DIABETES, ADVANCED GLYCATION, AND TENDINOPATHY

by

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To my parents.

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LIST OF ABBREVIATIONS

3DG.....3-deoxyglucosone
ADAM .....A disintegrin and metalloproteinase
AGE .....Advanced glycation end product
Ager.....Advanced glycosylation end product-specific receptor precursor
ATP .....Adenosine triphosphate
AU.....Arbitrary unit
Bak1 .....BCL2-antagonist/killer 1
Bax .....BCL2 associated X, apoptosis regulator
Bcl2 .....B-cell lymphoma 2, apoptosis regulator
Bcs1l .....BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone
BMI .....Body mass index
BWN .....Body weight normalized
Casp.....Caspase
CHOL/HDL .....Cholesterol to high-density lipoprotein ratio
CML.....N^{ε}-(carboxymethyl)lysine
Col1a1 .....Collagen alpha-1(I) chain
Col3a1 .....Collagen alpha-1(III) chain
COX-2.....Cyclooxygenase-2
Cox4i1 .....Cytochrome c oxidase subunit 4i1
CSA.....Cross-sectional area
Ctgf .....Connective tissue growth factor
Dcn.....Decorin
ddPCR.....Droplet digital polymerase chain reaction
DHAP.....Dihydroxyacetone phosphate
DHE .....Dihydroethidium
dUTP .....Deoxyuridine triphosphate
EAG .....Estimated average glucose
ECM.....Extracellular matrix
EdU .....5-Ethynyl-2´-deoxyuridine

EIF2C1 .....Protein argonaute-1

ERK.....Extracellular signal regulated kinase

FCCP.....Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

Fgf2 .....Fibroblast growth factor 2

GA3P .....Glyceraldehyde 3-phosphate

GAG.....Glycosaminoglycan

Glo.....Glyoxalase system

HbA1c .....Hemoglobin A1c

HDL .....High-density lipoprotein

HG.....High glucose

HIF-1 .....Hypoxia inducible factor 1

HOMA-IR .....Homeostatic model assessment of insulin resistance

IL-33 .....Interleukin-33

iNos .....Nitric oxide synthase 2

IκB .....Inhibitor of nuclear factor kappa B

JNK .....c-Jun N-terminal kinase

Keap1 .....Kelch-like ECH-associated protein 1

LDL.....Low-density lipoprotein

LOX .....Lysyl oxidase

MAPK .....Mitogen-activated protein kinase

MG .....Methylglyoxal

MG-H1 .....Methylglyoxal-derived hydroimidazolone

MGV .....Mean gray value

MMP .....Matrix metalloproteinase

mRNA .....Messenger RNA

MT-ATP6.....ATP synthase 6, mitochondrial

mtDNA.....Mitochondrial DNA

MTR.....Moderate treadmill running

Mybl2 .....MYB proto-oncogene like 2

MyD88 .....Myeloid differentiation primary response 88

NADPH.....Nicotinamide adenine dinucleotide phosphate (reduced)

ND1.....NADH-ubiquinone oxidoreductase chain 1

Ndufa1.....NADH-ubiquinone oxidoreductase subunit A1

NF-κB .....Nuclear factor kappa-light-chain-enhancer of activated B cells

NG.....Normal Glucose

NRF2.....Nuclear factor erythroid 2-related factor 2

OCR .....Oxygen Consumption Rate

Pcna.....Proliferating cell nuclear antigen

PG .....Proteoglycans

PGE_2.....Prostaglandin E_2

Pre-DM .....Prediabetes mellitus

Ptger2 .....Prostaglandin E2 receptor EP2 subtype

Ptges.....Prostaglandin E synthase

Ptgs2.....Prostaglandin G/H synthase 2

RAGE.....Receptor for advanced glycation end products

RNAseq.....RNA sequencing

ROS.....Reactive oxygen species

Scx.....Scleraxis

Sdha.....Succinate dehydrogenase complex flavoprotein subunit A

STZ .....Streptozotocin

T2-DM .....Type II diabetes mellitus

TGF-β.....Transforming growth factor beta

Tgm3 .....Transglutaminase

TIMP .....Tissue inhibitor of matrix metalloproteinase

TMRE .....Tetramethylrhodamine, ethyl ester

Tnmd.....Tenomodulin

VEGF .....Vascular endothelial growth factor

ΔpH .....Proton gradient

ΔΨm.....Mitochondrial membrane potential

ABSTRACT

Introduction: Diabetes mellitus is a major risk factor for tendon pain, injury, and pathology. Surprisingly, tendon problems persist in diabetic patients with superior blood glucose control (HbA1c<6.5), suggesting that alternative mechanisms contribute to this problem. Advanced glycation end products (AGEs) have been implicated in several diabetes-related complications, but their role in diabetic tendon pathology has not fully been explored. To expand our understanding of AGE-mediated tendon pathology, the following five studies were completed.

Study 1: Streptozotocin-induced diabetes alters transcription of multiple genes necessary for extracellular matrix remodeling in rat patellar tendon. RNA was isolated from the patellar tendon of non-diabetic (control, n=9), 1-week diabetic (acute, n=8), 10-weeks diabetic (chronic, n=7), and insulin treated 10-weeks diabetic (insulin, n=8) rats. Determination of mRNA transcripts was completed using droplet digital PCR (ddPCR). Our findings indicated that STZ-induced diabetes results in rapid and large changes in the expression of several genes that are key to extracellular matrix (ECM) remodeling, maintenance, and maturation.

Study 2: Advanced glycation end products suppress mitochondrial function and proliferative capacity of Achilles tendon-derived fibroblasts. Using an *in vitro* cell culture system, rat Achilles tendon fibroblasts were treated with glycolaldehyde-derived AGEs (0, 50, 100, and 200µg/ml) for 48 hours in normal glucose (5.5mM) and high glucose (25mM) conditions. Our findings demonstrate that tendon fibroblasts treated with AGEs display reduced ATP production, electron transport efficiency, and proliferative capacity. These impairments were coupled with alterations in mitochondrial DNA content and expression of genes associated with ECM remodeling, mitochondrial energy metabolism, and apoptosis.

Study 3: Descriptive transcriptome analysis of tendon derived fibroblasts following *in vitro* exposure to advanced glycation end products. Rat Achilles tendon fibroblasts were treated with glycolaldehyde-derived AGEs (200µg/ml) for 48 hours in normal glucose (5.5mM) conditions. Total RNA was isolated and the PolyA$^+$ library was sequenced. We demonstrate that tendon fibroblasts treated with 200µg/ml of AGEs differentially express 2,159 gene targets compared to fibroblasts treated with an equal amount of bovine serum albumin (BSA)-Control.

Our findings suggest that AGEs disrupt the tendon fibroblast transcriptome on a large scale and that these pathways may contribute to the development and progression of diabetic tendinopathy.

Study 4: Evaluation of tendon healing in a mouse model of elevated serum advanced glycation end products following tendon injury. Mice received daily BSA-Control or AGE-BSA injections (200µg/ml) for two weeks prior to creation of a tendon injury in the central third of both patellar tendons. Animals assigned to an exercise group began a moderate treadmill protocol (13 meters/min, five days/week, five weeks) one week following injury and all animals continued to receive injections until termination. We demonstrated that based on our injection dose and schedule, that serum AGEs are significantly elevated to ~200µg/ml, levels that are typically seen in type II diabetic patients. Additionally, a main effect for AGEs was observed in genes related to cell proliferation (Mybl2), mitochondrial function (Bcs1l), and growth factors (Fgf2). However, moderate treadmill exercise did not alter gene markers, such as Ctgf and Fgf2, which are makers of a tendon healing response. Our findings suggest that AGEs modulate tendon gene expression following patellar tendon injury, with no effect of moderate treadmill exercise.

Study 5: Serum levels of advanced glycation end products and their relationship to patellar tendon properties in diabetes. Subjects (n=32) from a full spectrum of diabetes status, including no history of diabetes were recruited for a cross-sectional study. A fasted blood sample was collected and magnetic resonance imaging (MRI) of the knee was completed. Both current HbA1c and previous diagnosis was used to stratify collected data. Additionally, a full correlation matrix of all measured variables was created to establish relationships that could be used to predict tendon pathology in diabetes. Our findings demonstrate that diabetes is associated with smaller patellar tendon dimensions, which is in disagreement with the literature. Further, we show that changes to body weight normalized (BWN) tendon cross-sectional area (CSA) occur independent of circulating N^{ε}-(carboxymethyl)lysine (CML) levels. These new data suggest that alternative mechanisms contributing to tendon pathology in diabetes deserve attention.

CHAPTER 1. REVIEW OF THE LITERATURE

1.1 Introduction

Diabetes mellitus is a major risk factor for tendon pathology. Tendons, which connect muscle to bone, are critical for optimal musculoskeletal function. The prevalence rates of tendon abnormalities and degeneration in diabetics have been reported as high as 90%, and many of these individuals are asymptomatic, despite the presence of structural tendon abnormalities (1-6). The functional integrity of tendon is largely dependent on the high collagen content and linear arrangement of collagen at the fibril level (7, 8). Both of these properties appear to be altered in diabetic tendinopathy (2-6). Diabetic tendinopathies are pervasive and disabling, but little is known about their pathogenesis, which has hampered the development of effective therapeutic interventions. While evidence describing changes to tendon with diabetes is documented, our understanding of this process is severely limited. In order to provide effective therapeutic options to reduce tendon degeneration and associate sequela in diabetic patients, it is important that we expand our understanding of the mechanisms contributing to tendinopathies in this large and growing patient population.

1.2 Tendon Structure and Function

Tendons are hierarchically organized, anisotropic, collagen-rich tissues with high tensile properties (9, 10). The primary function of tendon is to transfer large contractile forces generated by skeletal muscle to bone. Optimal structure and composition of tendon tissue dictates mechanical properties, which is directly correlated to musculoskeletal function (11). As a result, alterations to structure and composition of both collagenous and non-collagenous proteins in tendon can modify the complex viscoelastic properties (12-14). Uniquely, healthy energy-storing tendons, such as the Achilles and patellar, are able to resist deformation while maintaining elasticity, permitting the tendon to function like a spring under high load (15).

