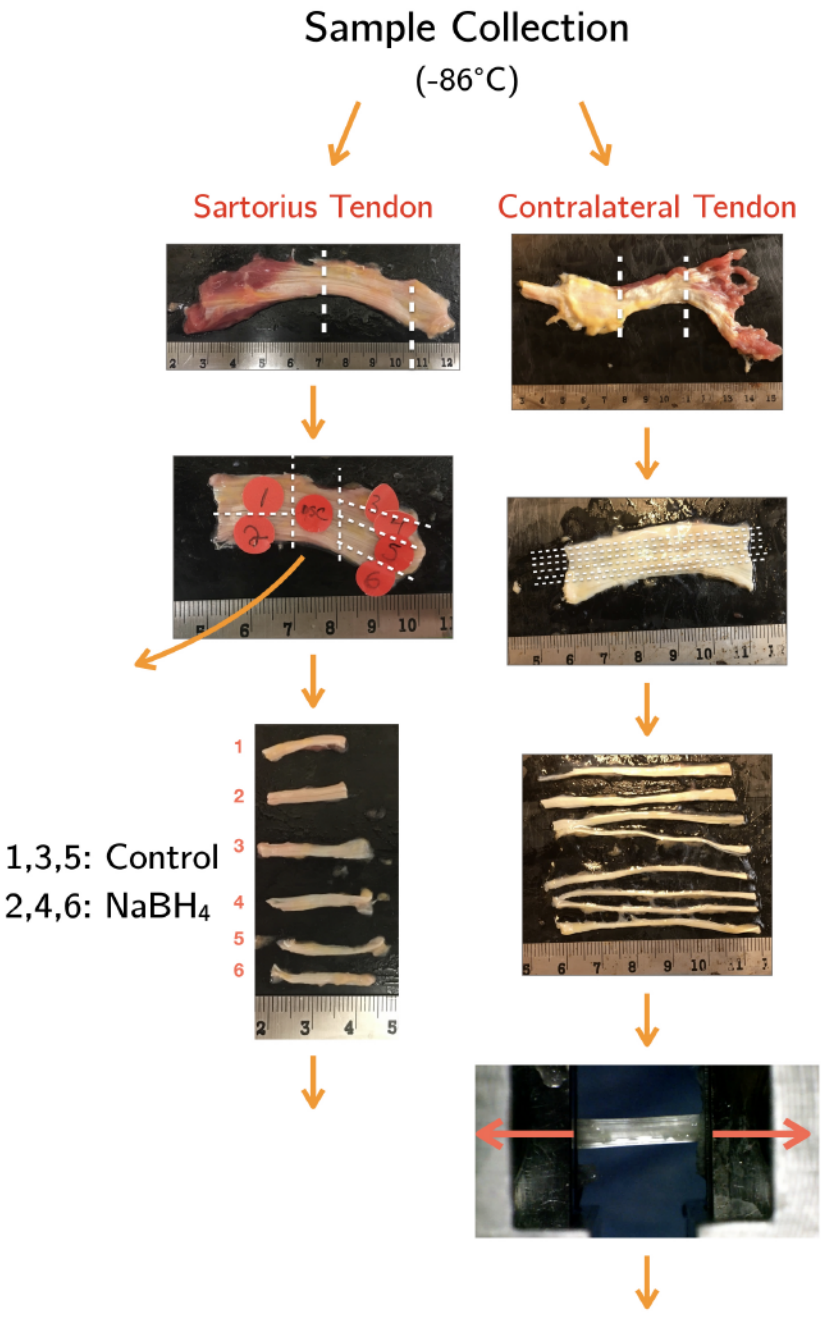

Figure 3.6: Sartorius tendon dissection and sectioning protocol. Contralateral tendons were used for assessment of thermal stability (via HIT and DSC) and nano-scale damage motifs with SEM following mechanical rupture

where r is the radius as measured from the photographs from each angle of rotation. This cross-sectional area was used to calculated the stress values from the load readings following mechanical overload.

3.3 Hydrothermal Isometric Tension (HIT) Testing

HIT testing was performed on male, non-diabetic, and non-overloaded tendon samples (n=12 donors with six HIT samples per donor) from young and old age groups (n_{young}=6 and n_{old}=6) to determine the denaturation temperature and maturity of crosslinking. Testing was done using a custom-made multi-sample apparatus, as previously described[81]. For each donor, the tendon was split up into six subsamples, three of which were stabilized using $NaBH_4$, while the other three served as control samples.

3.3.1 Sodium Borohydride ($NaBH_4$) Stabilization

Sodium borohydride was used to reduce crosslinks in tendon subsamples[40,164–166], as per Wells et al.[83]. Briefly, stabilized samples were placed in 100 ml of borate buffer (pH 9.0) containing 0.1 mg/mL $NaBH_4$ while control samples were placed in 100 ml of borate buffer. Both control and borohydride-treated samples were placed on a shaker for 15 minutes at 4°C, after which time the solutions were removed, and replenished with fresh borate buffer (control) and fresh borate buffer containing $NaBH_4$ (treated samples). This washing procedure was repeated for a total of four times, and was followed by a 15 minute rinse in PBS at 4°C for both the control and treated samples.

3.3.2 Experimental HIT Protocol

The samples were gripped at a constrained length and hung onto rigid rods attached to load cells. A preload (60 g) was applied to allow for stress relaxation to occur over a

15 minute period prior to beginning the test. Load, temperature, and time data were collected at 5 Hz using a custom-written LabVIEW program (version 7.1, National Instruments). This apparatus was placed in a distilled and deionized water bath that was gradually heated ($\sim$ 4°C/min) to 75°C. At this temperature, the rate of heating was decreased ($\sim$ 2°C/min) until the temperature reached 90°C. At the T_d, there is enough energy present within the system to break the hydrogen bonds that hold the collagen triple helix together. At this temperature, there is an energetic driving force for the triple helix to transition to a random coil which would cause a shrinkage of the structure[76,84]. In HIT, because the samples are isometrically constrained, this transition is does not occur but is captured as an increase in load. During the ramp to 90°C, thermally labile crosslinks begin to hydrolyze and depending on the maturity of the crosslinks, the sample may fail[76,84,164]. During the isothermal portion of the test, the peptide backbone of collagen molecules will gradually hydrolyze, resulting in load decay over time[69,87,90,167]. Sodium borohydride treatment stabilizes these thermally labile crosslinks, allowing for an assessment of total crosslinking present in samples by comparing samples that failed before and after 90°C and comparing $t_{1/2}$ values.

3.3.3 Data Analysis

The load, temperature, and time data were analyzed using Microsoft Excel and custom-written MATLAB codes (R2017a, MathWorks Inc.). The denaturation temperature (T_d) was determined to be the temperature at which there was a measurable increase in load, corresponding to the helix to coil transition characteristic of collagen denaturation (Figure 3.7).

Typically during the temperature ramp before denaturation, there is stress relaxation occurring in the sample and this is seen as a decrease in load prior to the denaturation temperature on the load versus temperature graph[81]. This relaxation did not occur in all sartorius tendon samples. Rather, some samples showed an increase in load prior to the onset of denaturation. To quantify this increase in force under isometric constraint, the fractional change in load before denaturation (FR_1) was calculated as per Equation 3.1 (where $Load_{T_d}$ is the load at T_d and $Load_{RT}$ is the load at room

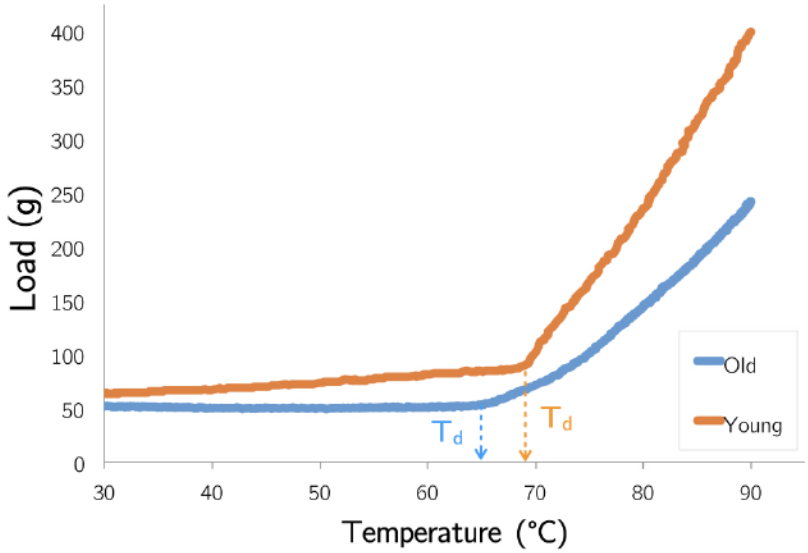

Figure 3.7: Representative load versus temperature plots from HIT testing of old and young samples. The denaturation temperature, T_d is shown as the first temperature where there is a measurable increase in load.

temperature).

$$FR_1 = \frac{Load_{T_d} - Load_{RT}}{Load_{RT}} \tag{3.2}$$

Following the onset of denaturation, it is expected that the sample with have an increase in force under isometric constraint as described above[76,87,92]. This energetic driving force towards contraction will result in an increase in load during the ramp from the T_d to 90°C. This was quantified by calculating the fractional change in load between the two points (termed FR_2) per Equation 3.2 where $Load_{End}$ is the final load reading prior to the beginning of the isotherm.

$$FR_2 = \frac{Load_{End} - Load_{T_d}}{Load_{T_d}} \tag{3.3}$$

Le Lous and Allain initially described the load decay in the isothermal portion of HIT

to be a Maxwellian exponential decay resulting from the hydrolysis of the peptide backbone in collagen (Equations 1.3 and 1.4)[90]. The half-time of load decay, $t_{1/2}$, can be calculated by:

$$t_{1/2} = \frac{ln(2)}{k} \tag{3.4}$$

The value of k was determined by calculating the slope over the linear portion of the $ln\frac{L(t)}{L_{max}}$ versus time plots[90,168], obtained over the last 6000 s of the HIT isothermal load data[83], where $L(t)$ is the load at any given time, and the L_{max} is the peak load. While load decay was expected to occur in all collagenous tissues, it was found that isothermal contraction occurred in some samples. For sartorius tendon samples that did not display load decay, it was not possible to calculate $t_{1/2}$ values. The proportion of subsamples with and without load decay was determined for each donor.

3.4 Differential Scanning Calorimetry (DSC)

DSC was performed on male, non-diabetic, and non-overloaded tendon samples (n=13 donors with at least five DSC samples from each) from both young and old age groups (n_{young}=6 and n_{old}=7) to determine the thermal stability of the collagen present.

3.4.1 Testing Protocol

DSC was conducted using a TA Instruments Q-200 differential scanning calorimeter (TA Instruments, New Castle, DE), as previously described[101]. Calibration of the system (both temperature and heat flow) was done using an Indium standard. Tissues were tested following immersion in isotonic PBS and subsequent blotting to remove excess moisture. They were then weighed and placed in aluminum DSC pans ensuring maximal contact area with the pan. The DSC pans were hermetically sealed and run against an empty reference pan. Equilibration was performed at 40°C followed by scanning at 5°C/min to 90°C (data recorded at 5 Hz)[101]. Following DSC testing, the aluminum pans were punctured and placed in a vacuum desiccator for 24 hours. The dry weight was measured and the water content of each sample was calculated.

3.4.2 Endotherm Analysis

Representative DSC endotherms for three different donors are shown in Figure 3.8. The endotherms were analyzed between 60°C and 85°C using Universal Analysis 2000 software (version 4.5A, TA Instruments) for onset and peak temperatures (T_{onset} and T_{peak}, respectively) as measures of thermal stability. In addition, full width at half maximum (FWHM) (a measure of the distribution of thermal stabilities) and specific enthalpy of denaturation (Δh) (a measure of amount of energy required for phase change) were extracted[85]. The T_{onset} is the temperature at which the least thermally stable collagen molecules begin to denature. On the endotherm, this parameter was determined as the intersection of the tangent of the endotherm's downward slope and the baseline preceding the endotherm. T_{peak} is calculated as the temperature of maximum heat flow. The Δh, or the area contained within the endotherm, was calculated initially using wet sample weight, and corrected for water content following drying. The FWHM is a parameter used to describe the distribution of thermal stabilities of all of the collagen molecules within each sample[94]. It was calculated as the difference between two temperatures on either side of the endotherm, where the heat flow was halfway between the baseline and the maximum.

In addition to the parameters explained above, the DSC endotherms were also analyzed using a custom-written MATLAB program (R2017a, MathWorks Inc.) for the temperature at which the denaturation process begins (i.e. the true onset temperature). Previously, this has been taken as the extrapolated value of T_{onset}, however, this value is often calculated by the software to be higher than the true onset of denaturation[92]. As such, the true onset temperature was determined to be the temperature at which the heat flow (F) begins to deviate from the baseline. This temperature, herein referred to as the deviation temperature (T_{dev}) was determined by taking the first derivative of the DSC endotherm. T_{dev} was the first temperature at which $dF/dT <$ -0.005. In theory, the HIT T_d should not precede T_{dev} in DSC, so to confirm that this was not the case, the average T_d as determined by HIT was plotted on each endotherm (Figure 3.9).

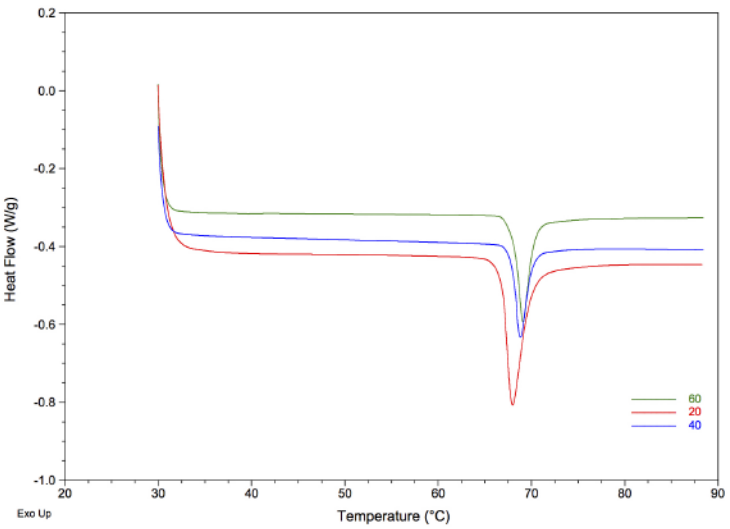

Figure 3.8: DSC endotherms from three different donors aged 20, 40, and 60 shown in red, blue, and green, respectively.

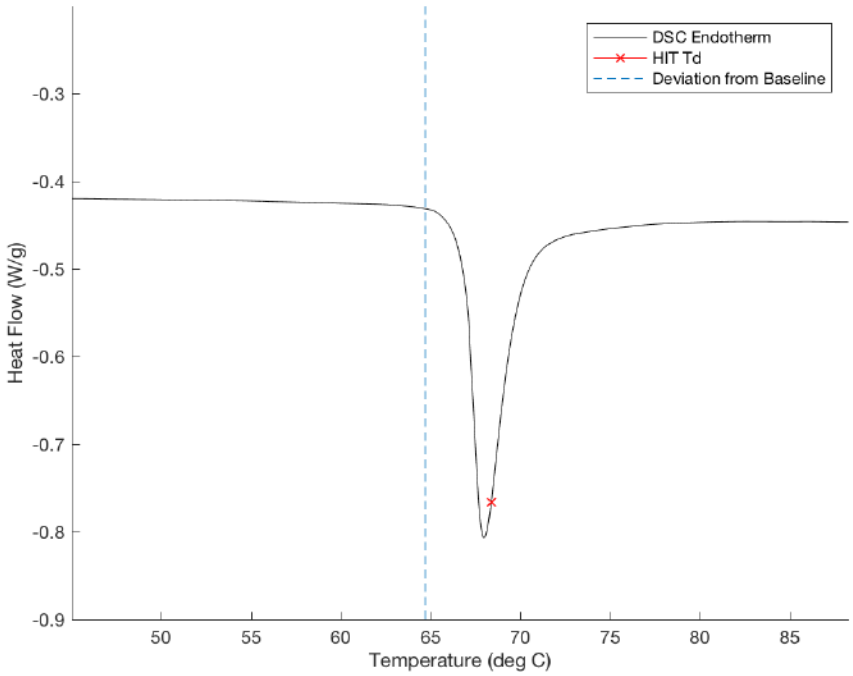

Figure 3.9: DSC Endotherm with HIT T_d marked and T_{dev} represented by the dotted line.