Although tendon was once thought to be an inert tissue, a large body of evidence indicating the ability of tendon to adapt and respond to mechanical stresses now exists (16-18). In response to mechanical stress, such as exercise, the adaptive process of tendon seems to be similar to that of skeletal muscle connective tissue (13, 14). Specifically, the extracellular matrix

(ECM) of tendon and skeletal muscle connective tissue appear to have coordinated collagen synthesis response following exercise (17). The ECM is vital to tissue health and serves many functions including cell adhesion, communication, and differentiation (19). In addition to this, the ECM provides structural and biochemical support to the surrounding cells (12). Tenocytes are specialized fibroblasts responsible primarily for production of collagen molecules in tendon ECM, and are analogous to fibroblast cells in the connective tissue of skeletal muscle (12, 20). The ECM of tendon tissue consists primarily of type I (Col1a1) and type III (Col3a1) collagen fibers surrounded by proteoglycans which assist with the assembly of collagen fibrils (21). Precise and linear arrangement of collagen fibrils is vital to tissue integrity, and therefore mechanical function (22).

Inclusion of multiple collagen isoforms allows the ECM to specialize and adapt to specific types of mechanical loading and functional responses (23). For instance, Col1a1 is a stronger form of collagen, whereas Col3a1 is weaker and generally upregulated in the early stages of tissue remodeling following exercise or during initial stages of healing (24, 25). Col3a1 is able to provide temporary tensile strength to the tissue assembly until it is later replaced by stronger type I collagen (26). Glycine-proline-X and glycine-X-hydroxyproline are two common amino acid sequences of collagen, where X can be any amino acid other than glycine, proline, or hydroxyproline. Lysine and hydroxylysine are also found within the collagen amino acid sequence and are responsible for cross-link formation. Oxidation of lysine and hydroxylysine, by lysyl oxidase, forms cross-links within collagen fibrils, which contributes to tissue integrity by increasing tensile strength and stabilizing the collagen fibril assembly (27-29). Strength and stability of the tissue assembly are important, especially given the high contractile forces tendons are responsible for transmitting from muscle to bone (30).

Much of the tendon literature has relied on measures related to collagen synthesis, content, and cross-link formation as an indication of tendon health. Collagen comprises ~60-85% of tendon dry weight and is largely responsible for tendon's ability to perform its physiological function (12, 18). However, the ECM and tendon fibroblasts are sensitive to external cues and signaling mechanisms likely influence tendon collagen to transition to a pathological state.

1.3 Tendon Pathophysiology

1.3.1 Overview

Tendon disorders, including tendinopathy and tendon rupture, occur with high incidence in young and older adults, significantly impacting the quality of life. Despite several decades of active tendon research, tendon pathophysiology and restoration remains a contemporary topic and represents one of the greatest challenges in musculoskeletal biology. In pursuit of strategies directed towards prevention, repair, and healing of tendon, a thorough understanding of the intrinsic and extrinsic factors contributing to tendinopathy are imperative. Specifically, understanding the influence of extrinsic factors, such as other chronic diseases (e.g. diabetes), dietary choices, body weight, and exercise frequency can help direct the development of successful treatments and preventative strategies.

Tendinopathy is poorly understood, but has been broadly characterized by three elements: pain, swelling, and functional impairment (31). Chronic tendinopathies are generally thought to be 'triggered' by an inflammatory cascade and then gradually propagate as degenerative conditions in the absence of inflammation (32-36). However, work from several groups has suggested that inflammation may not only play a prominent role in the initiation of tendinopathy, but also the progression of the diseased state (37). Despite competing theories regarding the mechanisms of tendon pathophysiology, the idea that degeneration and inflammation are not mutually exclusive, but instead co-contribute to the development and progression of the disease state has been favored (32, 37, 38).

1.3.2 Etiology

The pathological process of tendinopathy is not well understood, but several risk factors have been identified and can be delineated by observing the contributions of mechanical stress (low grade mechanical microtrauma) and metabolic diseases (e.g. diabetes, obesity, hyperlipidemia) (39). Other factors, such as the transition to menopause increases the risk of developing a tendinopathy (40). The normal aging process is also known to increase the risk for tendinopathy (39, 41). It is apparent that pathogenesis is both multifactorial and heterogeneous and therefore many conflicting reports exist in the literature. It has proven extremely challenging to study tendinopathy in the early phases of disease because tendinopathy occurs with gradual

onset and the ability to collect tissue specimens at this time point in the disease process is rare. Tendinopathy patients may also be asymptomatic in the early stages of disease. Classic tendinopathy is generally a result of overuse (repetitive microtrauma), but can also arise from genetic predisposition or inherited disorders. However, distinct characteristics of diabetic tendinopathy further complicate our understanding of the disease process.

1.3.3 Histopathological Changes

At a macroscopic level, the diseased tendon loses its white appearance and becomes amorphous (42). Tissue thickness is greater in tendinopathy, partially due to swelling but calcification of tendon has also been observed at the insertion and attachment points during later stages of the disease (42, 43). Further, the infiltration of many large blood vessels and nerves (glutamate) can be observed and contribute to pain. While blood flow to the tendon can increase up to ~7 fold in the Achilles tendon during exercise, an increase in blood flow at resting conditions generally implies presence of tendinopathy (44-46). Neovessel infiltration is considered a healthy healing response, but it is unclear why tendinopathy persists even after vessel infiltration (46).

Microscopically, collagen fibers become deranged, accumulate, and appear much smaller in diameter (47). In normal tendon, collagen fibrils are tightly packed in para-linear arrangement (48). However, in the diseased state fibrils become disorganized and contain twists, curves, and greater inter-fibrillar spacing (48). Enzymatic cross-linking between fibrils, which help to stabilize the assembly, can also be reduced (49). Given that tendon is a predominately extracellular matrix tissue, structural abnormalities dramatically interrupt optimal mechanical function (viscoelastic properties and ultimate stress) and increase the risk for complete and partial tendon rupture (42).

1.3.4 Cellular and Molecular Responses

A population of specialized fibroblasts, known as tenocytes, reside within and adjacent to collagen fibrils. Tenocytes can respond to both mechanical (stretch) and chemical stimuli to regulate collagen production and maintenance of the ECM (12, 20). Tendon, being predominately an extracellular matrix tissue, displays a very low cellular density and this small

population of cells is responsible for maintenance of non-cellular ECM components in tendon (50). As result, during pathology, tenocyte transcriptional, translational, and homeostatic (e.g. cell proliferation, energy production) responses can become altered (51). Further, an increase in cellular density is generally observed in pathological and injured tendon (7, 42). While it may be assumed that these changes are a compensatory response in effort to aid with reestablishment of tissue homeostasis, it is not fully understood whether these changes are always regenerative or further contribute to pathology, especially given the long observed duration of healing (52).

A hallmark of 'classic' tendinopathy is the infiltration of large blood vessels (44-46). In the healthy state, VEGF is inhibited due to interaction with endostatin, a collagen fragment with anti-angiogenic properties (53). In the diseased state, HIF-1 expression is increased and binds to the VEGF gene to increase VEGF expression and subsequently initiate neovessel formation (54). For proper vessel infiltration, some degradation of the ECM is required. In order to accomplish this, VEGF is able to partially regulate expression of ECM degenerative matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) (55). Specifically, VEGF has been shown to upregulate expression of MMPs and down-regulate expression of their reversible inhibitors, TIMP (55). It is possible that neovessel infiltration can further weaken the tendon assembly, in the early stages at least, and increase the risk for tendon rupture. Due to the partial regulation of MMPs and TIMPs by VEGF, and subsequent weakening of the tendon proper as result of invasion of blood vessels, the angiogenic response in tendon pathology remains a controversial topic, and has propelled the angiogenic tissue degeneration hypothesis proposed by Pufe *et al.* (56). This hypothesis may explain why tendon pathology persists even in cases of neovessel infiltration.

Aside from VEGF-mediated MMP regulation, MMP mRNA (-1, -2, -9, and -13) has been shown to be upregulated while TIMP mRNA is downregulated in the diseased state (57). The ECM environment is tightly controlled by regulation of these remodeling enzymes, however, this pattern suggests that an environment favoring ECM degradation could persist (27). ADAMTS2 and ADAMTS3 mRNA, proteinase enzymes necessary for collagen arrangement, are also increased in tendinopathy and may contribute to further degeneration of collagen fibrils (47). MMP and ADAM associated collagen degeneration is likely a contributor to tendinopathy, but the mechanisms by which these enzymes are disrupted remain minimally understood.

In a state of pathology, tenocytes increase mRNA expression of both Col1a1 and Col3a1 isoforms (58). Col1a1 is a mature collagen isoform, while Col3a1 is a weaker isoform generally upregulated in the early stages of healing (24, 25, 59). It is possible that the increase in mRNA levels of Col1a1 and Col3a1 serve as compensatory responses to excessive collagen degradation commonly noted in tendinopathy (38). However, collagen protein levels do not always mirror mRNA responses (47). While this is not well understood, translational expression of collagen is thought to be regulated by IL-33 and miRNA-29a to favor Col3a1 protein production (60). Similarly, increased IL-33 expression appears to occur concomitantly with early tendinopathy (60). Generally, tendinopathy is associated with a reduction in total collagen protein content, and an increased ratio of denatured collagen, suggesting issues with proteolytic driven clearance of damaged tissue (47, 61, 62). This dysregulation of collagen isoforms favors the histological data that also suggest that the ECM environment is weak and susceptible to rupture (47, 63, 64).

Proteoglycans (PGs), a group of glycoproteins that can serve structural, regulatory, and signaling roles in tendon, are known to become disrupted in tendinopathy. Decorin, a regulator of collagen fibrillogenesis and major contributor to the viscoelastic properties of tendon, does not change with tendinopathy (65). However, other proteoglycans such as aggrecan are expressed in greater amounts, while versican expression is reduced (66). Further, PGs are connected to glycosaminoglycan (GAG) side chains that are highly polar and can regulate the water content of the tendon. Overall, GAG content is increased in tendinopathy, and this may contribute to the swelling of the tendon that is observed in tendinopathy (67, 68). Overall, a disruption to collagen glycoproteins and their side chains have been observed with tendinopathy, but whether these changes are responsible for the altered tissue mechanics and viscoelastic properties, or if they serve a compensatory purpose, is not known. Given the multitude of functions and roles of PGs in connective tissue, further work in this area is needed.

1.3.5 Inflammation versus Degeneration

Tendinopathy of the energy-storing tendons has long been characterized as a degenerative disease, with inflammation only playing a role in the early stages of the disease process. However, in recent years chronic inflammation has been shown to contribute to the later stages of the disease process. Specifically, chronic inflammation in tendinopathy is mediated via the NF-κB and Stat6 inflammatory activation pathways (33). Further, *in vitro* work has

demonstrated that tenocytes isolated from diseased tendon tissue are primed for inflammation (32, 33). Tendon fibroblasts isolated from diseased tissues appear to have 'pro-inflammatory' memory that makes them more susceptible to cytokine mediated stimulation (32, 33). It is important to remember that even acute inflammation is not devoid of degeneration, and that degeneration during the later stages of tendinopathy may be driven by several sources (e.g. circulating cytokines and factors, oxidative stress, tenocyte apoptosis). This additional work has demonstrated that energy-storing tendons, such as the Achilles and patellar, have a similar pathological process to rotator cuff tendons, which have long been known to persist with chronic inflammation (32, 33, 38). Moving forward, therapeutic strategies may consider management of inflammation and prevention of acute pro-inflammatory events, to prevent diseased tenocytes from entering enter a noxious inflammatory-degenerative cycle accompanied with macrophage and T-cell infiltration (33). Data implicating chronic inflammation highlight the difficulties in tendon research. There may be several reasons why chronic inflammation signatures have been missed in previous studies of chronic tendinopathy, but the primary reasons point to the heterogeneous nature of the disease process and timing of tissue collection.

1.4 Diabetic Tendon Pathology

Tendinopathies affect the majority of diabetic patients, are chronic, and respond poorly to therapy (2, 69-77). Patients with type II diabetes (T2-DM) are nearly four times more likely to live with a tendinopathy and tendon fibril disorganization is present in as many as 89% of diabetic patients (78). Further, 42% of patients with Achilles tendinopathy are diabetic compared to only 13% of individuals with no Achilles tendon pain (72). The duration of diabetes also increases the risk of developing tendinopathies (78). Additionally, half of T2-DM patients who ceased participating in a chronic exercise intervention did so because of musculoskeletal discomfort, which included many cases of Achilles tendinopathy (79).

Syndromes, including shoulder tendinopathy, diabetic cheiropathy, calcification, Achilles tendon shortening, rupture, and general tendon pain are responsible for considerable reduction in the quality of life for many diabetic patients (2, 71, 77). Tendon abnormalities contribute to reduced joint mobility, ulcer formation in the foot, Charcot arthropathy, and a loss of muscle function (80-83). Treatment of diabetic tendinopathies is costly, often requiring surgical intervention and lengthy physical therapy (83, 84). However, the risk of complications associated

with surgical repair of the Achilles tendon is higher in diabetic patients (85). Tendon healing is impaired in diabetic animals, as is angiogenesis and the inflammatory response following tendon injury, deficits that likely contribute to the greater rate of post-surgical complications in patients with T2-DM (85-92). Tendinopathies due to T2-DM may also worsen other common degenerative musculoskeletal diseases (93). Diabetic tendinopathies persist similarly to chronic tendinopathy, with the exception of two major differences, they do not generally present with neovessel infiltration and have increased, rather than decreased, total collagen content (94, 95). The disease process is more difficult to treat as the severity of degeneration is typically more advanced in diabetic patients.

The development of diabetic tendinopathies has largely been attributed to the accumulation of advance glycation end-products (AGEs). It is most often believed that diabetic tendinopathy is due to AGEs forming non-enzymatic cross-links in long-lived ECM proteins, such as collagen in tendon, which can increase tissue stiffness and impair optimal mechanical properties (49). However, serum levels of AGEs are also increased in diabetic patients from either dietary sources (exogenous), endogenously glycated proteins due to elevated blood glucose levels, or excess glycolytic flux and these AGEs may act via alternative mechanisms to induce pathology (96).

1.5 Advanced Glycation

1.5.1 A Molecular and Chemical Basis of Advanced Glycation

AGEs are formed in a non-enzymatic process in which free amine terminals on proteins are modified by reactive glucose or other carbonyl containing molecules, ultimately resulting in a covalent and irreversible modification (97, 98). Generally, the amine terminal of lysine and arginine residues, which are abundantly expressed in most protein motifs, are common targets of protein glycation. The side chains for both lysine and arginine are long (4 carbon backbone) and end with an easily accessible $-NH_2$ group, rendering them accessible for glycation (99). Glycation products can be proteolytically cleaved from a protein and enter the free circulation. In addition to endogenous AGE sources, exogenous AGE sources such as highly processed food items and meat products cooked with high heat can also raise serum AGE levels (100). Several negative implications have been documented as a result of protein glycation. For example,

proteins become resistant to proteolytic cleavage and clearance, and interaction to other proteins and molecules via their native binding sites can become impaired. Once in the circulation, AGE products can also interact with the cell surface receptor for advanced glycation end products (RAGE) to initiate a noxious cycle of inflammation. RAGE activation can result in several diabetes-related complications such as cardiomyopathy, retinopathy, nephropathy, and endothelial dysfunction (101, 102).

AGE formation can categorically be separated into endogenous and exogenous sources. Exogenous sources, commonly termed dietary AGEs, are the result of consuming high-heat processed foods (100). The glycation process occurs at a much higher rate during cooking, and carbohydrates can rapidly glycate amino terminals via the Maillard pathway, commonly referred to as the browning reaction (100). Dietary AGEs and amino acids or peptides containing glycated residues can enter the circulation after consumption where they are able to interact with RAGE to initiate the pathway or accumulate in long-lived proteins, such as extracellular collagen.

Endogenous AGE can further be characterized by extracellular or intracellular origin. Extracellular AGEs are most commonly formed due to elevated blood glucose levels, something that is commonly observed in T2-DM. Reactive sugars can interact with amine terminals and form a Schiff Base via a condensation reaction (101). After further rearrangement, the sugar molecule can be linearized to form an Amadori product containing a carbonyl group. This can occur over several days to weeks. While Schiff Bases and Amadori products are both reversible, further rearrangement of the Amadori products, by oxidation for example, can form an irreversible AGE, which occurs over weeks to months (99, 101). These AGEs can be formed as bound AGE adducts or AGE cross-links which span two free amine terminals (99). Protein bound AGE adducts are available to be proteolytically cleaved and enter the circulation where they can bind to RAGE.

Intracellular formation of AGEs can also contribute to protein bound or circulating free adducts. The primary mechanism by which intracellular AGEs are formed is due to the accumulation of reactive intermediate oxoaldehydes such as methylglyoxal (MG), glyoxal, and 3-deoxyglucosone (3DG) (96, 103, 104). MG for example, is formed during glycolysis when dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P) accumulate. Likely due to deficiency in the triosephosphate isomerase, accumulated DHAP and GA3P spontaneously form MG (96, 104). MG is then typically cleared by the glyoxylase enzyme

detoxification system (Glo1/2) in the form of D-lactate (105). However, in cases of extreme glycolytic flux, a state of dicarbonyl stress can be initiated when reactive intermediates accumulate faster than the clearance rate of the Glo1/2 detoxification system. When MG accumulates, it is able to bind with arginine groups to directly form methylglyoxal-derived hydroimidazolone (MG-H1), an AGE product. MG-H1 can be then be proteolytically cleaved and enter the circulating pool. Similarly, N^{ε}-(carboxymethyl)lysine (CML) can be formed when glyoxal accumulation occurs. Specifically, in the case of diabetes, T2-DM patients have impairments to systems that are beneficial for prevention of AGE formation (105). For example the Glo1/2 detoxification system is impaired due to reduced Glo1 protein in T2-DM (105). As result, MG rapidly accumulates and spontaneous formation of MG-H1 results. Nuclear factor erythroid 2-related factor 2 (NRF2) promotes Glo1 protein expression, while kelch-like ECH-associated protein 1 (Keap1) inhibits NRF2 from serving its role as a transcription factor. Not only is T2-DM associated with reductions to Glo1, but T2-DM patients also have reduced NRF2 expression and enhanced Keap1 expression indicating that metabolic syndrome itself is responsible for endogenous AGE production (105-107).

Serum AGE levels in diabetic and obese individuals are greatly elevated and AGE-RAGE signaling has been implicated in many of the diabetes-related complications. For example, CML a common circulating AGE, can be 2-4 times higher (3-5μM) in a diabetic versus healthy patient (108, 109). CML can form in the ECM from fructosyl-lysine via the Maillard reaction, but also from intracellular glyoxal mediated glycation. Pentosidine, an AGE that forms cross-links between adjacent collagen fibrils is also 2-4 times more expressed in diabetic and aged tissue (109). Pentosidine accumulates in the patellar tendon with age, however, life-long exercisers have reduced levels of pentosidine compared to age-matched controls which can help to mitigate altered mechanical properties resulting from AGE cross-linking (110).

RAGE is a multiligand receptor with an extracellular domain and a cytosolic tail (111). RAGE can recognize circulating AGEs and initiate a noxious feed forward cycle of the downstream pathway (112). Once bound by AGE, RAGE, via the MyD88 adapter protein, can initiate ERK and JNK MAPK signaling, which in turn will phosphorylate Iκb and cause the release of NF-κB (113). NF-κB translocates to the nucleus of the cell and initiates transcription of its canonical pathway. Importantly, a binding site for NF-κB exists on the promoter of RAGE. Therefore, RAGE is a self-promoting receptor, and RAGE activation can increase the expression

of RAGE on the cell membrane (114). RAGE activation not only results in NF-κB mediated inflammation, but is also able to dysregulate energy producing systems, increase ROS production, and increase overall oxidative stress in the cell which can contribute to impaired cellular function and result in a shift from homeostasis (115).