3.5 Mechanical Rupture

Samples (n=12 donors; n_{young}=5 and n_{old}=7) with at least 5 subsamples for each donor were mounted into a servo-hydraulic materials testing system (458-series, MTS; Minneapolis, MN) between two diamond-coated waveform grips. The position of the actuator was measured with an LVDT and the load with an MTS 1-kN load cell. To ensure no slippage occurred during the test, small pieces of PBS-moistened cloth were placed between the grips. Following mounting, one actuator was retracted, increasing the distance between the grips until a small force (~0.5 N) was registered. Then, intergrip length was measured (~15-20 mm) while the sample was under tension. The sample was periodically moistened with drops of PBS while the test was occurring. A custom-written LabVIEW program (version 6.1, National Instruments) controlled the position of the actuator while measuring the resulting load (N), displacement (mm), and time (s). Initially, each sample was pulled to rupture at a strain rate of either 0.1%/s (to induce as much damage as possible) or 1%/s (previously reported by[86,94,101]). In order to be consistent with the initial BTT study where discrete plasticity kinks were seen, a strain rate of 0.25%/s was chosen[117]. A video camera (OEM-Optical HD133DV HD; OEM-Optical, Danville, CA) with a Zoom 7000 macro video lens (Nativar Inc., Rochester, NY) and video capture board (Intensity; Blackmagic Design, Burbank, CA) was used to monitor each test to confirm complete fascicle rupture of each sample. Following rupture, each sample was removed from the grips, and the gripped ends were removed (~10 mm per gripped side).

Calculation of Mechanical Parameters

Microsoft Excel and a MATLAB code (R2017a, MathWorks Inc.) were used to extract stress, strain, tissue modulus (E), ultimate tensile strength (UTS), and the strain at UTS (ϵ_{UTS}) (Figure 3.10). The stress, σ (MPa), was calculated using the load (N) and the cross-sectional areas (mm^2) (Equation 3.4).

$$\sigma = \frac{Load}{CSA} \tag{3.5}$$

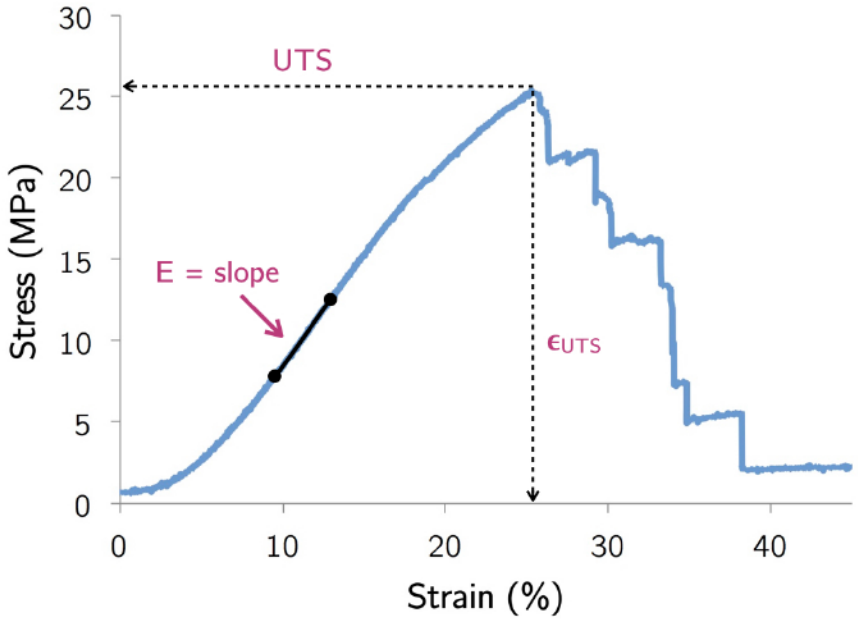

Figure 3.10: Stress-strain curve of ruptured sartorius tendon with mechanical parameters labelled.

The strain, ϵ, was determined using the initial sample intergrip length (l_o) and the displacement (Δl) (Equation 3.5).

$$\epsilon = \frac{\Delta l}{l_o} \tag{3.6}$$

The tissue modulus was calculated from the slope of the steepest linear part of the stress-strain curve and the UTS was calculated to be the maximum load reached by the sample.

3.6 Scanning Electron Microscopy

3.6.1 Sample Preparation and Imaging

Ruptured samples were fixed in a 2.5% glutaraldehyde (in isotonic PBS, pH 7.4) solution overnight, then bisected longitudinally, to expose the interior[117]. After rinsing

three times with distilled/deionized water, dehydration of the samples was done in graded ethanol by performing short rinses in ethanol solutions as follows: 75%, 90%, 95%, and 100%. Once the samples placed in 100% ethanol, they were dried in liquid CO_2 using a critical point dryer (Leica EM CPD300, Wetzlar, Germany). The fully dried samples were mounted onto aluminum stubs using carbon tape, ensuring with a dissecting microscope that the cut surface was facing upwards. The samples were coated with gold-palladium for 1 minute using a current (I) of 30 mA (Leica EM ACE200, Wetzlar, Germany). The cut side of each sample was visualized using magnifications up to 200,000X using an S-4700 SEM (Hitachi, Chula Vista, CA) operating at an accelerating voltage of 3 kV and a current of 15 μA[117]. To assess damage present, each sample was visualized a minimum of three times along it's length (Figure 3.11).

3.7 Statistical Analyses

Statistical analyses were performed using JMP (Version 12.1.0, SAS Institute Inc., Cary, NC) with p values ≤ 0.05 considered to be statistically significant. Outliers were removed if there was a methodological error or if data points fell outside of this range: (25th $\pm$ 1.5 X (interquartile range)) to (75th $\pm$ 1.5 X (interquartile range)). Values are expressed as averages plus or minus SD. Following removal of outliers, a Shapiro-Wilk test was performed to test for normality. Two T_d outliers were removed, seven $t_{1/2}$ outliers were found, and a total of 13 DSC runs were omitted from analysis as the pans were not completely sealed. One donor was considered an outlier in terms of the T_{onset} and T_{peak} values. For the mechanical data, one modulus outlier, three UTS outliers, and two failure strain outliers were removed.

All of the quantitative measures except for Δh values were normally distributed, as per the Shapiro-Wilk test. For the T_d values, t-tests were used to compare the means of borohydride-treated and control samples. The effect of donor age, sampling location, and borohydride treatment on the T_d as measured by HIT was done using a three-way ANOVA. Least-squares linear regressions were performed on the effect of

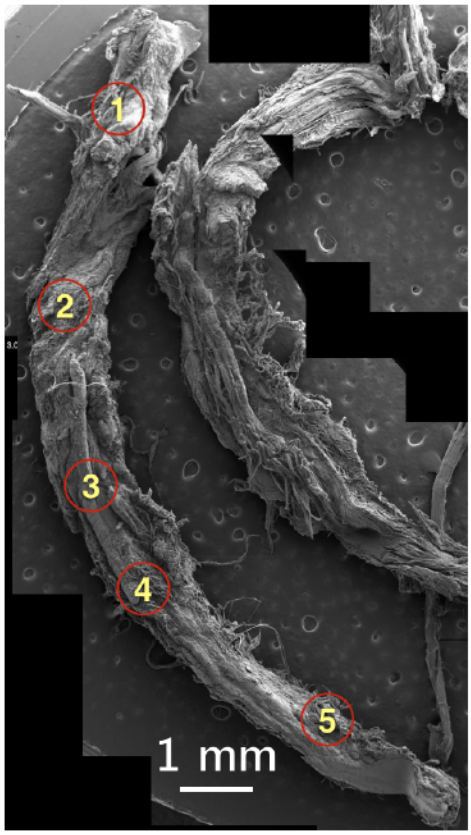

Figure 3.11: Low magnification overview of an SEM sample with sampling locations indicated.

donor age on the T_d, T_{onset}, T_{peak}, Δh, and FWHM, and tested for significance using a t-test. Differences in the proportion of samples with load decay in the isotherm between age groups were tested for significance using a χ^2-squared test. The likelihood of T_d preceding T_{dev} was also tested using a χ^2-squared test. The effect of strain rate and age on the mechanical parameters (modulus, UTS, and failure strains) were analyzed using a 2-way ANOVA. The age-dependence of the mechanical parameters was assessed using a least-squares linear regression.

Chapter 4

Results

4.1 Sartorius Tendon Anatomy is Heterogeneous and Mechanical and Thermomechanical Behaviours are Atypical

The sartorius tendon is flat, ribbon-like and fans out near the attachments on either side resulting in a non-uniform structure. The tendon is quite fascicular in nature, and upon incision into the epitenon, the entire structure begins to unravel, suggesting that there may be more levels of hierarchy than are visible to the naked eye – a feature perhaps unique to this tendon. There was a great deal of inter-person variability present within the studied population in terms of tendon length, which was a limiting factor for number of samples that could be tested using the outlined methodology. Not only were there variations in length, but also in width, and amount of excess tissue present. Sartorius muscle tissue often extended along the length of the entire tendon, which may have had an impact on the properties of the collagen within the tendon. While it was possible to excise this excess tissue, on occasion it was difficult to tell where exactly the tendon mid-substance was located, posing a multitude of dissecting problems and additional variables. Some tendons showed crosshatching of fascicles over the mid-substance of the tendon, and appeared as a mesh network of

fascicles, visible with the naked eye, rather than the uniform longitudinally arranged tendon structure that has been well characterized in previously used tendon models. It is possible that this is present because the sartorius muscle is involved in abduction and some rotation along with flexion. This tendon is involved in many locomotive motions, and therefore it is possible that uniaxial loading does not fully represent its *in vivo* loading environment.

There was a great deal of troubleshooting that went into determining how to best handle this tendon. It was difficult to create uniform samples for each experiment, in part due to the unique anatomy of the tendon yielding a heterogeneous population of samples. Since the sartorius tendon has a variable tendon belly length, but is relatively short when compared to other tendons, there was little margin for dissection error either during harvest or experimentation. Additionally, the relatively short tendon length brought into question whether or not the myotendinous and osteotendinous junctions impacted the tendon responses during thermal stability tests and mechanical rupture.

While all tendons are comprised of fascicles, this tendon has very well-defined fascicles which fail individually bundle by bundle prior to the failure of the entire structure (discussed further in Section 4.3). The response of this particular tendon following HIT was also unique (Sections 4.2.1 and 4.2.2). The typical dip in load (isometric relaxation) prior to denaturation[81] was absent in most HIT thermograms, regardless of age. Additionally, the 90°C isothermal portion did not show load decay in all samples; indeed, many samples showed an *increase* in load over the five hour isothermal period.

4.2 Sartorius Tendon Collagen is Heavily Crosslinked

The results from thermal stability analysis by HIT and DSC show that the constituent collagen within the human sartorius tendon is heavily crosslinked, with all denaturation temperatures (T_d, T_{onset}, and T_{peak}) being higher than those previously been seen in bovine models (Table 4.1 and 4.2). Additionally, from HIT it was found that all

of the human sartorius tendon samples survived past 90^{o}C, well into the isothermal portion of the experiment. This is beyond the temperature range where thermally unstable crosslinks would hydrolyze, and the survival to 90^{o}C suggests that many or most of the crosslinks present are thermally stable. This was confirmed with $NaBH_4$ treatment, which had no effect on any of the HIT responses, suggesting that all of the crosslinks present are thermally stable in nature. While the relationship between thermal stability and age will be discussed in a later section, some of the other observed results further demonstrate that the crosslinking is quite dense in sartorius tendon collagen.

Table 4.1: Average values of T_d (°C) calculated from HIT.

Age	**Average T_d (°C)**
20	68.03 ± 0.49
21	68.38 ± 0.39
24	66.82 ± 0.33
34	66.28 ± 0.31
38	66.60 ± 0.73
40	66.50 ± 0.43
48	65.68 ± 0.33
50	65.40 ± 0.09
52	66.22 ± 0.50
57	65.40 ± 0.53
60	65.77 ± 0.50

Table 4.2: Average values of T_{onset}, T_{peak}, T_{dev}, FWHM (all in °C) and Δh ($\mathrm{J\,g^{-1}}$) calculated from DSC.

Age	$\mathbf{T_{onset}}$	$\mathbf{T_{peak}}$	$\mathbf{T_{dev}}$	**FWHM**	$\mathbf{\Delta h}$
20	66.61 ± 0.49	67.91 ± 0.33	64.84 ± 0.18	2.10 ± 0.17	41.08 ± 5.47
21	65.20 ± 0.33	66.55 ± 0.32	64.08 ± 0.35	1.98 ± 0.20	24.13 ± 4.28
24	67.57 ± 0.12	68.80 ± 0.09	66.23 ± 0.02	1.63 ± 0.10	29.93 ± 2.42
29	67.04 ± 0.23	68.13 ± 0.27	65.35 ± 0.48	1.52 ± 0.13	21.00 ± 7.37
34	66.80 ± 0.25	68.02 ± 0.10	65.25 ± 0.00	1.71 ± 0.13	16.74 ± 3.13
38	67.42 ± 0.17	68.54 ± 0.15	65.87 ± 0.27	1.44 ± 0.10	16.32 ± 5.10
40	67.42 ± 0.89	68.61 ± 0.25	65.97 ± 0.54	1.54 ± 0.28	26.87 ± 4.87
48	67.88 ± 0.19	69.10 ± 0.15	66.19 ± 0.41	1.95 ± 0.10	36.14 ± 5.63
50	67.08 ± 0.31	68.33 ± 0.24	65.59 ± 0.41	1.52 ± 0.16	20.36 ± 3.58
51	67.60 ± 0.67	68.63 ± 0.37	64.98 ± 0.70	1.62 ± 0.38	N/A
52	67.42 ± 0.45	68.63 ± 0.31	66.01 ± 0.38	1.64 ± 0.25	21.35 ± 5.05
59	67.60 ± 0.17	68.57 ± 0.12	N/A	1.50 ± 0.18	17.45 ± 0.53
60	67.94 ± 0.20	68.89 ± 0.28	66.43 ± 0.03	1.32 ± 0.03	33.35 ± 0.64

4.2.1 Rise in Force Under Isometric Constraint Occurs Prior to Denaturation

The shape of the HIT curves revealed an interesting phenomenon. The typical dip in load that occurs prior to denaturation (that corresponds to stress relaxation within the sample)[81], is absent in many of the human sartorius thermograms. This indicates that there is some energetic driving force towards contraction that is happening within the sample, prior to the denaturation event. The process of denaturation will indeed result in an increase in load on HIT, however a steady increase in load prior to the onset of denaturation is puzzling. Changes in load before and after denaturation (FR_1 and FR_2, respectively) were calculated in an attempt to quantify this phenomenon. These parameters had a relationship with one another that was nearly significant (p=0.0556)(Figure 4.1), suggesting that it is possible that if the sample demonstrates an increase in load before denaturation, it may not have as large an increase in load post-denaturation.

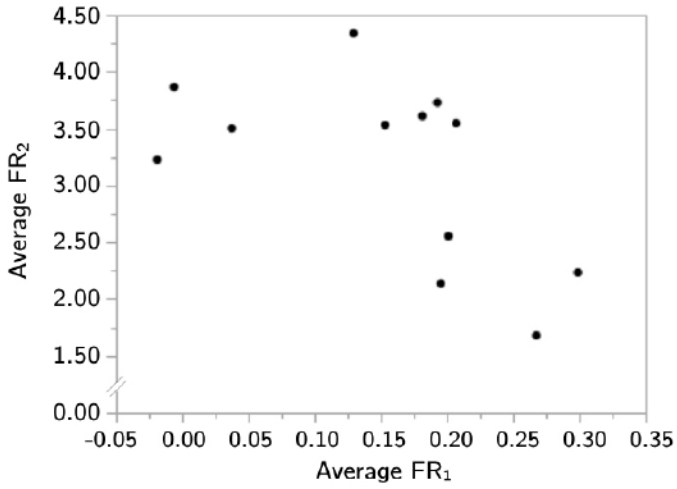

Figure 4.1: The fractional change in load was used to quantify the rise in force under isometric constraint present within the sample before and after denaturation. There was no correlation between the two parameters, suggesting that there is no 'net' amount of force that can be exerted by the sample under isometric constraint.

4.2.2 Contraction Occurs During Isothermal Portion of HIT

Typically, the isothermal data from HIT is used to calculate the half-time of load *decay*. In human sartorius tendon samples, it was found that the load did not decay in all samples (Figure 4.2), but that in some cases isothermal contraction occurred. This suggests that, despite hydrolysis of the peptide backbone, there is a network of dense crosslinking present within these samples. The $t_{1/2}$ was calculated for the samples where load decay occurred by measuring the slope of the linear portion of the $ln\frac{L(t)}{L_{max}}$ versus time plot over the last 6000 s of the HIT isotherm. Using a 3-way ANOVA, it was found that the $t_{1/2}$ was unaffected by age, borohydride treatment and sampling location (p=0.8298, 0.6160, and 0.7843, respectively). The average $t_{1/2}$ values can be found in Table 4.3, and while these values alone may not be very informative, the inability to calculate $t_{1/2}$ data for all of the samples is itself a useful piece of information, as it is not found in previous HIT studies of other tendons.

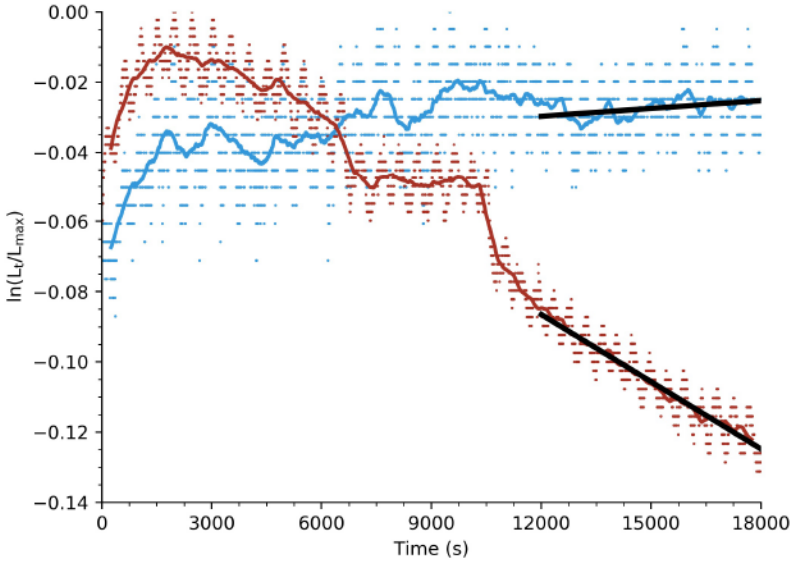

Figure 4.2: The slope of the HIT isothermal plot ($ln\frac{L(t)}{L_{max}}$ versus time) was calculated over the last 6000 seconds of the experiment to determine the presence or absence of load decay. Two representative plots showing positive (blue) and negative (red) slopes are shown above, corresponding to isothermal contraction and relaxation, respectively.

Table 4.3: Values of $t_{1/2}$, in hours, calculated where possible for samples where load decay was present.

Age	Average $t_{1/2}$ (hrs)
20	35 ± 24
24	77 ± 49
29	61 ± 42
34	29 ± 28
38	107 ± 145
40	79 ± 43
48	35 ± 43
50	58 ± 65
52	$40 \pm$ N/A
60	$31 \pm$ N/A

4.2.3 Structural Changes in Aging Tendon

While the results mentioned above confirm that the thermal stability of sartorius tendon collagen is high in all donor tendon samples (Table 4.1 and 4.2), the relationship between collagen thermal stability and donor age is unclear. Using a 3-way ANOVA, it was determined that borohydride treatment and sampling location did not have an effect on T_d (p=0.1519 and 0.8797, respectively), but the age of the donor had a significant effect (p<0.0001). Using a least squares linear regression was found that the T_d as measured by HIT decreases with age (p = 0.0004)(Figure 4.3). This may indicate that the tendon collagen is more constrained against molecular slippage and uncoiling in young age, as compared to old age.

The thermal stability of the collagen was found to increase with age, as measured by T_{onset} (p=0.0251) from DSC (Figure 4.4A). The T_{peak} values had a relationship with age that was nearly significant (p=0.0653), indicating that the peak heat flow may have a relationship with age (Figure 4.4C). The shape of the DSC endotherms did change with increasing age, and was captured with the FWHM parameter, which

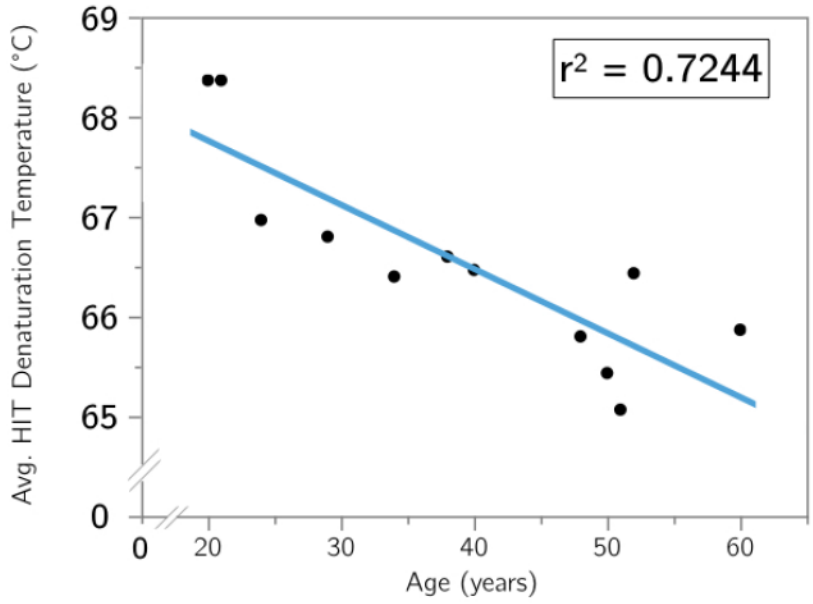

Figure 4.3: HIT testing was used to determine the denaturation temperature, T_d, which was used as a proxy for determining thermal stability of tendon collagen. The average values of T_d (^{o}C) from 12 donors were calculated and found to decrease with increasing age (years). This suggests that older tendon collagen is less constrained against uncoiling of the triple helix, and molecular slippage than young tendon collagen.

showed that there is a less heterogeneous collagen population present in old age (p=0.0201)(Figure 4.4B). The specific enthalpy of denaturation did not change significantly with age (p=0.4833)(Figure 4.4D).

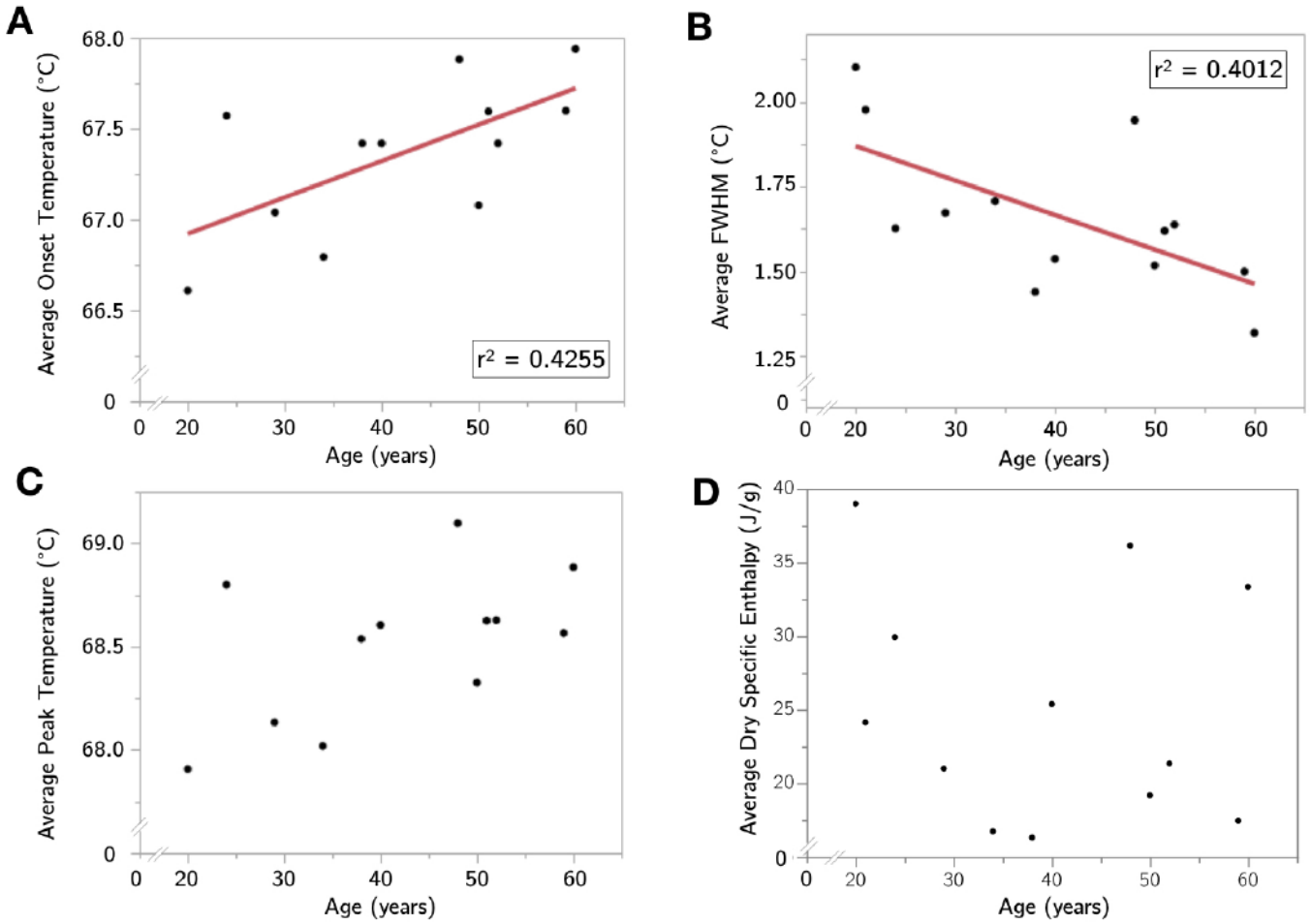

Figure 4.4: A variety of parameters were extracted from the endotherm data from DSC. The T_{onset} was found to increase with age, suggesting that the thermal stability increases over the span of four decades. The relationship between FWHM and age was significant, showing that the range of thermal stabilities of tendon collagen decreases linearly with age. The relationship between T_{peak} and donor age was nearly significant. The enthalpy of denaturation, Δh_{dry}, was not significantly affected by donor age. A: T_{onset} (^{o}C) versus age (years); B: FWHM (^{o}C) versus age (years); C: T_{peak} (^{o}C) versus age (years); D: Δh_{dry} (J/g) versus age (years).

It is known that increases in crosslinking can increase thermal stability via dehydration[43]. Despite potential increases in denaturation temperature, as seen by DSC, there was no relationship between the average water content and age (p=0.5909) (Figure 4.5). The average water content within the human sartorius tendon was determined to be 70.5 $\pm$ 5.6% (n=12).

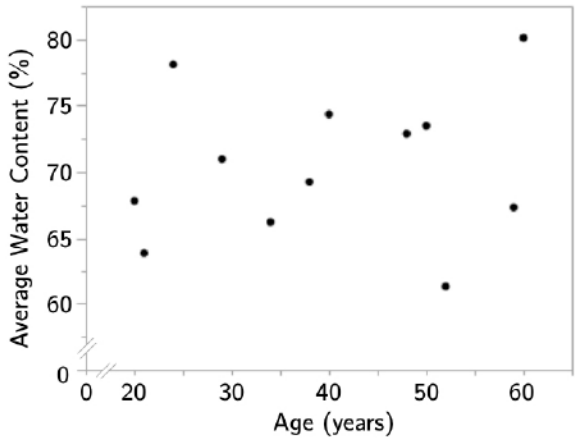

Figure 4.5: Water content was calculated for each DSC sample. The average water content (%) did not have a significant relationship with age (years).

In an attempt to further understand the relationship between thermal stability and age, the true onset temperature (T_{dev}) from DSC was calculated for all samples and plotted alongside the corresponding T_d from HIT on each endotherm. Interestingly, it was found that the the likelihood of T_d (as determined by HIT) preceding T_{dev} (from DSC) was different between the two age groups (p<0.0001) (Figure 4.6). A χ^2-square test showed that there is a greater probability of T_d preceding T_{dev} for the old age group (p=0.0004), implying that there is some age-related structural change within the tendon collagen molecules that is seemingly detectable by HIT prior to detection by DSC.

As mentioned above, the sartorius tendon samples did not always exhibit load decay during the isothermal portion of HIT, suggesting that the tendon is highly crosslinked. When the phenomenon was initially observed, it was thought that that younger sam-

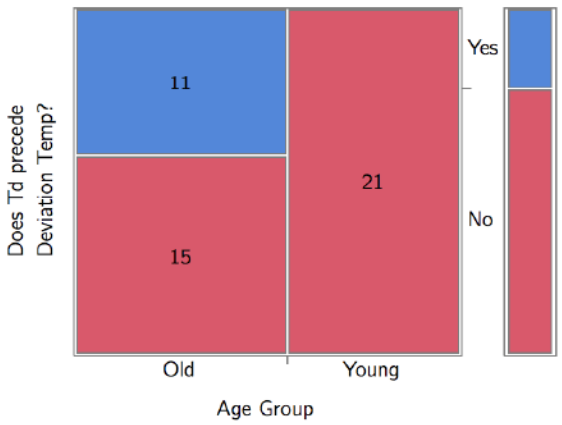

Figure 4.6: The true onset of denaturation was determined using DSC by calculating the temperature at which the initial deviation from the heat flow baseline began. This parameter was defined as T_{dev} and plotted alongside the corresponding T_d from HIT on each endotherm. The likelihood of T_{dev} preceding T_d from HIT was higher in the old age group than the young one, suggesting that there is some age-related change in the tendon collagen that is detected by HIT before DSC.

ples (that would be less crosslinked) would exhibit load decay; however it was found that isothermal contraction was not a feature related to age. The proportion of samples with and without load decay is shown in Figure 4.7. A χ^2 test showed that there was no relationship between age and the presence of load decay (p=0.2554).

The increase in force seen under isometric constraint prior to and after denaturation was also considered to be something related to crosslinking, which might increase with age. The two calculated parameters, FR_1 and FR_2, also did not vary with increasing age (p=0.2446 and 0.9314, respectively)(Figure 4.8). Together, these result further support the notion that sartorius tendon collagen is heavily crosslinked across all ages.

Mechanical Properties of Sartorius Tendon Do Not Change With Age

Interestingly, despite some age-related changes in tendon collagen thermal stability (i.e. crosslinking and/or packing) that were seen with HIT and DSC, the mechanical

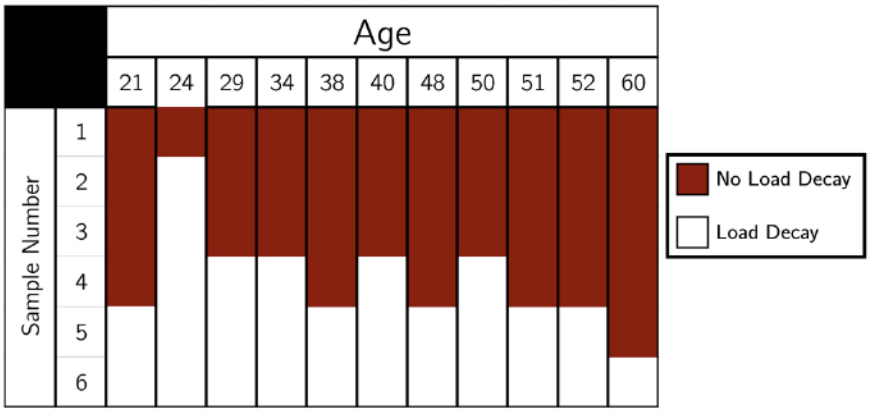

Figure 4.7: Surprisingly, load decay was not present in all tendon samples. Each HIT experiment used six samples from a single donor, and the effect of age on the presence or absence of load decay was determined. The presence and absence of isothermal load decay by donor age are indicated by white and red blocks, respectively. It was found that there was no relationship between the age of the donor and the presence of load decay during the isothermal portion of HIT.

properties of the sartorius tendon did not change with increasing age. Prior to using the final strain rate of 0.25%/s to rupture the tendons, different strain rates (0.1 and 1.0%/s) were used to rupture the tendons in an attempt to discern differences in the amount of damage present (which will be discussed in the following section). The effect of strain rate and age on each of the extracted mechanical parameters was done using a 2-way ANOVA and it was found that strain rate had an effect on the tissue modulus and UTS (p=0.0071 and 0.0038, respectively), but no effect on the strain at the UTS (p=0.6448). Using only the data from samples ruptured at 0.25%/s (n=9 donors) (since the sample size for the other strain rate was small) age was shown to have no effect on the tissue modulus or the UTS (p=0.4758 and 0.8822, respectively) (Figure 4.9A and B). Age also did not have an effect on the strain at the UTS (n=11 donors, p=0.0999)(Figure 4.9C).