1.5.2 Role of AGEs in Diabetes-Related Tendon Pathology

In diabetes, AGE cross-link formation due to chronic hyperglycemia has been shown to increase tissue stiffness (5, 116-122). Surprisingly, reducing blood glucose (HbA1c<6.5) does not reduce tendon stiffness to levels of euglycemic controls (5, 118). AGEs form at an accelerated rate in diabetes and these modifications can result in non-enzymatic cross-links in long-lived extracellular proteins such as tendon collagen, which increases tissue stiffness and alters tissue mechanical properties (101, 119, 120, 123, 124). Specifically, an AGE spanning two free amine terminals can result in a non-enzymatic cross-link. In adjacent collagen fibrils, free anime terminals on lysine and arginine are susceptible to AGE cross-link formation, which can increase stiffness properties of the tendon assembly. While tendon stiffness is positively correlated to function, very high tissue stiffness can result in loss of viscoelastic properties (11, 15, 30). A careful balance between tissue stiffness and viscoelasticity is imperative for optimal tendon function.

Additionally, dietary AGEs have been shown to accumulate in tendon of mice (125). AGE binding to free amine groups prevents the native protein from interacting with other proteins, enzymes, and molecules. For example, glycated collagen is more resistant to enzymatic degradation by MMPs due to inaccessibility of appropriate binding sites on the protein (98). Inability of MMPs to enzymatically degrade glycated collagen may explain the accumulation of collagen commonly observed in diabetic tendons (126). It is believed that this impaired sensitivity to collagenases due to AGE decoration results in collagen content accumulation, a hallmark of diabetic tendinopathy (47). This accumulating collagen is likely denatured or damaged, but MMP mediated collagen disposal is limited as result of AGE decoration (127-129). It is possible that MMP expression is enhanced to compete for site-specific binding via a compensatory feedback mechanism, but AGEs can also increase MMP expression (130). Enhanced MMP expression is noted in classic tendinopathy, but AGE-induced MMP upregulation may further enhance MMP expression beyond the ‘classic’ tendinopathy levels.

Although MMP binding sites appear blocked, overexpression of MMPs can result in excessive degradation if binding sites were to be exposed. It has been hypothesized that loading mediated micro-unfolding of the fibril assembly exposes new sites and because the tendon does not refold to the exact configuration that was observed prior to loading MMPs could potentially bind to these new unexposed sites (131). Exposed MMP sites following loading, in conjunction with over expression of MMPs can result in increased susceptibility to degradation. It is possible that that collagen degeneration noted in diabetic tendinopathies may be mediated via this mechanism, however it is not known why clearance of denatured collagen does not occur concomitantly. Definitive work in this area has not been completed, but several non-tendon studies of diabetic wound healing have shown that MMP inhibition is a promising approach (132).

Circulating AGE adducts are able to interact with the cell surface receptor RAGE to initiate a noxious feed forward cycle of sustained inflammation and tissue damage (133). AGE formation as result of chronic hyperglycemia and spontaneous oxidation of glycolytic intermediates contributes significantly to elevated levels of circulating AGE adducts in diabetic patients which may serve to activate RAGE in tenocytes (134, 135). Specifically, the extent to which circulating AGEs alter tendon properties independent of AGE collagen cross-linking has received little attention in the literature (136). Recent reviews have advocated the potential of serum AGEs to induce tendon dysfunction by impacting cell proliferation, apoptosis, and ECM degradation in addition to AGE cross-linking of collagen (136). In opposition of the AGE cross-link accumulation data, recent studies with humans have revealed that neither tendon AGE content nor stiffness are elevated in tendons of diabetic patients when compared to age-matched non-diabetics (4-6). In fact, some data indicate that tissue stiffness and toughness are lower in Achilles tendon samples from diabetic patients (4). Consistent with these data, disorganization of collagen fibrils and reduced tenocyte proliferation, rather than increased tendon stiffness, are common features of diabetic tendinopathies (2-4).

Findings of reduced stiffness and fibril disorganization with diabetes is supported by several recent studies in various diabetic rodent models, lending the idea that RAGE activation may contribute to tissue degeneration in diabetic tendinopathy (137-140). Specifically, collagen fibrils from Achilles tendon of diabetic individuals are smaller, which would further promote the idea that RAGE activation contributes to tissue degeneration. In fact, RAGE activation increases catabolic activity in cartilage leading to cartilage degeneration and acceleration of osteoarthritis

(141). The consequences of RAGE activation in diabetic tendon pathology remain to be fully explored.

1.6 Overarching Study Goals

It was hypothesized that circulating AGEs, independent of hyperglycemia, induce limitations to tendon homeostatic and healing properties. To test our major hypothesis, the following five studies were performed:

1. Role of chronic uncontrolled and controlled hyperglycemia on patellar tendon ECM gene expression in a T1DM rat model.
2. Role of AGEs on cell proliferation, energy production, and oxidative stress in an Achilles tendon cell culture model.
3. Transcriptome analysis of tendon fibroblasts following exposure to AGEs.
4. Role of circulating AGEs on tendon healing properties in a mouse patellar tendon injury model.
5. Role of circulating AGEs on patellar tendon dimensions and signal intensity (pathology) in subjects with no history of DM, Pre-DM, and T2-DM.

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CHAPTER 2. STREPTOZOTOCIN-INDUCED DIABETES ALTERS TRANSCRIPTION OF MULTIPLE GENES NECESSARY FOR EXTRACELLULAR MATRIX REMODELING IN RAT PATELLAR TENDON

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2.1 Abstract

Introduction: Tendon collagen fibril disorganization and degradation is commonly seen in tendons of diabetics, but the mechanisms responsible for these changes remain to be elucidated. We have demonstrated that streptozotocin (STZ)-induced diabetes increases tendon cell proliferation and collagen content. In the present study, we evaluated that impact of STZ-induced diabetes on mRNA transcripts involved with collagen fibril organization, extracellular matrix (ECM) remodeling, apoptosis, and proliferation.

Methods: Rats were divided into four groups: non-diabetic (control, n=9), 1-week of diabetes (acute, n=8), 10-weeks of diabetes (chronic, n=7), and 10-weeks of diabetes with insulin (insulin, n=8). RNA was isolated from the patellar tendon for determination of mRNA transcripts using droplet digital PCR (ddPCR).

Results: Transcript counts for Col1a1, Col3a1, Mmp2, Timp1, Scx, Tnmd, Casp3, Casp8, and Ager were lower in acute relative to control and insulin rats ($p \leq 0.05$). With the exception of Scx, transcript counts for Col1a1, Col3a1, Mmp2, Timp1, Tnmd, Casp3, Casp8, and Ager were also lower in chronic when compared to control ($p < 0.05$). Transcript counts for Col1a1, Col3a1, Mmp2, Timp1, Tnmd, Casp3, Casp8, but not Ager, where not different between control and insulin ($p > 0.05$). Transcript counts for Dcn, Mmp1a, Mmp9, Pcna, Tgfbr3, Ptgs2, Ptger2, Ptges, and iNos were not altered by diabetes or insulin ($p > 0.05$).

Conclusion: To the best of our knowledge, this is the first investigation utilizing ddPCR technology to quantify RNA transcripts in tendon. Our findings indicated that STZ-induced diabetes results in rapid and large changes in the expression of several genes that are key to ECM remodeling, maintenance, and maturation.

2.2 Introduction

Debilitating tendon pathologies, collectively referred to as tendinopathies, affect the majority of patients with type 1 and 2 diabetes (1, 2), significantly limiting mobility and reducing the quality of life for this patient population (3, 4). Diabetes is associated with an increase in

connective tissue stiffness (5), tendon degeneration and fibril disorganization (1, 2, 6-10), and increased tendon thickness (1, 11-13). Although the increase in tendon stiffness with diabetes has been linked to advanced glycation end-product (AGE) cross-linking of collagen fibrils (14-18), factors contributing to tendon degeneration and collagen fibril derangement with diabetes have remained largely unexplored. In rats, we have demonstrated that induction of diabetes with streptozotocin (STZ) results in a rapid rise in tendon collagen content along with a significant rise in cell proliferation and cellular density (19), work that is supported by others (13). The rapid rise in tendon collagen content and changes in cell proliferation suggest that hyperglycemia may also cause changes to the transcription of genes which encode proteins involved with regulation of tenocytes and extracellular matrix (ECM) remodeling. In fact, Lin *et al.* (20) have demonstrated that treatment of tendon-derived stem cells with glucose results in a dose-dependent decrease in the expression of scleraxis (Scx), collagen 1 (Col1a1), and tenomodulin (Tnmd). In contrast, when compared to non-diabetic controls, Tnmd expression was elevated in the rotator cuff tendon of diabetic patients (21). Although in conflict, these limited findings suggest that changes to collagen, the primary structural component of tendon, and Scx and Tnmd, which regulate tendon development and stem cell fate, may contribute to the development of the diabetic tendon phenotype.

Tendon ECM remodeling is also regulated by a series of enzymes known as matrix metalloproteinases (Mmps) and their inhibitors, tissue inhibitors of matrix metalloproteinases (Timps). An imbalance between Mmps and Timps could lead to excessive degradation of tendon ECM. Chung *et al.* (21) have recently demonstrated that Mmp9 expression is greater in tendons isolated from diabetic humans when compared to controls, suggesting an increased state of ECM degradation in diabetic tendons. Similar results for Mmp9 expression were noted by Tsai *et al.* (22) when tenocytes were incubated with glucose. Collectively, this small set of studies suggests that a state of enhanced degradation may exist in tendons exposed to elevated concentrations of glucose. However, more comprehensive *in vivo* studies are lacking.

Interestingly, recent reports suggest that controlling blood glucose ($HbA_1c<7$) does not reduce tendon stiffness to levels of euglycemic controls (5, 14), nor does it correlate with the extent of tendon abnormalities in diabetic patients (2). Simply controlling blood glucose levels alone does not appear effective in preventing changes to tendon mechanical properties. The lack of improvement in tendon properties with glucose control suggests that the diabetic tendon

phenotype may be driven by additional mechanisms. Specifically, serum AGEs are also elevated in diabetic individuals (5). In chondrocytes, Huang *et al.* (23) and Nah *et al.* (24) have demonstrated that AGEs activate cyclooxygenase-2 (COX-2) causing a dose-dependent rise in PGE_2. While the production of PGE_2 in response to AGE treatment has not been studied in tendons, exposure of tendon tissue and tendon fibroblasts to PGE_2 reduces fibril diameter and disrupts collagen fibril architecture (25, 26).