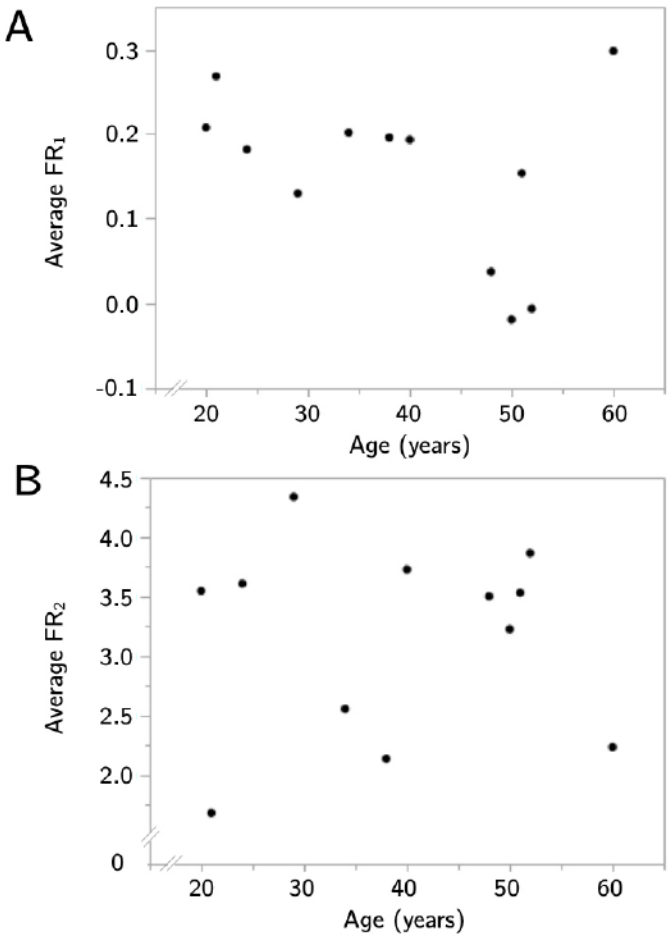

Figure 4.8: The fractional change in load was used to quantify the amount of force the samples could exert under isometric constraint before and after denaturation. These parameters, FR_1 and FR_2, respectively, were found to have no significant relationship with age (A and B, respectively).

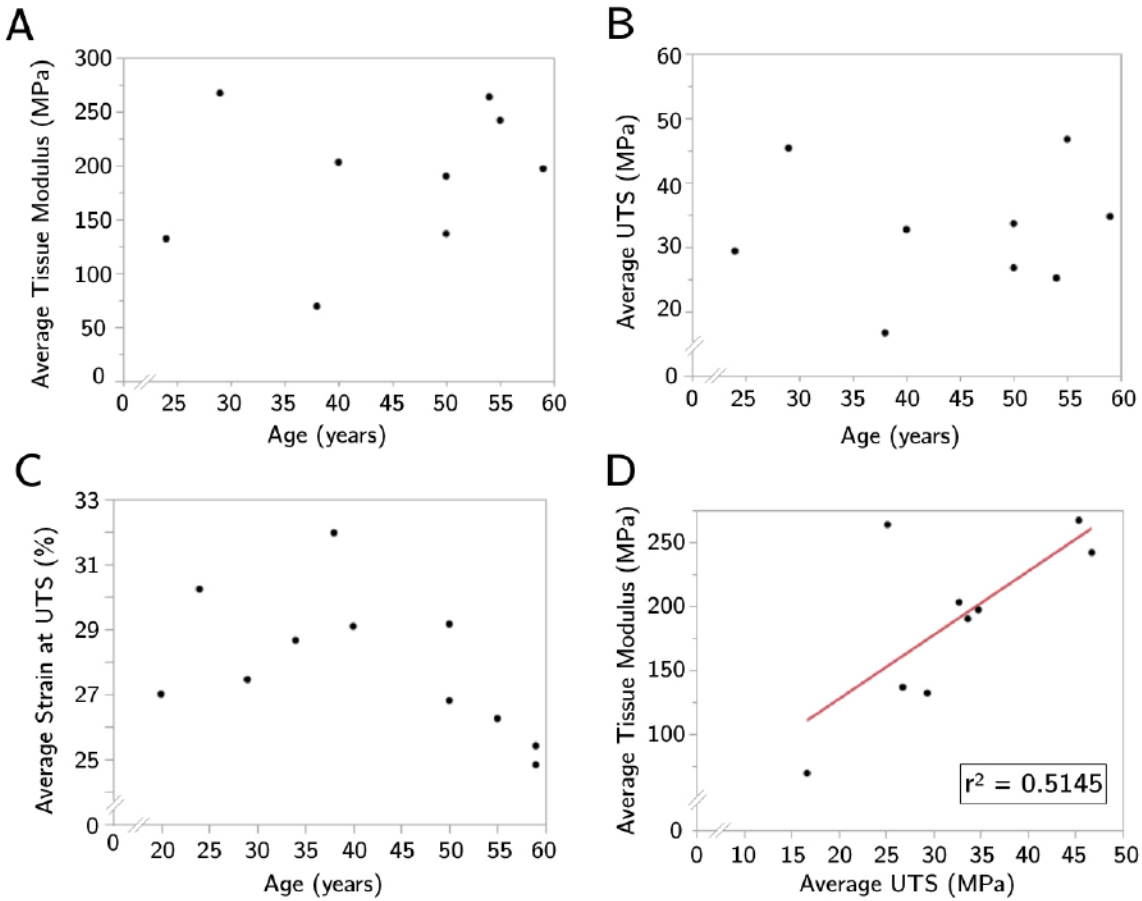

Figure 4.9: The tissue modulus, ultimate tensile strength, and strain at the ultimate tensile strength were obtained from each mechanical rupture performed at a strain rate of 0.25%/s. Averaged values of tissue modulus (MPa), UTS (MPa), and strain at UTS (%) were plotted versus age (years) (Panels A, B, and C, respectively). None of the extracted parameters was found to change with increasing age.

4.3 Failure of Individual Components Occurs Prior to Complete Failure

The fascicular and ribbon-like structure of the sartorius tendon was visible within the subsamples that were loaded into the MTS for rupture. During the loading to rupture, the videos that were captured revealed that individual fascicles or fibres were failing prior to the failure of the entire sample. The rupture of these components occurred as a series of pops or snaps that were audible during overload, suggesting that the fracture was an elastic recoil. The videos revealed elastic recoil of the individual components as they fractured, implying that this was an elastic process.

This serial 'snapping' of smaller tendon components was also evident in the corresponding stress-strain curves that were constructed using the load and displacement data following mechanical rupture. From these stress-strain curves (an example can be seen in Figure 4.10), it is evident that sartorius tendon samples do not undergo one fracture event. Instead, the individual fascicles of the tendon sample fail in sequence as the displacement is increased, until there is no mechanical integrity remaining within the sample. The notches on the stress-strain curve correlate with the sequential rupture as seen in the corresponding video, confirming that failure does not occur as one event, but rather as a cumulative failure.

4.4 Sartorius Tendon Does Not Show Failure via Discrete Plasticity

One of the main goals of this thesis work were to investigate the possible presence of discrete plasticity in a model human tendon. Contrary to what was expected, discrete plasticity kinks did not form in this particular model, regardless of age or strain rate used. There was no evidence of the documented repeated nanoscale kink-like structures along the length of collagen fibrils. Initially, two different strain rates were used (0.1 and 1.0%/s) in an attempt to incur as much tendon damage as possible.

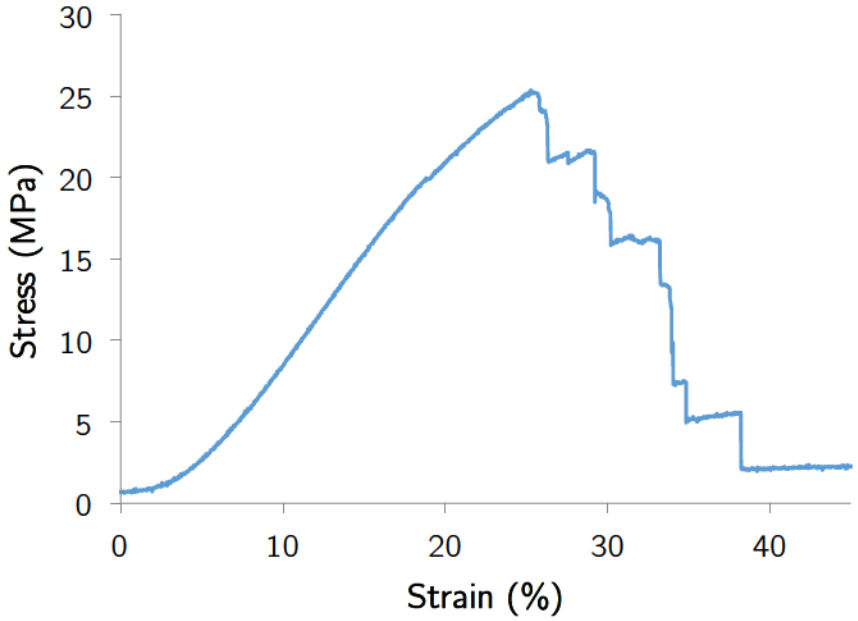

Figure 4.10: Mechanical rupture of sartorius tendon samples revealed an interesting phenomenon. Rather than breaking as one large bundle, individual fascicles were rupturing bundle by bundle prior to the failure of the entire tendon subsample. A representative stress-strain curve of a ruptured sartorius tendon sample is shown above, demonstrating that all of the fascicles contribute to the mechanical integrity of the sample. This was further confirmed with a video capture system that clearly shows individual fascicles snapping in sequence.

After determining that both strain rates did not affect the type or amount of damage seen, a final strain rate of 0.25%/s was chosen to match the initial discrete plasticity studies in bovine tendons[117]. Early on, it was noted under SEM that nanoscale damage motifs were quite rare, irrespective of strain rate. Moreover, it was noted that the native structure of non-ruptured sartorius tendons was quite disorganized at low magnifications, yet contained properly packed and aligned collagen fibrils.

4.4.1 Control Samples Show D-Banding and Tight Packing on Fibril Level

Non-ruptured control samples of human sartorius tendons appeared disordered at low magnifications, yet contained longitudinally aligned collagen fibrils that were orga-

nized into individual bundles. D-banding was present in all of the control sample collagen (n=13), and representative micrographs from control tissue can be seen in Figures 4.11C and 4.12B and D. The collagen fibrils within these samples also showed registry of the D-bands with neighbouring fibrils, indicating that there is tight packing of fibrils bundles present within native sartorius tendon collagen (best seen in Figure 4.12D). The diameters of the fibrils in control samples appeared relatively uniform and there was no exposure of substructure (i.e. subfibrils) present.

Sartorius tendon collagen at high magnifications clearly shows proper packing and collagen structure; however, the tendon appears disorganized at low magnifications. There appear to be many bundles of fibrous material without apparent organization, laid down as a mesh network or in twisted bundle configurations. Other areas appeared to have matrix components that were not collagenous. These matrix materials are finer than collagen fibrils, lack D-banding, and appear to connect collagen fibrils and fibril bundles in certain areas.

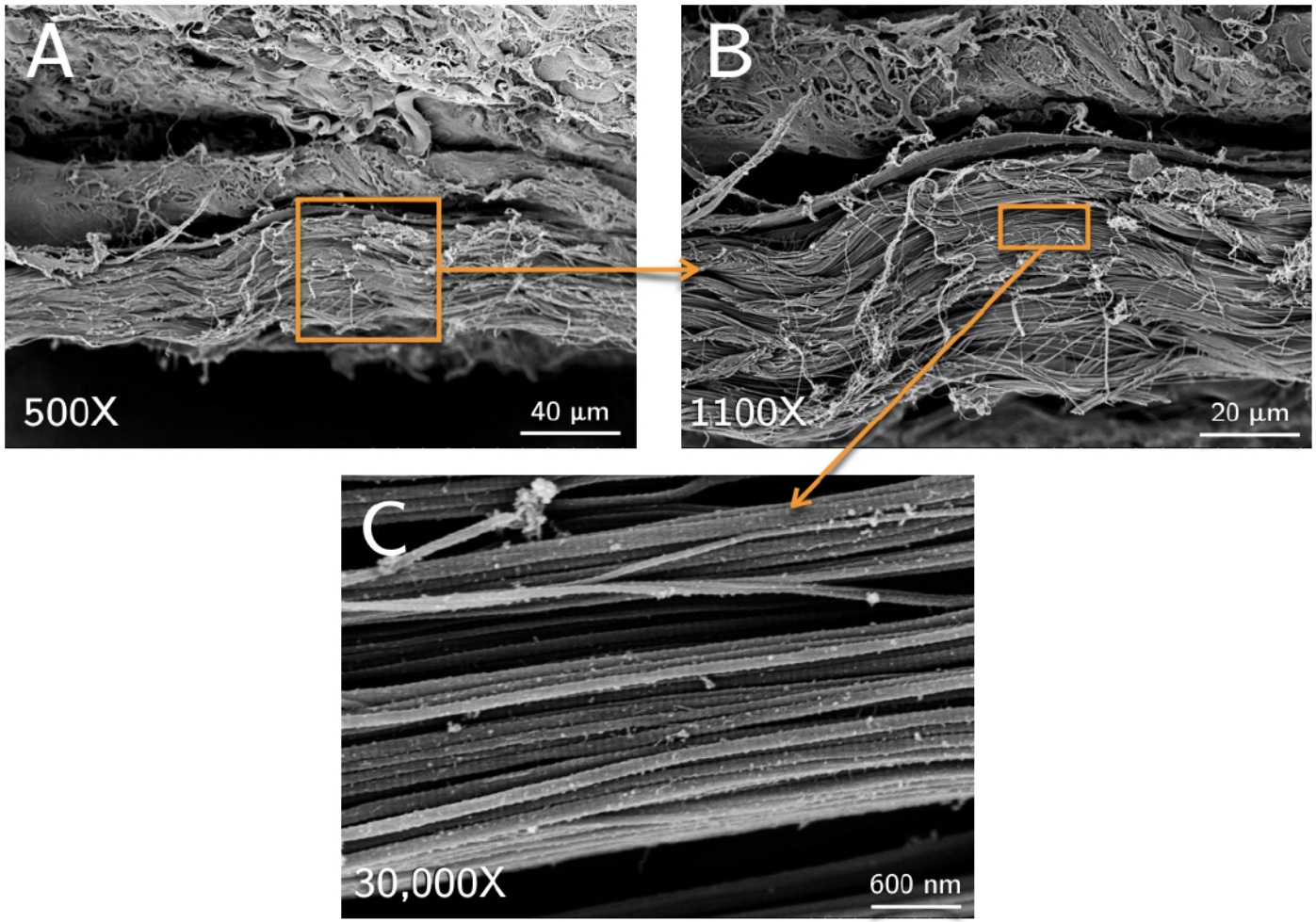

Figure 4.11: Micrographs of an undamaged control tendon sample from a 20-year old male donor show that even at low magnifications there are non-longitudinally aligned matrix components present (top of panels A and B). Bundles of collagen fibrils are present, and at 30,000X the D-banding can clearly be seen. Moreover, the collagen fibrils are in close proximity with one another and the D-banding is in registry, suggesting that native sartorius tendon collagen fibrils are packed tightly.

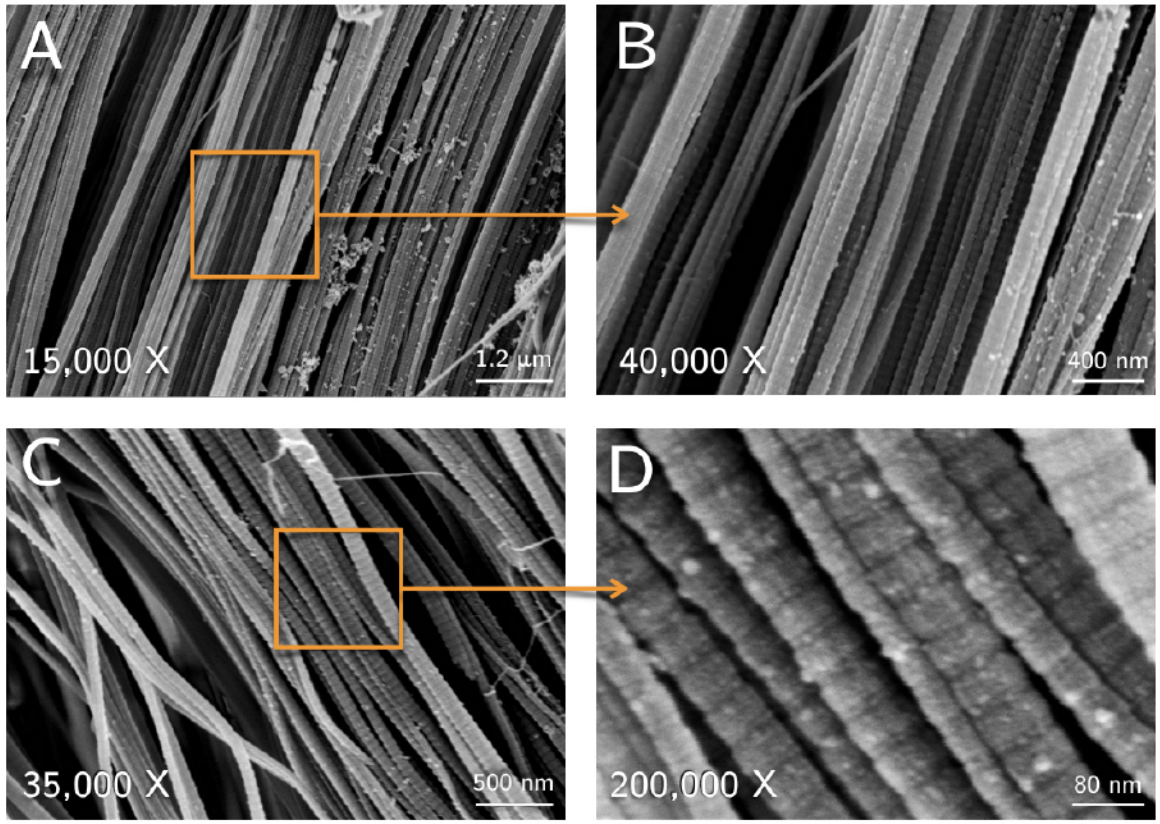

Figure 4.12: Even samples from middle aged and older individuals (34- and 55-year old male donors (A/B and C/D, respectively.)) show longitudinally aligned collagen fibrils with D-banding present. At 200,000X (panel D) the D-banding registry and close proximity of neighbouring fibrils is clearly evident, showing that control samples contain properly packed collagen.

SEM Reveals Failure of Fibre Bundles and Unique Damage Motifs

As mentioned above, the control samples revealed that the ultrastructure of the sartorius tendon is comprised of many different components and is heterogeneous in nature. Following rupture, the disorder within the sample increased, with the matrix materials being more loosely packed and unraveled. In comparison to age-matched control samples, there appeared to be an even higher level of disorder and disorganization (Figure 4.13 and 4.14). The bundles of collagen appeared frayed or unraveled, and the lateral spacing between fibrous elements was larger. Within some samples, it appeared that the bundles of collagen fibrils had recoiled following rupture, further supporting the notion that the rupture of the sartorius tendon fascicles was an elastic recoil process (Figure 4.14 A and B). Within these areas of disorder, the collagen fibrils themselves typically still showed D-banding, however there were certain areas where changes in nanoscale structure were evident.

Nanoscaled damage within human sartorius tendons was visible in the form of kinks or turns within collagen fibrils. The collagen within the kinks typically did not have D-banding present, but D-banding was preserved on either side of the kink itself (Figure 4.15). In some samples, there was propagation of kinks along the length of the fibril along with propagation widthwise through a bundle of fibrils (Figures 4.15 and 4.16). Despite damage being present, neighbouring fibrils remained in close proximity with D-banding remaining in registry neighbour-to-neighbour (Figures 4.15 and 4.16). Both within damaged fibrils and within neighbouring fibrils, there was exposure of some substructural elements (Figure 4.17D). Examples of this type of damage can be seen in Figure 4.17D (within kink site) and in Figure 4.16D (along the length of a fibril). Along with the kinks, other motifs were also visible. Within some samples, twists were present, accompanying the kink sites. In other samples, there were hairpin turns, twists, knots, and loops found on the fibrillar level (Figures 4.17A-C, 4.18, and 4.19). Some of these local structural disruptions did propagate through the length of fibrils, but the density of the damage was not high and did not typically propagate past 100 microns along the fibril's length.

Interestingly, there was no evidence of discrete plasticity damage in human sartorius

tendons. While there were local, discrete sites of damage present on the nanoscale, the type of damage seen was not the same. There were certain characteristics that were shared with discrete plasticity, but the amount or density of nanoscale damage was far lower in the human samples than in the bovine models where the mechanism was first seen. Similar to discrete plasticity, there was propagation of kinks across neighbouring fibrils and local, discrete sites of damage, with D-banding being preserved between.

In contrast to what was expected, there was also no change in the type and amount of damage present with increasing age. It was expected that the discrete plasticity mechanism would disappear in older populations but that it would be present in younger populations. There was, however, no evidence of this type of damage. Even the damage that was present occurred infrequently and the main feature seen in damaged tendons was the unraveling and disordering of higher scale structures such as fibril bundles. The damage that was seen on the nanoscale, while interesting, was scattered, not nearly so widespread as was expected. There was also no single motif that was characteristic of a certain age group.

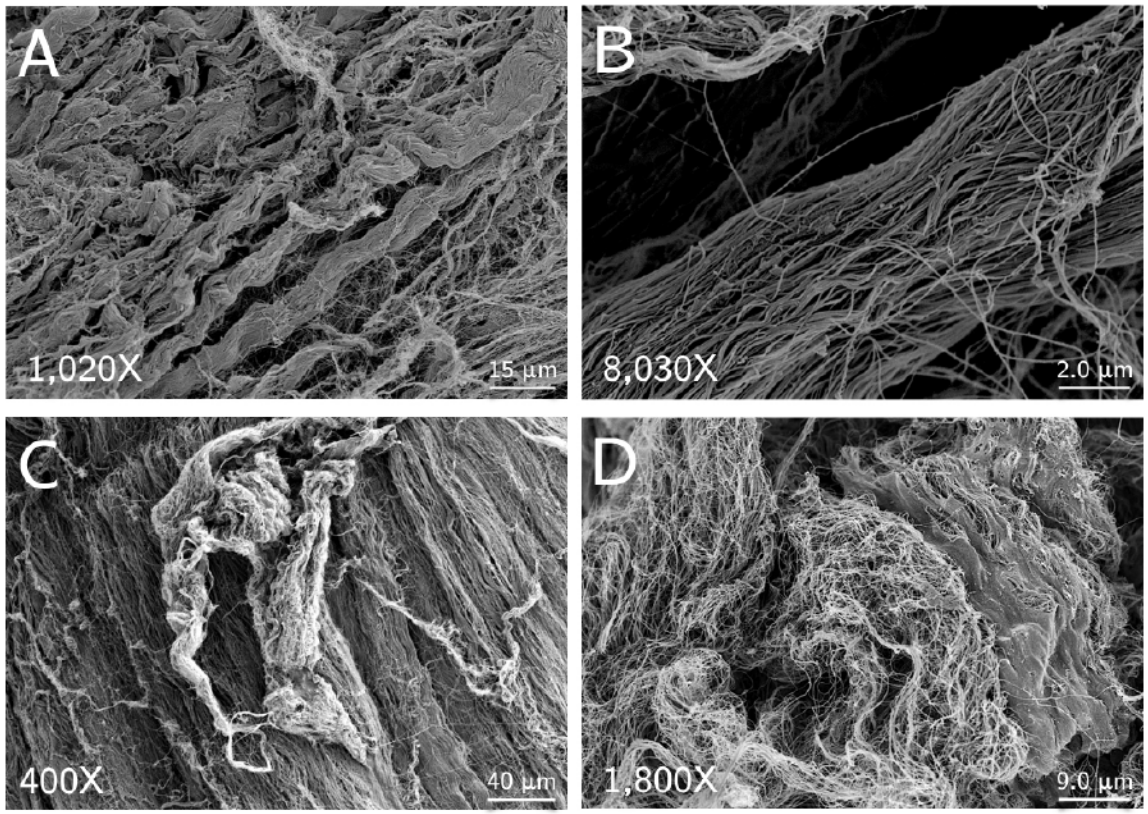

Figure 4.13: At relatively low magnifications, disruption of collagen fibril organization is evident in young and old samples. These micrographs are from two ruptured male tendons, aged 29 and 59 (panels A/B and C/D, respectively). The collagen fibrils form larger bundles (A) that are not as uniform as control samples, and the fibrils within these structures are no longer as homogeneous and as tightly packed (B). The samples appear to fail as a bundle of collagen fibrils as shown in panel C. The collagen within these bundles has a disrupted and disorganized structure (D).

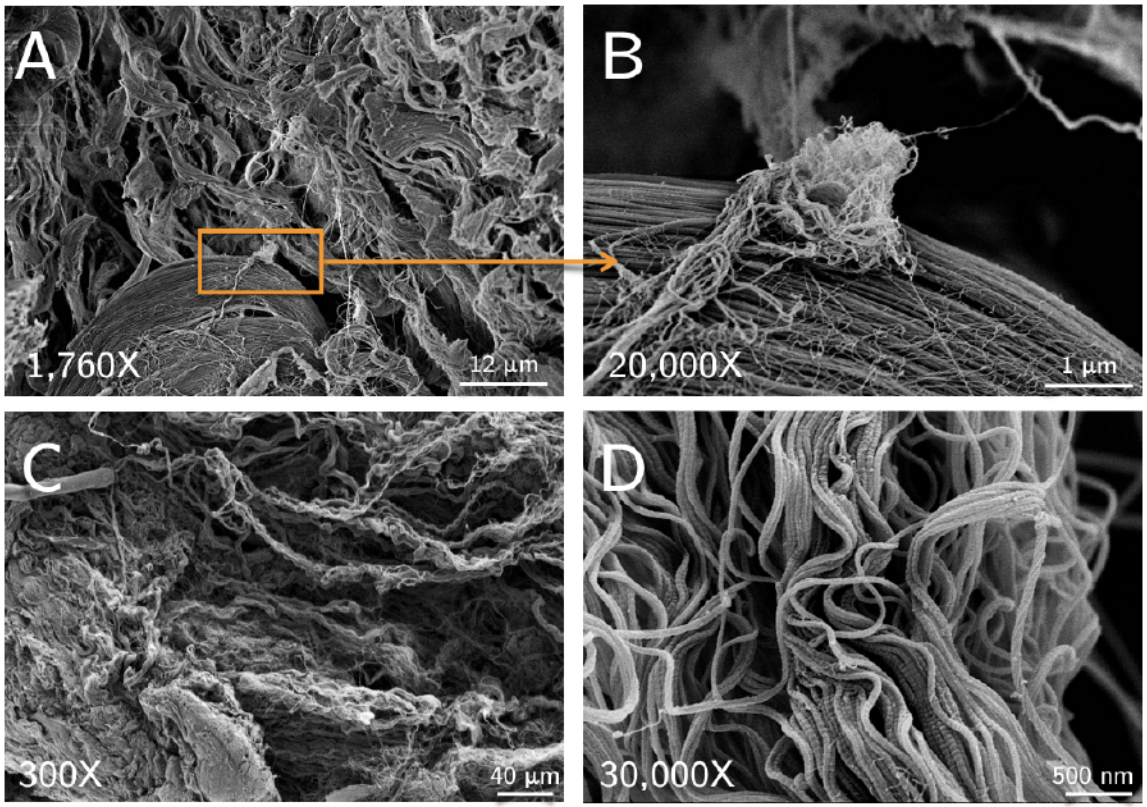

Figure 4.14: The varying scales of damage within the sartorius tendon can be seen above in samples from a 40-year old male donor. The lack of longitudinally aligned fibrils is clearly visible in panel A, where there are multiple bundles of collagen fibrils that appear unorganized. If inspected more closely (panel B) it can be seen that a smaller bundle of fibrils appears to fray into smaller subcomponents, while being near an area of well aligned homogenous fibrils. Panel C once again demonstrates heterogeneity within the tendon, with individual bundles being composed of D-banded, but loosely arranged collagen fibrils (panel D). It is possible that damage to sartorius tendons occurs on the nanoscale and on a larger scale, where the bundles of collagen fibrils serve as their own functional units, leaving the collagen within fibrils properly packed but disorganized.

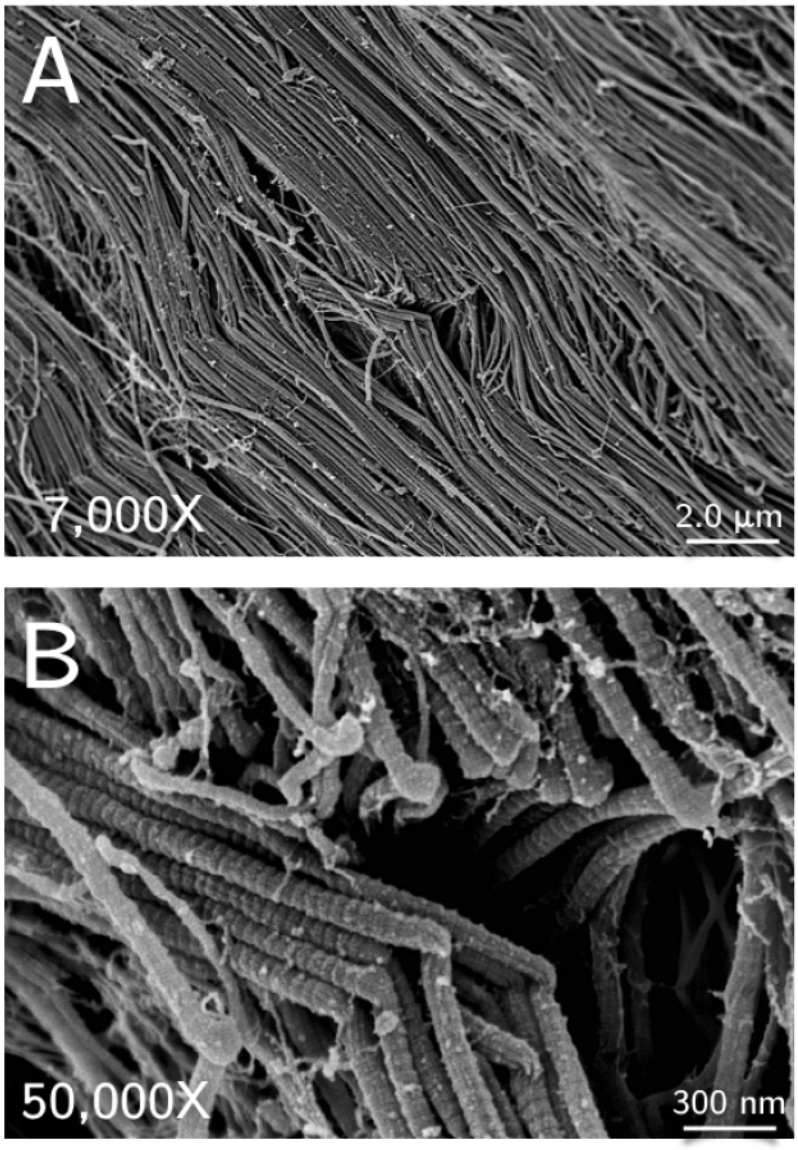

Figure 4.15: Nanoscale damage can clearly be seen above in the micrographs from a ruptured tendon from a 20-year old donor. There are local sites of damage within collagen fibrils, that appear to occur at discrete sites, potentially along a plane through a bundle of fibrils (A). The collagen fibrils have kinks and turns that form while the collagen on either side remains intact, as indicated by the presence of D-banding. Within the kink sites there appears to be exposure of some substructure, suggesting that the collagen fibrils may be unwinding locally to expose subfibrillar structure. The fibrils remain in close proximity with one another, despite the presence of damage.