The purpose of this study was to use a STZ rat model to investigate the transcriptional changes that occur to steady state levels of several encoding transcripts involved with the regulation and maintenance of tendon ECM after short (1-week) and long-term (10-weeks) type 1 diabetes. A separate group of type 1 diabetic rats was treated with insulin daily to assess the potential corrective effects of insulin therapy on patellar tendon gene expression. Further, we explored the impact to mRNA transcripts which encode proteins involved in the COX pathway to better understand the role of COX-2 and PGE_2 in diabetes related tendon degeneration. We hypothesized that STZ-induced diabetes would alter the expression of genes that regulate ECM remodeling and apoptosis, as well as relevant aspects of the COX pathway as they pertain to PGE_2. Specifically, we hypothesized that STZ-induced diabetes would upregulate the gene expression associated with ECM degradation and the COX-2 pathway. Additionally, to the best of our knowledge, we are the first to utilize digital droplet PCR (ddPCR) to quantify transcript counts in tendon tissue.

2.3 Materials and Methods

2.3.1 Study Protocol

A detailed description of the animal care and treatment has been published previously (19). Male 8-week-old Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Rats (8-week-old) were housed in pairs, allowed *ad libitum* access to standard lab chow and water, and maintained on a 12:12-hour light-dark cycle. After 1 week of acclimation, rats were randomly divided into one of the following four groups: nondiabetic (control, n=9), one-week of diabetes (acute, n=8), 10 weeks of diabetes (chronic, n=7), and 10-weeks of diabetes with insulin therapy (insulin, n=8). These rats were a sub-set of animals from our previous work (19). Tissue was not available from all of the rats reported in our previous

publication (19). Intravenous injection of STZ (60 mg/kg in citrate buffer, pH 4.5) via the penile vein under isoflourane anesthesia after 1 week of acclimation (19) was used as a model of type 1 diabetes. This dose of STZ is well established and induces necrosis of the pancreatic β-cells, which results in low insulin levels and hyperglycemia (27). The one-week (acute) group was injected with STZ at the beginning of week 9 of the experiment, such that all animals were euthanized at 19 weeks of age. Control animals were given a vehicle injection of citrate buffer. The insulin group received 0.6–0.8 U/100 g (28) Humulin-N (Moore Medical, Farmington, CT) subcutaneously twice daily to assess the potential corrective effects of insulin therapy on patellar tendon gene expression. Insulin dosing was adjusted weekly based on animal body weight. Hyperglycemia was confirmed by testing urine glucose (Diastix®, Bayer) throughout, and by plasma glucose measures at the time of termination (19). After 10 weeks, rats were euthanized by decapitation after CO_2 inhalation. Patellar tendons were carefully dissected from the animal, removed of any excess fascia and muscle, flash frozen in liquid nitrogen, and transferred to -80°C. This study was approved by the Institutional Animal Care and Use Committee of Midwestern University.

2.3.2 Tendon Gene Expression

Total RNA for gene expression analysis was isolated from patellar tendons. First, ~10 to 20 mg of frozen tendon tissue was pulverized to a fine powder under cryogenic conditions (OPS Diagnostics, Lebanon, NJ). The pulverized tissue was transferred to a pre-chilled 2.0 ml reinforced tube containing 2.8 mm ceramic beads (Omni, Kennesaw, GA) and lysis buffer provided with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). A bead mill homogenizer (Omni) was used to disrupt samples. Samples were treated with Proteinase K (Omni) prior to proceeding with RNA isolation per the RNeasy Fibrous Tissue handbook. On-column DNase digestion was completed on all samples prior to elution of RNA (Qiagen). RNA concentration was determined using a NanoDrop 2000 (Thermo Scientific). Quality of RNA was assessed using the 260/280 and 260/230 ratios.

Reverse transcription (iScript, BioRad, Hercules, CA) was completed to produce complementary DNA from 300ng of RNA. Absolute quantification of mRNA targets transcripts was completed using digital droplet PCR (ddPCR; BioRad) according to the manufacturer's protocol using validated ddPCR expression probe-based assays purchased from BioRad. A list of

measured gene targets is provided in Table 1. Utilizing ddPCR offers many advantages over traditional qRT-PCR including increased target sensitivity (sensitive to one transcript), absolute quantification of gene targets without the need of standard curves, and the ability to report data without the need of an endogenous control gene. ddPCR reactions were prepared in duplex, in a final volume of 20µl with 2x ddPCR Supermix for Probes (No dUTP) (BioRad), 20x reference probe FAM, 20x reference probe HEX, cDNA (1ng or 15ng, Table 2), and nuclease free water. One nanoliter-sized droplets were generated from prepared PCR reactions using Droplet Generation Oil for Probes (BioRad) on a QX200 Droplet Generator (BioRad), carefully transferred to a deep-well 96 well plate, and amplified on a C1000 thermal cycler (BioRad) according to the recommendations provided with the validated probe assays. Following thermal cycling, absolute quantification of PCR products was completed on a QX200 Droplet Reader (BioRad) with QuantaSoft Sotware Version 1.7 (BioRad) and reported as positive counts per 20µl reaction. According to the validated probe assays (BioRad), it was suggested that 1ng of cDNA is sufficient for transcript detection via ddPCR. However, we have noted that targets found in lower abundance require a higher content of input cDNA. Therefore, for targets found less abundantly in tendon tissue, we increased the input cDNA to 15ng. Table 2 provides details on target specific cDNA loading information.

2.3.3 Statistical Analysis

Our statistical analyses on contrasts between acute, chronic, insulin, and control groups proceed via regression models. Specifically, Normal linear regression models were fit for those genes that had continuous measurements, and Negative Binomial regression models were fit for those genes that had integer measurements. The independent variables in all of the regression models were indicators for the acute, chronic, and insulin groups, and the control group served as the baseline. All regression models were fitted using the lm and glm.nb functions in the statistical software environment R, and were diagnosed by consideration of standardized and deviance residuals. For those genes that did not satisfy the assumptions for the standard Normal linear regression model, we fit regression models on the square roots of the measurements and also weighted linear regression models on both the original and square root-transformed measurements (29), and assessed which model provided the most appropriate fit to the data in terms of satisfying the assumptions. We then performed tests for the contrasts using the model

parameters' estimates and standard errors, and implemented Bonferroni adjustments for the multiple tests separately across genes. For the case of weighted linear regressions or Negative Binomial regressions, the tests were performed using the parametric bootstrap (30). Our chosen significant level was $\alpha = 0.05$ throughout. Data are presented as mean±standard error.

2.4 Results

Compared to control (134±29 mg/dL) and insulin (82±12 mg/dL) groups, blood glucose values were elevated ($p<0.05$) in both acute (428±39 mg/dL) and chronic (426±31 mg/dL) groups at the time of termination (19). Chronic (242±13 g) diabetic animals were unable to increase body mass over time and had lower body mass than control (431±11 g) and insulin (443±9 g) groups at time of termination ($p<0.05$). The acute group was able to increase body mass similarly to the control and insulin groups until STZ injection at week 9. At time of termination the acute (354±12 g) group had a lower ($p<0.05$) body mass when compared to control and insulin groups.

Induction of type 1 diabetes with STZ resulted in a rapid and sustained decline in Col1a1 and Col3a1 transcript counts relative to non-diabetic controls (Figure 1 and 2, $p<0.05$). In contrast, insulin therapy maintained Col1a1 and Col3a1 transcript counts to similar levels as non-diabetic controls (Figure 1 and 2, $p>0.05$). Although following a similar pattern as Col1a1, neither STZ-induced diabetes nor insulin therapy altered transcript counts of the proteoglycan decorin (Dcn, Figure 2, $p>0.05$). Transcript counts for Mmp1a and Mmp9 were not altered by induction of diabetes or insulin therapy (Figure 3, $p>0.05$). In contrast, Mmp2 and Timp1 transcript counts decreased rapidly after induction of diabetes, and remained reduced after 10-weeks of diabetes (Figure 3, $p<0.05$). As with Col1a1 and Col3a1, insulin therapy maintained Mmp2 and Timp1 transcript counts to similar levels as non-diabetic controls (Figure 3, $p>0.05$).

Neither STZ-induced diabetes nor insulin therapy altered transcript counts for Pcna (Figure 4, $p>0.05$). However, transcript counts for Scx and Tnmd were rapidly suppressed in animals treated with STZ relative to untreated controls (Figure 4, $p<0.05$). The suppression of Tnmd was still apparent in the untreated rats at 10-weeks (Figure 4, $p<0.05$) but the difference in Scx was not significant in untreated rats at 10-weeks. Insulin therapy maintained Scx and Tnmd transcript counts at the level of non-diabetic controls (Figure 4, $p>0.05$). Further, neither diabetes or insulin therapy altered Tgfbr3 counts (Figure 4, $p>0.05$) but counts for Casp3 and Casp8 were

lower in rats treated with STZ (Figure 5, $p<0.05$). In contrast, insulin-treated rats maintained Casp3 and Casp8 counts to levels of the untreated control rats (Figure 5, $p>0.05$).

In contrast to our hypothesis, Ptgs2, Ptger2, Ptges, and iNos gene transcripts were not altered by diabetes or insulin therapy (Figure 6, $p>0.05$). However, Ager transcript counts were lower ($p\leq0.05$) in STZ-treated rats when compared to non-diabetic controls (Figure 6). Insulin therapy prevented any declines in Ager transcript counts (Figure 6, $p>0.05$). The genes Mybl2, Tgm3, Tgm4, and Tgm7 were not detected using ddPCR.