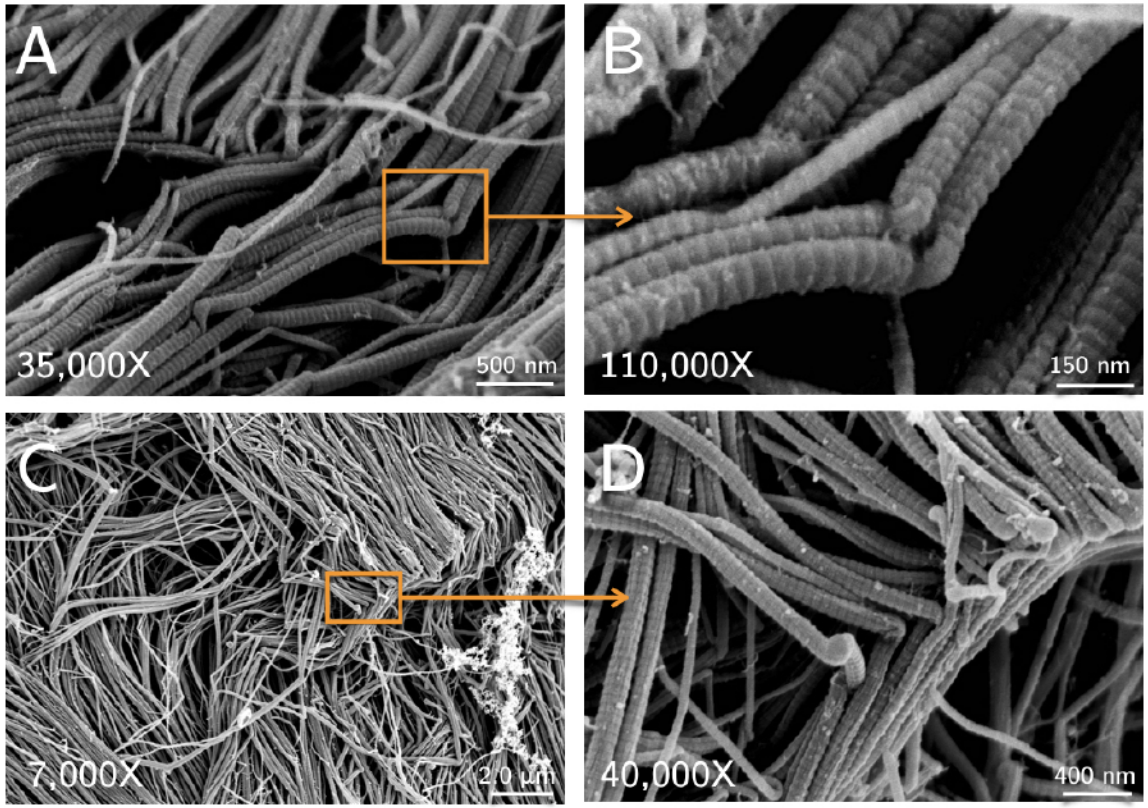

Figure 4.16: The plane of propagating local damage can be seen in these micrographs from ruptured 34-year old sartorius tendon. In panel A, it is also visible that there are multiple sites of damage within a single fibril. While the kinks within these damaged fibrils lack D-banding, it is evident that the collagen is only discretely damaged while the other portions remain intact (B). At a low magnification, disorganization and kink propagation is visible (C), while a large portion of the sample remains properly packed and undamaged when inspected more closely (D). The left of panel D also shows a collagen fibril with striations along its length, suggesting that despite intact D-banding, the structure is unwinding in some way.

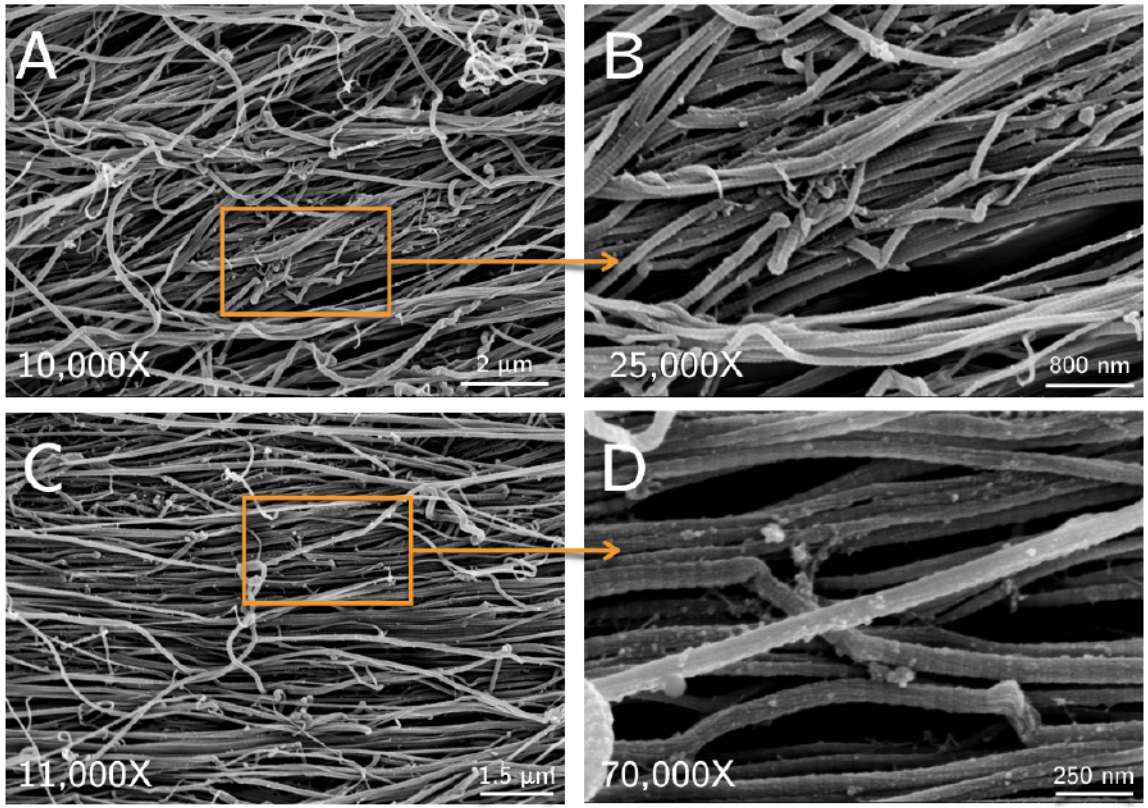

Figure 4.17: The disruption of structure can be seen in samples from middle aged tendons as well. Micrographs of ruptured 40-year old sartorius tendon reveal loose packing of collagen fibrils at relatively low magnifications (A and C) and nanoscale damage (B and D). The local sites of damage within panel B show that kinks may be coming in and out of the plane of the micrograph, and show that there is some twisting of collagen fibrils as well. The exposure of subfibrillar elements is clearly visible within the damaged fibrils shown in panel D, specifically within the kink sites themselves.

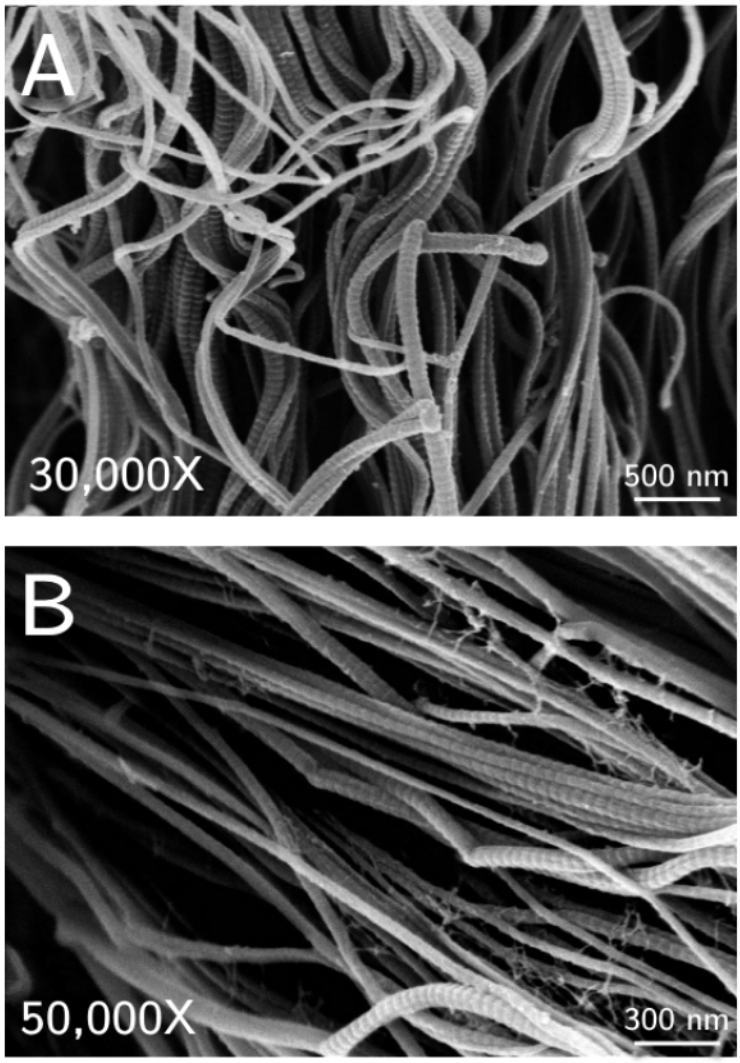

Figure 4.18: Even within older samples (such as the 50-year old ruptured sartorius tendon sample shown here), nanoscale damage is rare, but kinks sites are found within sections of loosely organized collagen fibrils (A). Kink sites are also formed in regions where collagen fibrils are longitudinally aligned and intact, which may suggest that there are invidual fibrils that are more likely to fail than others (B).

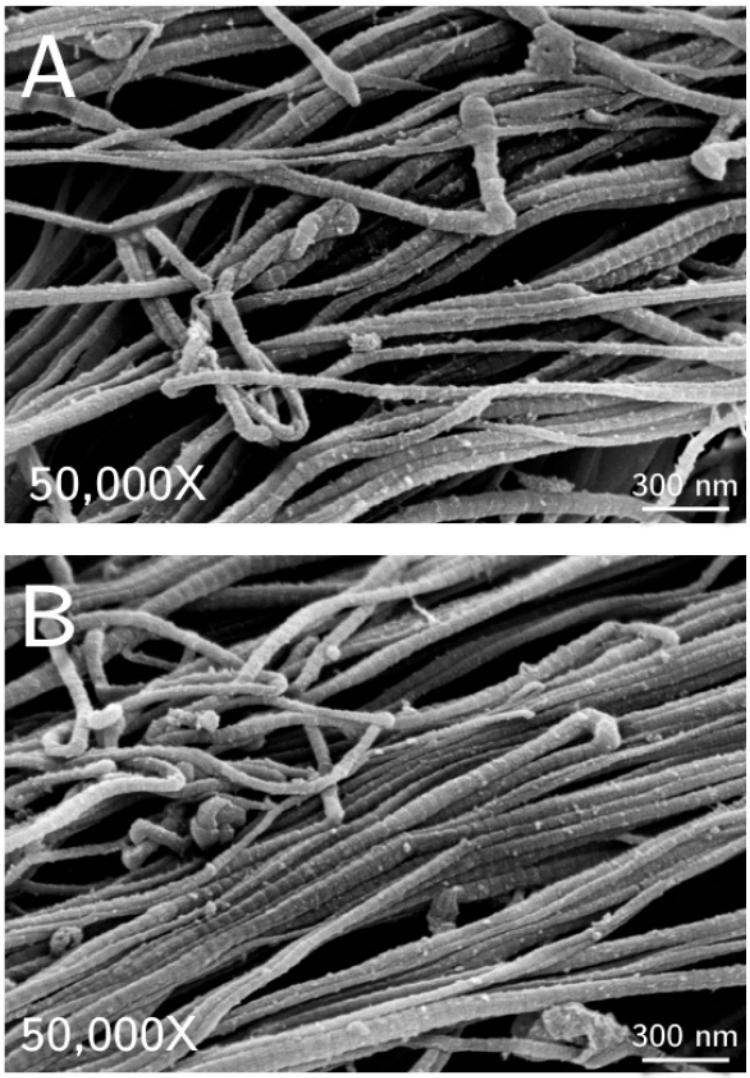

Figure 4.19: Some samples revealed some unique motifs that were unlike the previously seen kinks or turns. This samples from a ruptured 55-year old sartorius tendon shows some twisted fibrils with intact D-banding (A) and a fibril with a hairpin turn present (B). Panel B also shows fibrils in the top left that have no apparent organization and appear twisted together, suggesting that damage motifs may come in many forms.

Chapter 5

Discussion

This is the first study of its kind to systematically investigate the failure mechanisms present within aging human tendons at the nanoscale. Moreover, this is the first study to report age-related changes to thermal stability in human tendon collagen with both HIT and DSC. It was discovered that human sartorius tendon collagen is thermally stable in young adulthood, and that this stability continues to change with age. Interestingly, it was found that the mechanical properties of the sartorius tendon did not change with age, and that the damage motifs present at the nanoscale differ from previously seen discrete plasticity kinks. The results of this thesis suggest that the collagen within this particular tendon is heavily crosslinked, inhibiting the mechanism of kink formation. Instead, there is disruption of larger scale tensile load bearing units and change in collagen thermal stability with age.

5.1 Age-Related Changes in Thermal Stability

Collagen thermal stability as measured by HIT and DSC can be used as a proxy for the amount and type of crosslinking present within soft-tissues. Other factors affecting thermal stability include molecular packing and tissue hydration, as these would both impact the activation of thermal denaturation within the thermally labile domain of

collagen molecules. The results of this thesis work show that thermal stability of sartorius tendon collagen changes with age, as shown by the HIT and DSC results presented in Section 4.2. It is interesting, however, that the two techniques show seemingly different age-related patterns of change with respect to molecular packing and thermal stability.

5.1.1 Collagen Thermal Stability Increases with Age

From DSC it was found that the denaturation temperature T_{onset} increased with age suggesting that there is higher thermal stability in older tendons. An increase in the quantity and/or density of crosslinks may decrease the lateral spacing, thereby decreasing the size of the 'box' as per the polymer in a box theory[72,74,79]. This decreases the amount of energy required to activate the thermally labile domain[74]. By reducing the lateral dimensions of the box via crosslinking or dehydration, there are fewer possible molecular configurations. In turn, this lowers the configurational entropy and increases the ΔG of activation.

Maturation and aging will cause an increase in trivalent crosslinking and glycation-driven AGE formation, which will cause tighter molecular packing as compared to divalent enzymatic crosslinking[43]. All of this together will increase the stability and in turn, the denaturation temperature. The denaturation temperatures of the human sartorius tendon as measured by DSC are higher than what has been reported for bovine forelimb and tail tendons (Table 5.1), suggesting that the human sartorius tendon samples are more heavily crosslinked than previously studied tendons, even at ages as young as 20. The denaturation temperatures are also higher than previously studied human tendons, implying that this tendon may have higher crosslinking or molecular packing than the Achilles, patellar, and quadriceps tendons[99]. Without performing a biochemical crosslink assay, it is not possible to say this with certainty. Additionally, the study on the thermal properties of human tendons used a different scanning rate compared to this thesis work (0.3 °C/min versus 5.0 °C/min)[99]. While the scanning rate used in the study of the sartorius tendon was relatively low to avoid superheating, it has been demonstrated by Miles et al. that scanning rates have an

impact on the DSC response and the extracted parameters[70].

Table 5.1: Thermal properties of various soft tissues whose thermal stability has been studied with DSC and/or HIT

Reference	Source and Tendon Type	T_{onset} (°C)	T_{peak} (°C)	FWHM (°C)	Δh (J/g)	T_d (°C)
Herod et al. (2016)[102]	Bovine; Common Digital Extensor	63.1 ± 1.0	65.4 ± 0.7	2.7 ± 0.5	58.0 ± 13.7	62.7 ± 0.4
	Bovine; Superficial Digital Flexor	64.4 ± 0.7	65.8 ± 0.8	1.7 ± 0.1	40.7 ± 10.1	65.4 ± 0.7
Willett et al. (2007)[101]	Bovine; Tail Tendon	66.1 ± 1.2	N/A	N/A	N/A	65.5 ± 1.1
Willett et al. (2008)[94]	Bovine; Tail Tendon	64.49 ± 0.51	66.27 ± 0.46	4.2 ± 1.4	65.7 ± 7.3	N/A
Willett et al. (2010)[86]	Bovine; Tail Tendon (Young)	58.5 ± 1.7	61.73 ± 0.42	N/A	N/A	62.4 ± 1.5
	Bovine; Tail Tendon (Old)	58.1 ± 1.7	61.76 ± 0.77	N/A	N/A	62.5 ± 1.6
Wiegand et al. (2010)[99]	Human; Achilles	N/A	59.7 ± 0.1	N/A	8.54 ± 0.45	N/A
	Human; Quadriceps	N/A	63.3 ± 0.1	N/A	6.27 ± 0.3	N/A
	Human; Patellar	N/A	61.7 ± 0.1	N/A	4.36 ± 0.2	N/A
Miles et al. (2005)[43]	Rat; Tail Tendon (Control)	N/A	65.1	N/A	58.6	N/A
	Rat; Tail Tendon (MDA)	N/A	69.3	N/A	N/A	N/A
	Rat; Tail Tendon (Glut)	N/A	84.1	N/A	N/A	N/A
	Rat; Tail Tendon (HMDC)	N/A	74.1	N/A	N/A	N/A

The denaturation temperature from DSC (T_{onset}) suggested that there is an increase in enzymatic and/or non-enzymatic crosslinking with age. Crosslinking has been found to increase thermal stability by dehydration[70]. The FWHM is an indicator of the range of molecular stabilities present[72] and can also provide information about

the heterogeneity of molecular packing within the sample[94]. As reported by Miles et al. dehydration would cause a reduction in the number of water bridges that could form within collagen molecules, which would increase the size of the thermally labile domain and this would be registered as a decrease in the width of the endotherm (i.e. lower FWHM)[70,72,169]. A narrowing of the endotherm would also be caused by low rates of collagen turnover. If no new collagen is being laid down, then there would be a decrease in the amount of immature divalent crosslinks and an accumulation of mature trivalent and glycation-derived crosslinks[51].