2.5 Discussion

In this investigation, we utilized ddPCR technology to evaluate the acute and chronic impact of uncontrolled STZ-induced type 1 diabetes on the expression of several genes key to tendon ECM remodeling. Furthermore, we determined whether STZ-induced diabetes altered the expressions of several genes associated with the COX pathway. We have previously demonstrated that tendon STZ-induced diabetes yields a rapid increase in collagen content along with an increase in cellular proliferation and cell density (19). In this follow-up investigation, we discovered that Col1a1 and Col3a1 transcript counts were drastically lower (8-fold) in rats treated with STZ, when compared to non-diabetic controls. Although following a similar trend as collagen, STZ treatment did not significantly alter Dcn transcripts. Further, Pcna expression, a marker of cell proliferation, was not altered by STZ-induced diabetes even though we have previously noted an increase in tenocyte proliferation with STZ-diabetes. In general, several genes associated with ECM regulation and maturation were lower in STZ-induced diabetic rats when compared to non-diabetic controls. In contrast to our hypothesis, STZ-induced diabetes did not impact the expression of genes associated with the COX pathway or iNos. However, Ager expression was lower in STZ-treated rats. Insulin therapy successfully prevented the effects of STZ-induced diabetes on tendon gene expression, implying that hyperglycemia was the main driver for suppression of gene expression.

Several studies have reported an increase in tendon collagen content with STZ-induced diabetes (13, 19, 31), suggesting that hyperglycemia stimulates an increase in tendon collagen synthesis by an unknown mechanism. However, the large reduction in collagen transcription noted in the current investigation appears to contradict our previous findings of an increase in tendon collagen content with STZ-induced diabetes (19). It is plausible that the reduction in

collagen transcription observed in the current investigation is a negative feedback response to a post-translational-mediated increase in tendon collagen synthesis. In contrast to our work with STZ-diabetic rats (19), Tsai *et al.* (22) reported that incubation of tendon cells with glucose concentrations similar to those obtained in our rats neither increased tenocyte cell proliferation nor altered collagen gene expression. In Tsai *et al.* (22), tendon cells were only exposed to high glucose for 24 hours, which suggests that the duration of glucose exposure should be considered when evaluating the effects of glucose on tenocytes. Such a concept fits with recent work indicating that the duration of diabetes increases the risk of developing tendinopathies (32). Further work exploring the impact of diabetes duration on the regulation of tendon collagen synthesis and cellular activation is needed.

Surprisingly, we did not detect a significant effect of diabetes on Dcn transcripts. Treatment of tendons with high concentrations (25 mM) of glucose for 2-weeks led to a large decrease in decorin and lumican RNA expression and protein content (33). The reduction in decorin noted by Burner *et al.* (33) is similar in magnitude to the current investigation, suggesting that a larger sample size may have been needed to discern a statistically significant difference in Dcn transcripts or that our adjustments for multiple comparisons were overly conservative in terms of Type I error rate control. Further, we did not have sufficient tissue to measure proteoglycan protein content, thus we cannot exclude the possibility that Dcn protein levels declined in our model. Dcn is a key regulator of collagen fibrillogenesis and cell proliferation, thus changes in Dcn, if present, could account for any structural abnormalities in tendons from diabetic individuals. We previously did not note any changes in Achilles tendon fibril structure in the STZ model (19), but extensive degeneration has been noted in the tendon of diabetic humans (6, 10). More detailed work is needed in type 2 diabetes models and in humans to delineate the mechanisms contributing to tendon collagen fibril disorganization in diabetic individuals.

Previous work with tendon cell culture models have indicated that exposure to high glucose concentrations increased Mmp-9 expression and enzyme activity but did not impact Mmp-2 (22). Work in humans also suggest that Mmp-9 expression and protein content in rotator cuff tendons is elevated in diabetic patients when compared to those without diabetes (21). In contrast, our *in vivo* data indicate that hyperglycemia does not alter tendon Mmp-9 or Mmp-1 gene transcripts, but instead rapidly suppressed Mmp-2 and Timp-1 transcripts. Although the

suppression of Mmp2 could be interpreted as a reduction in ECM degradation with hyperglycemia, the coinciding reduction in Timp1 in conjunction with no changes in Mmp1a or Mmp9 expression suggest an imbalance in the ratio of factors promoting ECM degradation and those which inhibit degradation. Our conclusions are limited as we cannot exclude the possibility that STZ-diabetes altered post-translational effects of Mmp or Timp enzyme activity.

Consistent with previous work in which tendon-derived stem cells were exposed to glucose (20), we noted a large and rapid decrease in the expressions of Tnmd and Scx. Tnmd and Scx expressions are critical for tenocyte differentiation (34, 35), tendon development (36), and healing (37, 38), A loss of Tnmd expression could account for the lower rates of tendon healing in cases of diabetes (39), as Tnmd is essential for proper early tendon healing and differentiation of stem cells to tenocytes for tendon regeneration (34, 35). Tnmd gene knockout models also present with reduced tendon stem/progenitor cells proliferation potential (40). Further, lower Tnmd and Scx expressions are associated with small brittle tendons in mice (41). The reduced Tnmd and Scx expressions suggest a lower rate of cellular proliferation, which is, however, not consistent with our previous work using the STZ-rat model (19). Additionally, it is plausible that the reduction in Tnmd and Scx do not have a large impact on baseline cellular proliferation in the diabetic state, but instead limit responses to tendon injury. Regardless, the dramatic reduction in Tnmd and Scx transcription suggest that the diabetic tendon may be less able to increase cellular proliferation in response to tendon injury or other perturbations. While others have noted increases in tendon cell apoptosis in diabetic models, similar to Tnmd and Scx, we observed a large decrease in transcription of Casp 3 and 8, suggesting a reduction in cellular apoptosis. As with our Mmp and Timp findings, we cannot exclude the possibility that hyperglycemia altered Casp enzyme activity, but our previous work in these animals (19), e.g. increased cell density, would suggest that apoptosis was suppressed in the diabetic animals. It is possible that the reduced Casp gene expression is indicative of a compensatory response, which may allow the tendon tissue assembly to increase cellular density. A higher cellular density in the tissue assembly may allow the tissue to more adequately respond to stresses such as uncontrolled hyperglycemia. Alternatively, the increased cellular density and reduced Casp expression may be contributing factors to abnormal extracellular matrix remodeling, which is often observed in diabetic tendinopathies.

In order to further evaluate potential mechanism contributing to diabetic tendinopathies, we choose to follow-up on cell culture work by Huang *et al.* (23) and Nah *et al.* (24) in which addition of AGE's to chondrocytes induced an upregulation of inflammatory mediators COX-2 and PGE_2 as well as iNos. Elevated levels of iNos and PGE_2 have been associated with tendon degeneration and collagen fibril disorganization (26, 42). In contrast to our hypothesis, STZ-induced diabetes did not alter the expression of prostaglandin and COX-2 related genes or iNos. However, we did observe a large reduction in Ager expression ($p \leq 0.05$), an effect that was not observed in the insulin treated group. These findings demonstrate that hyperglycemia, induced by STZ, has little impact on transcription of genes associated with the COX pathway but, as with our other findings, we cannot exclude the possibility of post-translational effects on COX enzyme activity or NOS. In addition to the genes discussed above, we also attempted to quantify the impact of STZ-induced diabetes on tendon expression of Mybl2, which is involved with cell cycle progression, and several transglutaminase genes. AGEs, which are elevated with diabetes (43) have been shown to alter Tgm activity in tenocytes, most likely via a post-translational mechanism (44). Surprisingly, we were unable to detect transcripts for Mybl2, Tgm3, 4, or 7. In preliminary work in our laboratory using RNA sequencing, we have confirmed that these genes are not expressed in rat tendon under baseline conditions (unpublished observation), suggesting these genes may not have a significant role in tendon.

Although our findings using a STZ model of diabetes are not completely consistent with the limited available studies, they suggest that hyperglycemia results in a rapid suppression of several genes vital to the maintenance and remodeling of tendon ECM as well as tenocyte proliferation. Our interpretation is limited because we did not have sufficient tissue to measure protein content or enzyme activity. We also cannot exclude the possibility that post-translational events are influenced by hyperglycemia. Our previous work in the STZ rat model suggest that post-translational changes are likely involved, as in contrast to our gene results, collagen content increased in tendons from diabetic animals, as did cellular proliferation. Timing may be an important consideration when assessing the impact of diabetes on tendon properties. For example, recent work with tenocyte cultures have demonstrated time-dependent effects of hyperglycemia and insulin treatment on several important ECM genes (45).

While tendinopathies are pervasive in both type 1 and type 2 diabetics, the STZ rodent model is representative of type 1 diabetes. It is possible, however, that tendon transcriptome

characteristics may either overlap or differ between type 1 and 2 diabetes. Further, the STZ diabetic model is an extreme model of hyperglycemia. There was one case of mortality in the chronic group, and the remaining animals in the chronic group became ill over the duration of the 10 weeks. In diet-induced uncontrolled type 2 diabetes, blood glucose values in patients will reach and/or exceed 300 mg/dL. Often, recurring instances of very high blood glucose levels (>300mg/dL) will prompt a physician to initiate corrective insulin therapy. Therefore, our model of hyperglycemia is not representative of typical type 2 diabetic patients due to the duration of uncontrolled hyperglycemia. Further, tendinopathies are thought to occur over a longer duration of diabetes. Long-term effects of type 2 diabetes on the musculoskeletal system, such as loading differences on tendon (e.g. lack of physical activity), may also influence the mechanical properties (46) and the progression of the diabetic phenotype.

Additionally, as this was a follow up study to Volper *et al.* (19), we were unable to complete further analyses in these animals such as immunohistochemistry, enzyme activity, and protein expression assays. It may have also been beneficial to evaluate other clinically relevant tendons, such as the Achilles, which is also susceptible to diabetes related tendinopathies. Lastly, although our study was powered appropriately, a larger sample size may have been beneficial, as we were unable to discern statistical significance in some of the measured targets such as Dcn and Ptges.

In summary, we have evaluated patellar tendon gene expression after STZ-induced diabetes with or without insulin therapy in the rat. In general, our findings suggest that STZ-induced hyperglycemia results in a large suppression to steady state transcript counts of several genes critical to tendon ECM remodeling, effects that were not observed in insulin-treated rats. Further work evaluating the post-translational effects of hyperglycemia on Mmp/Timp, COX, and iNos enzymes would be beneficial in understanding the pathological changes that occur to tendon with diabetes. Given the prevalence of tendon abnormalities in diabetic patients, it is important that we further develop our understanding of the pathophysiology and progression of tendinopathies, specifically in regards to long-term type 2 diabetes.

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Table 1: ddPCR Probes

Gene Symbol	Description	Gene ID
Ager	Advanced glycosylation end product-specific receptor precursor	81722
Casp3	Caspase-3	25402
Casp8	Caspase-8	64044
Col1a1	Collagen alpha-1(I) chain	29393
Col3a1	Collagen alpha-1(III) chain	84032
Dcn	Decorin	29139
iNos	Nitric oxide synthase 2	24599
Mmp1a	Matrix metallopeptidase 1a precursor	300339
Mmp2	Matrix metallopeptidase 2	81686
Mmp9	Matrix metalloproteinase 9	81687
Mybl2	Myb-related protein B	296344
Pcna	Proliferating cell nuclear antigen	25737
Ptger2	Prostaglandin E2 receptor EP2 subtype	81752
Ptges	Prostaglandin E synthase	59103
Ptgs2	Prostaglandin G/H synthase 2	29527
Scx	Scleraxis	680712
Tgfbr3	Transforming growth factor beta receptor type 3	29610
Tgm3	Transglutaminase 3	366189
Tgm4	Transglutaminase 4	64679
Tgm7	Transglutaminase 7	102552199
Timp1	Tissue inhibitor of matrix metalloproteinase 1	116510
Tnmd	Tenomodulin	64104

Table 2: Transcript Counts

Gene of Interest	Control	Acute	Chronic	Insulin
Col1a1	43683±11495	394±160*	5323±3420*	22816±7420
Col3a1	2066±463	72±30*	246±97*	1804±610
Dcn	51203±15094	11047±5485	19666±9095	36778±14463
Mmp1a1	39±10	41±10	37±11	38±11
Mmp2	1228±387	88±29*	261±102*	615±192
Mmp9	46±12	50±12	42±10	42±10
Timp1	1019±307	223±61*	146±49*	722±257
Pcna	1349±274	767±246	795±280	1317±357
Scx	3520±983	158±34*	617±284	2032±645
Tnmd	2136±703	58±23*	555±346*	1006±411
Tgfbr3	2619±606	1010±367	1814±667	2285±638
Casp3	262±56	69±22*	83±25*	214±54
Casp8	179±23	77±12*	83±18*	156±28
Ager	104±12	53±8*	45±12*	112±21
Ptger2	60±11	66±15	50±11	53±12
Ptges	63±17	27±7	32±9	59±17
Ptgs2	81±14	54±11	53±13	58±14
iNos	105±16	74±16	67±17	112±27

All counts are per 15 ng of cDNA except Col1a1, Col3a1, Tnmd, and Dcn, which are per 1 ng of cDNA. *$p \leq 0.05$.

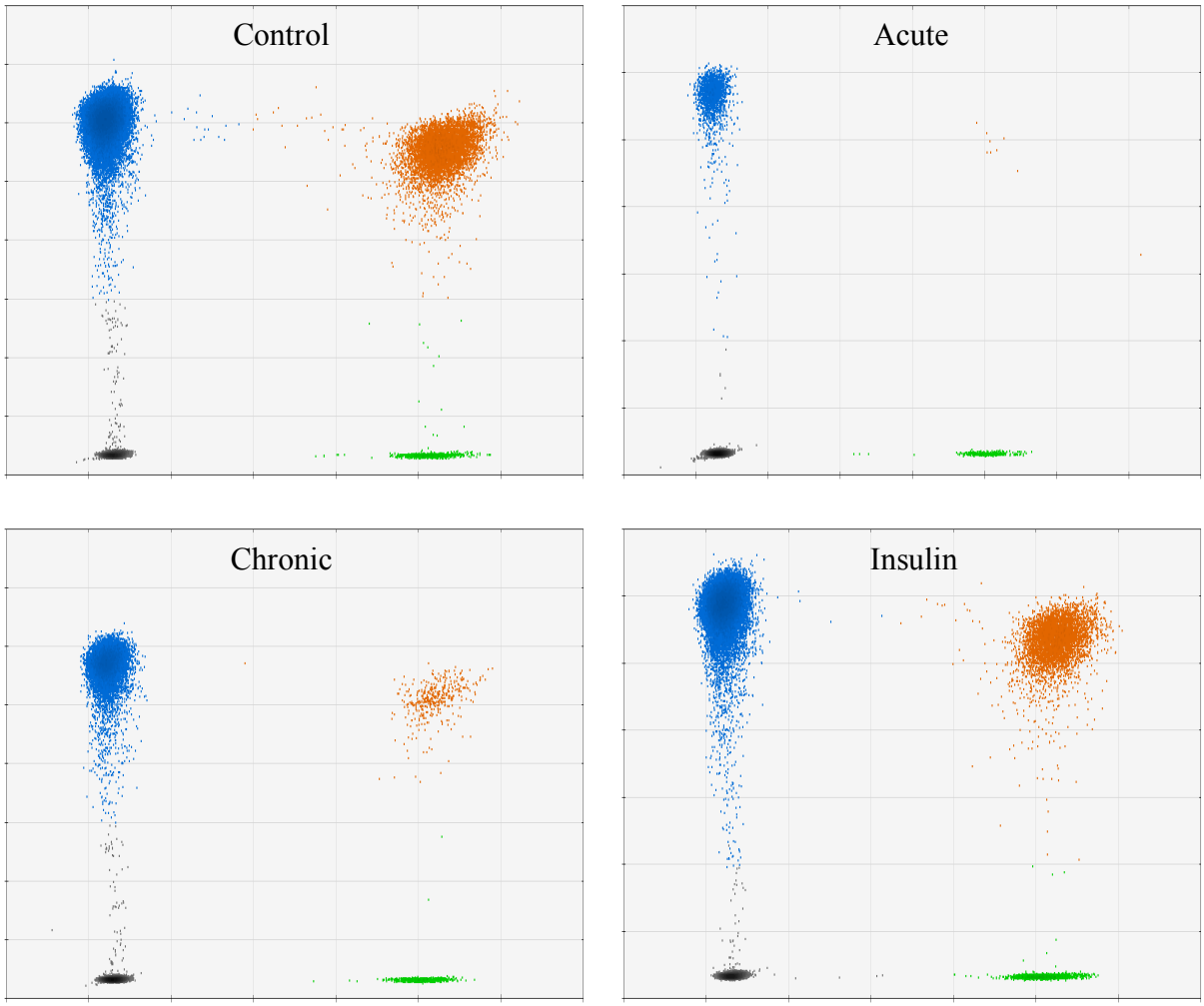

Figure 1: ddPCR Amplitude Chart

Amplitude Chart from ddPCR analysis provides absolute quantification of transcripts in duplex reactions. Images are representative of Col1a1 and Col3a1 ddPCR analysis. Each "droplet" represents an individual end-point PCR reaction. Blue droplets are positive for Col1a1. Green droplets are positive for Col3a1. Orange droplets are positive for both Col1a1 and Col3a1. Grey droplets represent a negative reaction for either gene. The loss of patellar tendon Col1a1 and Col3a1 transcript counts with acute and chronic uncontrolled hyperglycemia can be seen by reductions in droplet density within clusters. Figure generated using QuantaSoft Software Version 1.7 (Bio-Rad Laboratories, Inc).

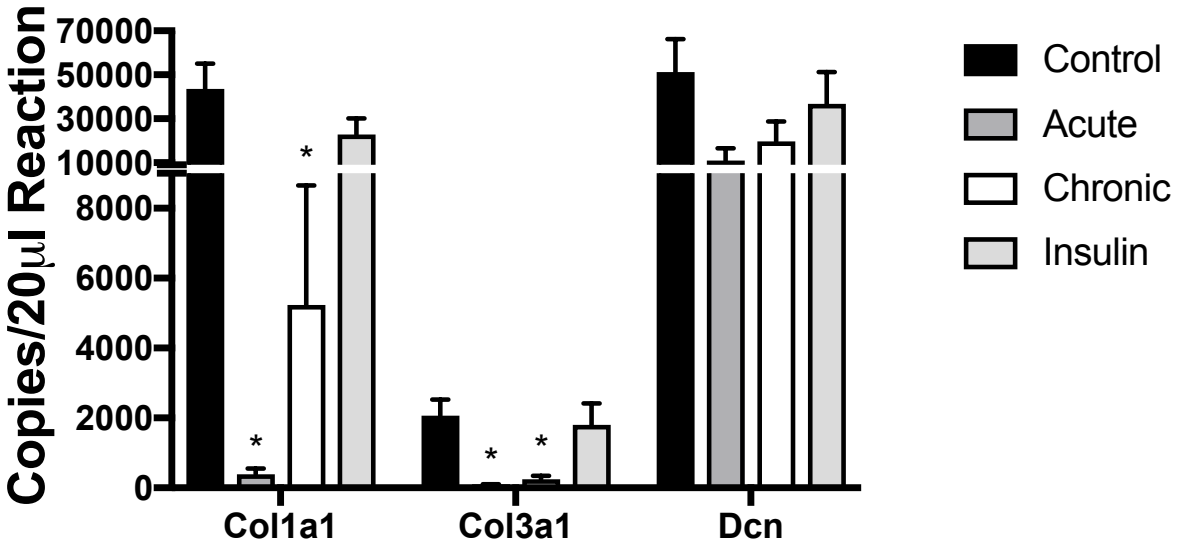

Figure 2: Col1a1, Col3a1, and Dcn transcript counts

Control n=9, Acute n=8, Chronic n=7, Insulin n=8. Data presented as mean± standard error. *$p<0.05$ vs. Control.