In vitro crosslinking studies correlate well with the findings from the human sartorius tendon, suggesting that the age-related increase in T_{onset} and decrease in FWHM may is due to increased crosslinking. Studies have shown that endogenous crosslinking of tail tendons using reducing sugars results in significant increases in collagen thermal stability[170,171]. *In vitro* glycation crosslinking using ribose or glucose produce AGEs such as pentosidine and glucosepane, respectively[172]. Tissues treated with these sugars result in increased T_{onset} and T_{peak} values compared to untreated samples, indicating a change in the amount of energy required to denature the collagen triple helix[171]. This is likely because the intrahelical glycation-derived crosslinks stabilize the molecular packing, while also inhibiting the unfolding of the thermally labile domain. The presence of crosslinks would also decrease the number of possible molecular conformations, thus decreasing the configurational entropy within the system.

DSC is a sensitive technique that can be used as a proxy for crosslinking. Together, the increase T_{onset} and decrease in FWHM show that sartorius tendon collagen thermal stability increases in age. This is likely due to increased crosslinking, which dehydrates collagen and brings neighbouring molecules closer together thus increasing the amount of energy needed to activate the thermally labile domain.

Water Content

As mentioned above, it is thought that sartorius tendon collagen increases in thermal stability due to crosslink-induced dehydration. Interestingly, the amount of water

present in the human sartorius tendon did not change over the span of four decades. It has previously been found that the water content within tendons changes from 80-85% at birth to 30-70% in old age[134]. While this is a rather large variation, other studies have demonstrated that there is in fact a difference in water content between different tendons in the body. One study found that there was a significant difference in the water content between human Achilles and anterior tibialis tendons, and that this had an effect on the mechanical properties of each[173]. The water content of the Achilles and tibialis anterior were 69.0 and 56.7%, respectively, and were calculated using tendons from donors aged 48-84[173]. This suggests that functionally distinct tendons may have varying matrix properties, and thus have different water contents. The water content within human sartorius tendons did lie within the range cited above, and is similar to that of the Achilles tendon.

It is possible that there is some level of dehydration occurring within the sartorius tendon samples with age but that this dehydration was not detected in this study. An increase in crosslinking would cause dehydration on the molecular level[70] and while this may have an impact on the responses via DSC, it is unlikely that it would be detectable through weighing DSC samples before and after testing.

5.1.2 Sartorius Tendon Collagen is Heavily Crosslinked

The denaturation temperatures of the sartorius tendon, as measured by DSC and HIT are higher than what has previously been seen in other tendons and soft tissues (shown in Table 5.1), suggesting that this tissue is significantly more crosslinked. It was found that the T_d decreases linearly with age in sartorius tendon collagen. This is intriguing because it has been well documented that there is increased enzymatic and non-enzymatic crosslinking of soft-tissues with age[49,50,134,150,174]. This increase in crosslinking should theoretically increase the denaturation temperature, as the crosslinks would decrease the entropy thus decreasing the number of possible molecular configurations, and increasing the energy required for activation of denaturation, as was seen by DSC.

Previous aging studies using BTTs, showed no significant differences in T_d (HIT) in young and old samples despite statistically significant differences in the percentage of mature and thermally stable crosslinks[86]. There were also differences in the temperature of maximum force ($T_{F_{max}}$) between the two groups. $T_{F_{max}}$ is thought to increase with increased crosslink density and thermal stability, suggesting that older BTTs are more mature and stable than young BTTs[86]. That particular study did not measure the presence of AGEs but suggested that the increased $T_{F_{max}}$ in older samples could be due to glycation. The results presented herein do not support this hypothesis, as the thermal stability of sartorius tendon collagen was found to increase with age.

The presence of thermally stable crosslinks is confirmed by the fact that all of the samples survived to the isotherm (i.e. 90°C), regardless of age. This suggests that the crosslinks present may be HHL, Hyl-Pyr, pyrrole, or glycation-derived crosslinks such as pentosidine or glucosepane[53–56,165]. The fact that even young tendons survived to 90°C implies that immature crosslinks (e.g. deH-HLNL, deH-LNL, or HLKNL) have already mostly been converted to their respective trivalent, mature forms by the age of 20. Typically, $NaBH_4$ increases the total amount of thermally stable crosslinks, increasing the $t_{1/2}$ of load decay[175]. This treatment did not show any differences in maturity of crosslinks between old and young donors as there was no effect of treatment on the rate of load decay, further confirming that there is a large amount of heat-stable, mature crosslinking present even at young ages. Perhaps the most striking observation within the isothermal data is the absence of load decay, which is expected to occur following the scission of the peptide backbone. The $t_{1/2}$ values that were calculated are widely distributed, with some values being comparable to previously studied tissues while others are significantly higher.

The isothermal data showed that the typical Maxwellian-type decay was not present in all samples, but rather that the load was increasing over time. It has previously been found that glutaraldehyde-crosslinked heart valve tissue shows isothermal contraction[91], which may suggest that this feature is characteristic of tissues that are heavily crosslinked. Surely the hydrolysis of the peptide backbone is occurring within collagen molecules, but there must be another mechanism involved that overshadows

this effect. The absence of load decay did not have a relationship with age, suggesting that sartorius tendons are heavily crosslinked even at young ages. Mature crosslink concentration has been shown to plateau at a young age in animal models and any changes in amounts of crosslinking thereafter are typically attributed to glycation[51].

The presence of these glycation derived crosslinks (either pentosidine or glucosepane) could also be responsible for not only the isothermal contraction, but also for the rise in force under isometrics conditions that was seen in HIT before denaturation (the increase in load after denaturation is because of helix to coil transition). While the fractional changes in load were not related to age, there was a negative linear correlation between the fractional change in load before and after denaturation that was marginally significant (p=0.0556). This could mean that the more the load increases prior to denaturation, the less that occurs after. This may suggest that there is a net amount of force that can be created under isometric constraint that can happen within the sample. While it may be possible that isothermal contraction is due to the presence of some type of chemical reaction, it is more likely that the decay is absent simply because the material contains a dense network of crosslinks. The load decay that occurs as a result of peptide bond hydrolysis may be neutralized by dense crosslinking, which results in a material that is able to sustain load over an extended period of time.

5.1.3 Age-Related Change in Molecular Spacing May Result in Decrease in T_d

It is difficult to construct a coherent picture about age-related changes in thermal stability from the data present herein. HIT and DSC showed opposite correlations between their respective denaturation temperatures and age. It is important to note that HIT and DSC measure different aspects of denaturation, as mentioned in previous sections. The denaturation temperature as measured by HIT, T_d, is the temperature at which sufficient thermal energy has been transferred to the system to overcome the energetic barrier associated with the configurational transition from he-

lix to coil[58,81,85,86]. The random coil is of a lower energy state, however the sample is isometrically constrained so full uncoiling of the collagen helix does not occur. The T_d is the temperature at which there is an energetic driving force towards contraction as denaturation begins. This is registered as a sharp increase in load by the system. Conversely, DSC measures the amount of thermal energy delivered to the system during denaturation, and the T_{onset} (calculated as the intersection between the baseline and the upward slope of the endotherm) is the temperature at which enough thermal energy has been put into the system to activate the thermally labile domain[176]. This is thought to be the onset of denaturation. The extrapolated T_{onset} is often an overestimate[92], thus the true onset of denaturation should be taken as the calculated value, T_{dev}. In theory, the T_d should be greater than the T_{onset} because HIT only captures events that occur after thermal activation of denaturation, an event captured by DSC. By that same logic, the T_{dev} should be less than or equal to the HIT T_d. It is possible that the T_d is higher than both the T_{onset} and T_{dev} because isometric constraint increases the order of the collagen structure, thus decreasing the molecular entropy of the system and increasing the T_d.

From DSC, it would appear that collagen thermal stability is increasing with age due to increased crosslinking. Conversely, HIT indicates that there is a decrease in the molecular packing and/or crosslinking, with less constraint in older samples as compared to young ones. Based on the T_d values from HIT alone, it is tempting to suggest that a decrease in crosslinking occurs with age. In contrast, the isothermal data and survival of samples to 90°C confirms the presence of thermally stable crosslinks. There is no isothermal load decay and enough crosslinks are thermally stable (either mature trivalent crosslinks or AGE-derived crosslinks). This observation correlates well with the literature and with the DSC data, where the T_{onset} is increasing with age. A recent study conducted by Gautieri et al. found that the molecular spacing increased within tendon collagen from semitendinosus and gracilis tendons from donors aged 16-90[62]. It was suggested that this increased molecular spacing resulted from diffusion of glucose into the fibrillar structure. This may explain the observed relationship between HIT T_d and age seen in the sartorius tendon. An increase in tendon collagen molecular spacing with age would result in less molecular constraint and allowing for

more space for uncoiling of individual molecular and a decrease in T_d in old age. The relationship between crosslinking, molecular spacing, and aging within human sartorius tendons is still unknown. Without biochemical analysis of crosslinking, these results are speculative and not coherent.

5.2 Mechanical Characterization of Sartorius Tendons

5.2.1 Mechanical Rupture Indicated Elastic Recoil During Fracture of Individual Fascicles

The stress-strain curves that were obtained from the rupture of human sartorius tendons showed a response typical of collagenous materials: an increasing modulus in the toe region of the curve, a linear portion at higher strains, followed by the yield point, where plastic deformation begins and ultimately leads to failure[18,177]. The initial apparent stiffening is typical of tendons, and occurs following alignment of collagen fibres. The nonlinearity of the stress-strain curves is typical of many tendons, but one interesting feature found in the sartorius tendon is the mechanism of failure. Within the sartorius tendon, there are many individual failures prior to the failure of the entire structure. The individual components bear load until they fail individually, after which other fascicles engage. This was confirmed by the videos captured during rupture, where it was evident that failure occurred as a series of audible snaps, suggesting an elastic roil of the structure.

5.2.2 Mechanical Properties of Sartorius Tendon

Tendons increase in stiffness during development due to increasing amounts of mature trivalent crosslinks and during maturation due to increases in tendon cross-section, collagen content and changes in the arrangement of collagen fibrils[178,179]. While many properties of the collagen fibrils (e.g. molecular packing, density, etc.) impact how tendons function, the collagen fibrils define how the tendon will perform mechanically;

this is impacted by the amount of crosslinking present. Trivalent crosslinks are responsible for stabilizing the fibrillar structure of collagen and contribute to the mechanical properties of tendons, but the amount of these crosslinks has been found to plateau following maturation in animal models. Collagen is turned over slowly and any age-related increase in stiffness has historically been attributed to further crosslinking via glycation[49], yet the exact relationship between age and tendon mechanical properties remains unknown. In this thesis work, it was found that age had no effect on the mechanical properties of the human sartorius tendon. This correlates well with other *in vivo* human studies[68,151], which have found that there are no age-related changes in modulus and strength.

Comparison to Aging Animal Models

Many studies have been conducted in an attempt to discern the correlation between the type and density of native collagen crosslinking with the mechanical properties of tendons. The results have been inconsistent[68,148,173,178,180,181]. While *in vitro* crosslinking of tendons has been used to mimic aging in animal models, there have also been studies comparing old and young tendons from animals. A recent study also found that the mechanical properties of three different murine tendons does not change with age, and that the cellular population does not change significantly[182].

More specifically, *in vitro* crosslinking has been used as a proxy for mimicking aging in tendons, as it is known that crosslinking increases with age. The importance of enzymatic crosslinking in collagen stability and integrity has been demonstrated in tissues where the activity of lysyl oxidase has been inhibited, resulting in tissues with lowered strength[183]. The enzyme-derived crosslinks plateau at maturation, but the stiffness of tendons continues to increase with age[51]. It is thought that this is a result of the formation of AGE-derived crosslinks, which form between a reducing sugar and amino acid side chains within tendons[51]. Due to their elevated glucose levels, diabetics are more susceptible to the formation of these covalent crosslinks[184].

In order to determine the relationship between AGEs and mechanical properties without taking into consideration age, lifestyle, and/or disease, *in vitro* studies have found that incubation of tendon with a reducing sugar results in an increase in tendon stiffness[52,135,185], but the correlation of crosslink density with tissue stiffness remains weak. Studies that use *in vitro* glycation mention that they are mimicking both aging and diabetes. This makes it difficult to draw conclusions about specific *age*-related changes in stiffness that are due to AGE formation. *In vitro* glucose-mediated glycation of rabbit Achilles tendons found that glycated tendons were 21% stronger, 72% stiffer, and more brittle[186]. Other studies have been conducted using RTTs that were also endogenously crosslinked using ribose. Following mechanical rupture, it was found that crosslinked RTTs exhibited a loss in post-yield plastic behaviour as compared to the control samples, but that there were no significant differences in elastic modulus[187]. Interestingly, the ultimate stress increased, and the failure strain decreased following crosslinking. The small-angle X-ray scattering (SAXS) data determined that there was diminished fibril-fibril sliding following glycation (as previously seen[67]), that led to a change in the mechanism of failure to favour brittle fracture[187].

Brown et al. found that the tensile parameters remained unchanged in ribose-treated BTTs, but the mechanical energy parameters had changed with ribose crosslinking[171]. The total amount of strain energy and post-yield energy were larger in control samples than ribose-crosslinked ones. This suggests that control tendons were able to dissipate strain energy more effectively through the formation of discrete plasticity kinks, as compared to the ribose-crosslinked tendons[171]. In this thesis work, the tensile properties of the sartorius tendon did not change with age. Discrete plasticity kinks were not seen in sartorius tendon samples so there must be another reason that there were no age-related changes in the mechanical properties. It is likely that the incredibly high density of enzymatic crosslinking (seen even in early adulthood) masks any increases in AGE-crosslinks thus preserving the mechanical properties despite any age-related increases in glycation.

Comparison to Aging Human Tendon Models

The relationship between mechanical properties and aging suggest that despite changes in thermal stability/crosslinking, mechanics of the sartorius tendon are preserved. Despite differences in absolute age, these findings are consistent with previous *in vitro* animal and human studies, and *in vivo* human studies. It is possible that the internal tendon structure (i.e. cellularity) is also not changing in the sartorius tendon and that the effects of crosslinking (either enzymatic or non-enzymatic) are neutralized by decreased collagen content, decreased molecular density, and the presence of microtraumas.

There have been many studies done on the age related changes in mechanics of tendons and ligaments. Animal studies allow for the control and knowledge of disease, nutrition, and activity – factors that cannot be controlled in studies of human tissues. *In vitro* human studies have found that there are no difference in stiffness, failure strain or failure load in donors aged 16-44. A study conducted by Hubbard et al. found that fresh-frozen palmaris longus and extensor hallucis longus harvested from the tendons of the hands and feet, respectively, did not change with respect to their tendon moduli in individuals between 16 and 88 years of age[177]. They did find that subject age had an effect on hysteresis and relaxation of the sample, something that was not evaluated in the presented work. A study on fresh-frozen human patellar tendons from two age groups (aged 29-50 and 64-93) found no age-related differences in in modulus, but found a 17% decrease in UTS in the older group[18]. While there was no difference in UTS detected in the older sartorius tendon group, it is important to note that all donors were below 60 years of age. It is possible that the UTS changes in older age groups such as the one measured in the study conducted by Johnson et al.[18].

While the presence of microdamage has not been fully investigated in human tendon mechanics studies, it has been suggested instead that mechanics of tendons are maintained over a life span due to decreases in the amount of collagen and the molecular density of fibrils within aging tendons[68]. That same study investigated old (67 $\pm$ 3 years) and young (27 $\pm$ 2 years) male patellar tendons *in vivo* and found that

there were no differences between the two groups with respect to their stiffnesses, deformations, strains, and Young's moduli[68]. Tendon biopsies were taken from both age groups and examined for their crosslink population and it was discovered that young tendons had significantly lower concentrations of HP, LP, and pentosidine but higher collagen concentrations[68]. This is an interesting finding because it would be expected that these age-related differences in tendon composition would lead to altered mechanical properties[68]. This study did not look into fibril length[188,189], fibril diameter[190], and changes in PG and GAG contents[158,191,192], which have all been shown to contribute to the mechanical properties of tendons.

The effect of glycation and formation of AGE-crosslinks on the mechanics of aging human tendon have been investigated using SAXS-couple mechanical tests[187]. Scraps of fresh-frozen semitendinosus and gracilis tendons from donors aged 16-89 were used to determine the level of glycation (by fluorescence analysis) and the mechanical properties (i.e. elastic modulus, yield stress and strain, failure stress and strain)[187]. It was found that the extracted mechanical parameters were not correlated to donor age, despite a strong correlation between donor age and pentosidine-related fluorescence[187]. Using SAXS, it was determined that older human tendons have decreased D-period lengths and increased molecular distances at rest[187].

Tendon degeneration and changes in tendon structure have previously been associated with an imbalance in synthesis and degradation of ECM[193]. A recent study has examined the structure and composition of aging tendons that are anatomically proximate to the sartorius tendon. The semitendinosus and gracilis tendons are the other two tendons of the pes anserinus, the complex from which the sartorius tendons used in this thesis study were taken[9]. This study investigated the differences between 10 adult (41.8 ± 13.3 years) and 6 old (72.7 ± 7.0 years) tendon samples by analyzing morphological differences using histology. Interestingly enough, the researchers found that there was no change in the structure between the two groups, and no change in the collagen turnover pathways, meaning that aged tendons have the same ability as young ones in terms of remodelling[9]. While the presented thesis work did not look at histological and molecular differences within aging sartorius tendons, the outcome

clearly shows no difference in mechanical properties, which may be attributed to constant cellular potential to maintain homeostasis or negligible collagen turnover.

5.2.3 Effect of Strain Rate on Mechanical Parameters

It has been well-established that the tensile and viscoelastic properties of soft-tissues are dependent on strain rate, with higher strain rates producing higher failure strains and ultimate tensile strengths[148]. The relationship between sensitivity to strain rate and age was studied by Haut, and it was found that the sensitivity of failure properties of RTTs to strain rate depended on age[145]. More specifically, maturation was found to cause a drop in strain-rate sensitivity and this was attributed to changes in the amount of mucopolysaccharides present. Aorta and ligament studies have shown that there is no relationship between failure strain and strain rate[194], but Haut found that there is a dependence on strain rate and the failure strain in his RTT samples. The relationship between strain rate and mechanical properties of the human Achilles tendon was investigated by Wren et al., who demonstrated that there was no difference in modulus between tendons tested at 10%/s and 1%/s[195]. They did note that there was a 15% increase in failure stress and strain at the higher rate[195]. These results would support the results from this thesis work, which suggest that there are only modest changes in mechanics between the strain rates used. It is likely that the use of quasistatic strain rates resulted in an undetectable difference in modulus and UTS.

5.3 Damage Motifs in Human Sartorius Tendons

Discrete plasticity, as described previously in the literature, was not found in ruptured human sartorius tendons. The motifs seen in the sartorius samples most closely resemble those found in tendons that are known to be more heavily crosslinked, such as bovine flexors[102] and glycated BTTs[171]. It is thought that discrete plasticity kinks form as a result of the slipping of molecules, resulting in the loss of D-banding –

a characteristic feature of properly assembled collagen fibrils. Brown et al. found that the introduction of glycation crosslinks through endogenous ribose crosslinking resulted in the inhibition of discrete plasticity damage[171]. It is known that AGE-crosslinks form between the helical portions of neighbouring collagen molecules rather than at the telopeptide ends, where enzymatic crosslinks are formed. The formation of these helix-to-helix crosslinks may prevent molecules from slipping past one another, inhibiting the formation of discrete plasticity kinks. Trivalent mature crosslinks may prevent molecular slippage using the same mechanism, as seen in the bovine flexor tendons[102]. Similar to both of those studies, it is possible that sartorius tendons are so heavily crosslinked that the fibrils cannot dissipate excess strain energy into the formation of discrete plasticity kinks, resulting in fracture with substantial elastic rebound[171]. This is supported in the literature, since it has been found that increased crosslinking causes stiffening of soft tissues[67,187].

The human sartorius samples failed bundle-by-bundle, as seen in the videos taken during mechanical rupture. It is possible that heavy fibril crosslinking results in the formation of larger functional units within the tendon, as previously seen in *in vitro* ribose crosslinked fibrils where it was found that they function as larger tensile load bearing units[135]. This may also serve as an additional explanation for the inhibition of discrete plasticity within human sartorius tendons. Fibrils may remain intact due to crosslinking, while failure occurs at a higher level within bundles of fibrils. The nature of the crosslinks are unknown from DSC and HIT testing, however it may be speculated that they are glycation-crosslinks. Since the AGE-crosslinks are formed helix-to-helix, fibrils may not be able to slide past each other, while fibril bundles are capable of doing so. This is supported by studies from Gautieri et al., who have demonstrated that glycation occurs on the outside of collagen fibrils[187]. This would result in the crosslinking of neighbouring fibrils, disorganization, and fragmentation on a higher order within tendons[135], exactly what was seen in sartorius tendons. Repetitive loading that causes this higher-order damage could result in the accumulation of stress concentrations and abnormal loading.

Future studies using molecular probes or enzymatic digestion to investigate the pres-

ence of denatured collagen would be interesting and potentially helpful in understanding what damage within human tendons look like. Collagen mimetic peptides, or collagen hybridizing peptides (CHPs) have been used to probe for molecular-level damage in collagen[196–198]. These small synthetic peptides mimic the molecular structure of native collagen and have the primary amino acid repeating sequence of (Gly-Pro-Hyp)$_n$. These molecular probes have previously been used to study the molecular folding and structure of collagens[197]. Moreover, CHP has been shown to bind to unfolded portions of collagen molecules following enzymatic or thermal denaturation[197]. Most recently, it was found that fluorescently labelled CHP can be used to detect and localize molecular level damage in mechanically overloaded RTTs[198]. This study supports those of Veres et al., which have shown that there is denaturation of collagen molecules following mechanical overload[85].

Taken together, all of the results of the sartorius tendon model point to the fact that discrete plasticity may not be physiologically relevant in highly loaded adult human tendons. Discrete plasticity kinks form readily in overloaded animal tendons that are less crosslinked than higher load bearing tendons (tail and extensor vs. flexor tendons). It is possible that human tendons are heavily crosslinked due to higher loads *in vivo*. The mechanism of discrete plasticity may only be a feature of low load, positional tendons that contain fewer crosslinks than load bearing tendons. Similar to the flexor model and the ribose-treated tail tendons, heavy crosslinking is present in human sartorius tendons that inhibits molecular slippage and discrete plasticity. It is possible that the mechanism may indeed be present in low load bearing human tendons such as the extensors in human hands. It would be interesting to study how crosslinking develops in a range of human tendons from early childhood into adulthood, as it was found in other animal models that there is a plateau in the amount of crosslinking following maturation[51]. Along with biochemical crosslink assays, this would be extremely useful in studying the type of crosslinking present over the course of a human life span.

5.4 Limitations to Sartorius Tendon Model

The wide array of outcomes regarding mechanics and changes with age suggests that there are many factors impacting the results, including physiological differences between species, tissue types, genotypes, and lifestyle factors. While many studies clearly show that there are changes in enzymatic and non-enzymatic crosslinking within aging human tendons, it remains difficult to draw a conclusion regarding age-related changes in mechanics. While human biological variability certainly plays a role in the heterogeneity of samples and their properties, it is also possible that dissection and handling of human tissue impacts mechanics. Animal models are often used because the geometry of individual tendons favours relatively easy characterization. These tendons have typically been RTTs, BTTs, flexors and extensors (bovine or equine), and these all share the common feature of being uniform, cylindrical, and not containing much excess fat and muscle tissue. By contrast, human tendons are typically surrounded by significant amounts of muscle and fat (as seen in the presented model). The removal of the excess tissues may actually impart some shear loading on the tendon. Human tendon, however, often needs to be subdivided into fascicles which are subject to tissue damage. The mechanical samples that are cut are reproducible geometrically, but may lead to variability in the mechanical testing of tendons.

The characterization of tendons (e.g. crosslinking profile, failure mechanisms, mechanics, to name a few) is difficult to study because tendons from different species and with different functionalities show variability in their composition and structural organization. Simplified tendon models such as the BTT and RTT models are limited in that they differ widely from clinically relevant human tendons in terms of tendon type, which influences the amount of crosslinking and the mechanical properties. On the other hand, tendon models from a wide variety of species have structural heterogeneity which impacts the specific properties of the tendon. Adding in other factors like injury, disease, and age only further complicates the ability to compare tendon properties both within and among species.

The specific anatomy of the sartorius tendon does not lend itself easily to handling and dissection. While morphological differences due to genetic variability surely exist in other tendons (e.g. Achilles, patellar, semitendinosus, and gracilis tendons), these are typically uniform along their length and contain fewer distinct fascicles. Additionally, there are no data in the literature that have previously described any properties related to the sartorius tendon. Because of this, it is difficult to validate the results reported herein. The aging trends observed by DSC are supported by the literature while HIT revealed some new phenomena that have not previously been reported. Combined, the two show that features of crosslinking present in sartorius tendons change with age and that there is a potential change in molecular packing that occurs in old age. The mechanical properties of tendons have no definite relationship with age, as supported by the literature and previous *in vitro* glycation work in our lab. The results of the structural study of damage motifs present in human sartorius tendons share a number of similarities with heavily crosslinked tissues like bovine flexors and ribose crosslinked BTTs. This supports the notion that the mechanism is specific to tendons that are lightly or sparsely crosslinked. The failure mechanism present is similar to that reported by Bai et al., whereby larger load bearing units are formed from increased crosslinking and fail as larger bundles, leaving the fibrils intact[135].

5.5 Summary of Age-Related Changes in Human Sartorius Tendons

5.5.1 Age-Related Changes in Thermal Stability

DSC

Hypothesis: The peak and onset temperatures and the enthalpy of denaturation will increase with age, due to increased crosslinking, while the FWHM will decrease due to a lesser potential for remodelling in older tissues.
Conclusion: The onset temperature did increase with age, and the FWHM decreased,

supporting the above hypotheses. The peak temperature and enthalpy of denaturation did not change with age.

HIT

Hypothesis: The denaturation temperature and half-time of load decay will be higher in older tendons than younger ones due to an increase in glycation-derived crosslinking.
Conclusion: Surprisingly, the denaturation temperature decreased with age, and was found to precede the onset temperature (DSC) in older samples. This could mean that there is a change in molecular spacing with age. The isothermal data revealed that contraction was occurring, likely due to very high amounts of crosslinking.

5.5.2 Age-Related Changes in Mechanics

Hypothesis: The strength and stiffness of sartorius tendons will increase in old age, due to the increased presence of crosslinks.
Conclusion: There were no age-related changes in the mechanics of the sartorius tendon. Any increases in stiffness that may occur as a result of AGE crosslinking are masked by the high density of enzymatically derived crosslinks that are present even in early adulthood.

5.5.3 Mechanism of Failure

Objective: Determine the mechanism of failure within human sartorius tendons and how it compares to discrete plasticity.
Conclusion: The mechanism of failure within the sartorius tendon is different than that of discrete plasticity. The damage motifs that are seen are most similar to those found in bovine flexor tendons. The amount of damage present is not as widespread as discrete plasticity, with a large portion of fibrils appearing undamaged. At low magnifications, the samples appear to fail as larger fibre bundles that show elastic

recoil and fraying. The inhibition of discrete plasticity kink formation is supported by previous work using endogenously ribose-crosslinked BTTs and bovine flexor tendons, which contain mature, trivalent crosslinks.

Relationship With Age

Hypothesis: Discrete plasticity kinks will form in younger samples, and will disappear with age, as it would no longer be evolutionarily advantageous to promote survival past reproductive age.
Conclusion: Discrete plasticity was not found in any samples, and therefore has no relationship with age. There was also no relationship between age and the relative amount of damage present within sartorius tendons.

Chapter 6

Conclusion

The study presented herein has been the first to study age-related changes in the nanoscale structure of ruptured human tendons. It has also provided the first systematic look into the changes in thermal stability in fresh human tendons, identifying that human tissues are heavily crosslinked even at 20 years of age. There may also be a change in the molecular packing of collagen with age that affects the collagen thermal stability. The damage motifs present in human sartorius tendons are similar to heavily crosslinked tissues such as *in vitro* glycated BTT and bovine flexor tendons, suggesting that the formation of discrete plasticity kink sites as we know them is inhibited by crosslinking. It is proposed that the failure mechanism of human sartorius tendons occurs on a level higher than the fibril, and that bundles of fibrils fail by brittle fracture leaving the nanoscale structure largely unchanged. Despite all of the new findings in this thesis, more research is required to elucidate the exact nature of the crosslinks present, how these may change with age, and whether the sartorius tendon failure mechanism is true of all human tendons or specific to the presented model.

6.1 Future Work

6.1.1 Crosslink Identification and Quantification

Both DSC and HIT serve as proxies for crosslinking. While sodium borohydride treatment provided valuable information regarding the maturity of the crosslinks, the exact type and amount of crosslinking present within these tendons is unknown. High performance liquid chromatography and other biochemical techniques may be used to investigate the amount of enzymatic and non-enzymatic crosslinks. Knowing the type and density of crosslinks will help better our understanding of the age-related changes in in tendon structure and this may help explain the failure mechanisms present.

6.1.2 Histology

The structure of the sartorius tendon was examined extensively using SEM, which provided valuable information about the nanoscale structural features of damaged and control tendons. SEM is limited in that it examines the dried metal coated tendon subsample and relies on the bisection of small samples, while only providing topological information. Histological examination of these human sartorius tendons would provide insight on the elastin content, proteoglycan and glycosaminoglycan content, cellularity, and collagen type among other things. All of these features may influence the mechanical behaviour of tendons and knowing the potential changes that occur with aging assist in understanding tendon injury and healing.

6.1.3 Calculation of Energetic Parameters and Subrupture

Other studies have demonstrated that the tensile mechanical parameters of human tendons does not change with aging but rather that the energetic parameters such as toughness, total strain energy, and post-yield energy are affected. It is possible that differences in mechanics of aging tendons are not apparent until after the yield point. Younger and older tendons may have differences in their ability to resist deformation

and may dissipate strain energy differently. Subjecting tendons to multiple subrupture overload cycles may incur more damage to the constituent collagen than the single pull-to-rupture resulting in an increased density of damage motifs.

6.1.4 Different Tendon Model

The sartorius tendon model used in this thesis study employed fresh tissues from a wide age range of donors, which allowed for its systematic characterization – something that has never been done before. A great deal of troubleshooting occurred to optimize the experimentation and despite this, another tendon model would be interesting to study. Investigating damage motifs in a different tendon would add to the knowledge we've gained regarding damage motifs and collagen thermal stability but may also be easier to handle. A thinner, more cylindrical tendon with a longer midsubstance may eliminate the issues seen with dissecting away the excess muscle and fat in the sartorius tendons. Using a tendon that is not ribbon-like and less fascicular would help in creating more uniform samples for mechanical rupture. Moreover, it would be of great interest to see whether other human tendons exhibit the same damage motifs characteristic of high-energy elastic rebound as seen in the sartorius tendons. A low-load tendon such as the extensor in the hand would be worthwhile to investigate, since it may contain fewer crosslinks and thereby have a different mechanism of failure.

6.2 Concluding Remarks

This thesis work has furthered our understanding of what damage to tendon collagen looks like within a human tendon. The sartorius tendon is a heterogeneous tissue containing well-defined fascicles that snap upon rupture, and the damage motifs seen reflect this fracture with significant elastic recoil. While the thermal stability of the constituent collagen was examined, the exact type and amount of crosslinking present remains a mystery. The model established herein is not without its flaws, but has certainly laid down the framework for future human tendon studies. Understanding

what damage to *human* tendons looks like in different tendon types is crucial in developing new treatment strategies and improving tendon injury prognoses.

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