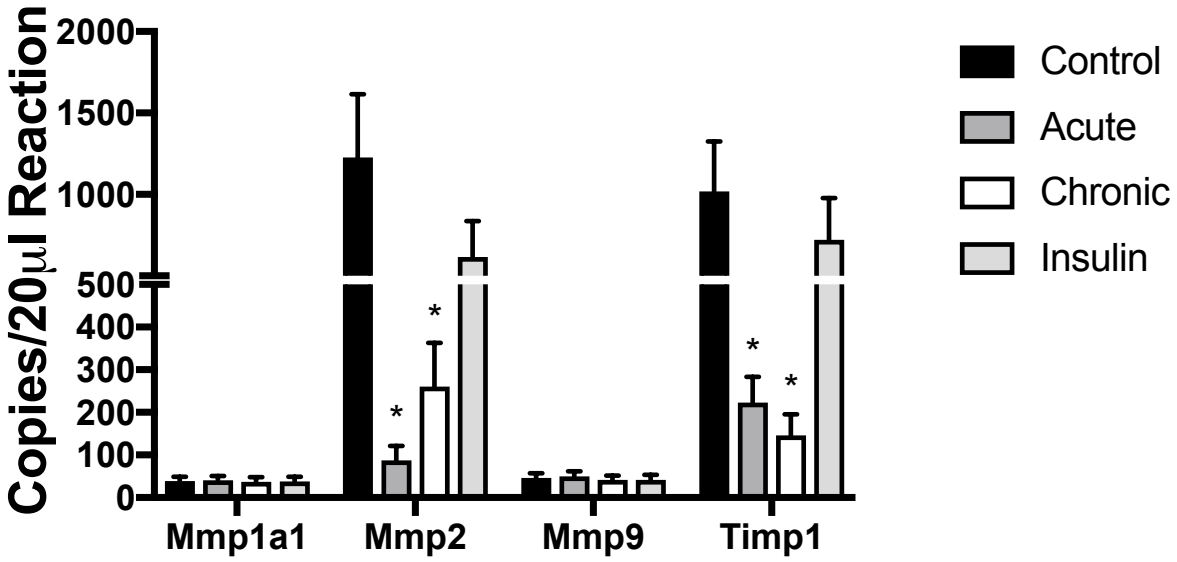

Figure 3: Mmp-1a1, Mmp-2, Mmp-9, and Timp-1 transcript counts

Control n=9, Acute n=8, Chronic n=7, Insulin n=8. Data presented as mean± standard error. *$p<0.05$ vs. Control.

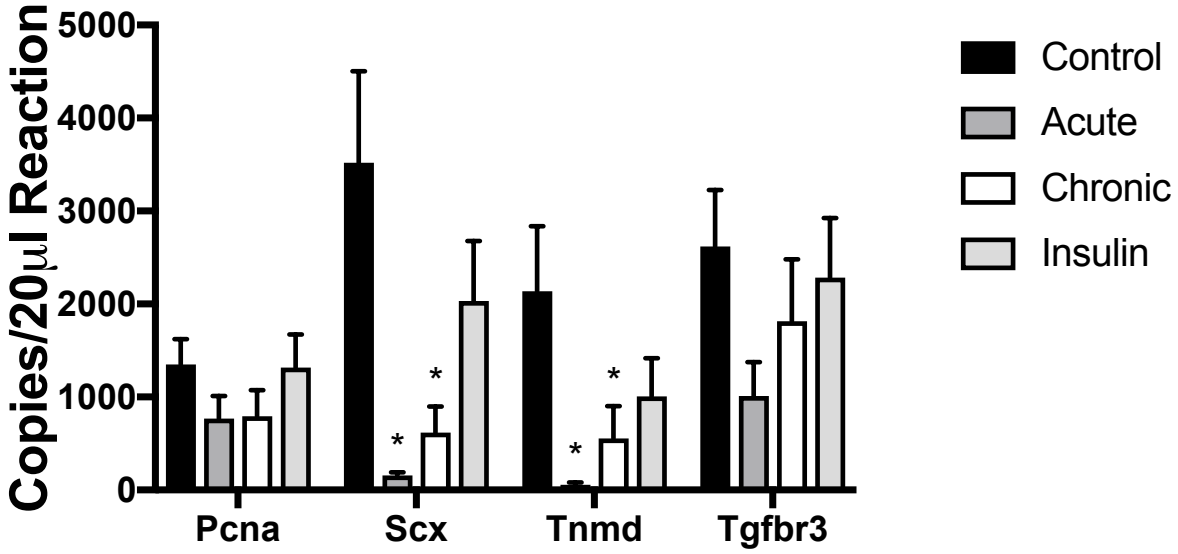

Figure 4: Pcna, Scx, Tnmd, and Tgfbr3 transcript counts

Control n=9, Acute n=8, Chronic n=7, Insulin n=8. Data presented as mean±standard error. *$p<0.05$ vs. Control.

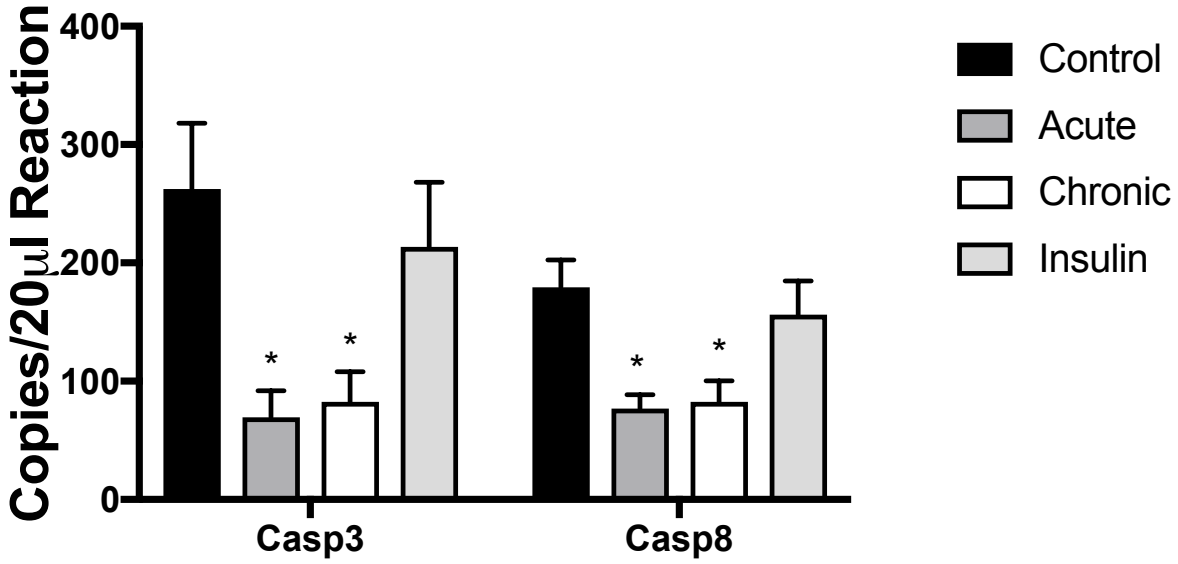

Figure 5: Casp3 and Casp 8 transcript counts

Control n=9, Acute n=8, Chronic n=7, Insulin n=8. Data presented as mean±standard error. *$p<0.05$ vs. Control.

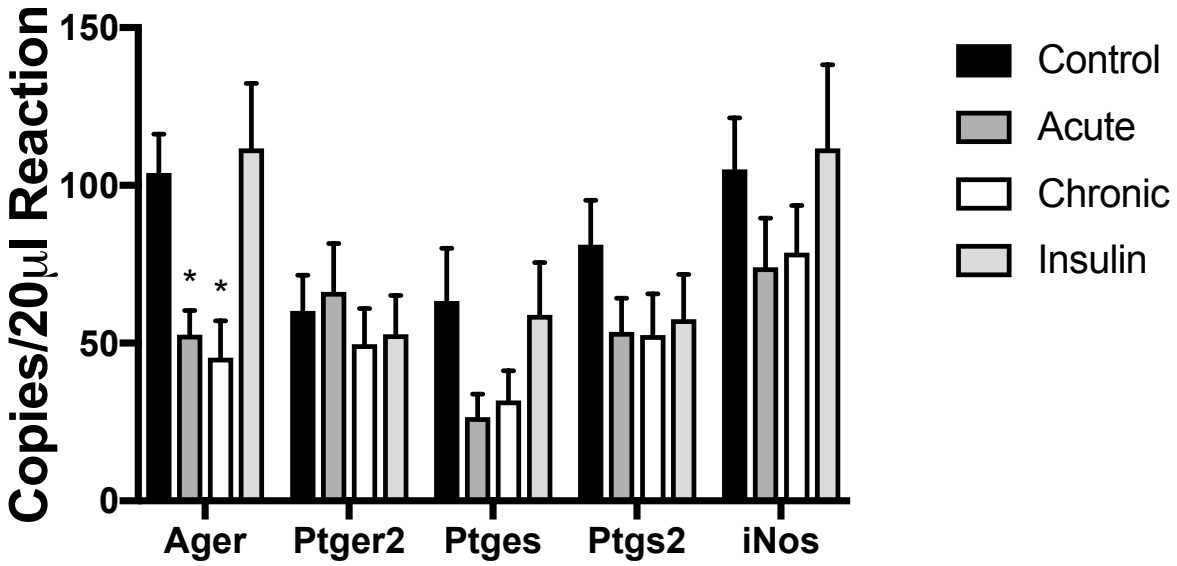

Figure 6: Ager, Ptger2, Ptges, Ptgs2, and iNos transcript counts

Control n=9, Acute n=8, Chronic n=7, Insulin n=8. Data presented as mean±standard error. *$p \leq 0.05$ vs. Control.

CHAPTER 3. ADVANCED GLYCATION END-PRODUCTS SUPPRESS MITOCHONDRIAL FUNCTION AND PROLIFERATIVE CAPACITY OF ACHILLES TENDON-DERIVED FIBROBLASTS

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3.1 Abstract

Introduction: Debilitating cases of tendon pain and degeneration affect the majority of diabetic individuals. The high rate of tendon degeneration persists even when glucose levels are well controlled, suggesting that other mechanisms may drive tendon degeneration in diabetic patients. The purpose of this study was to investigate the impact of advanced glycation end-products on tendon fibroblasts to further our mechanistic understanding of the development and progression of diabetic tendinopathy. We proposed that advanced glycation end-products would induce limitations to mitochondrial function and proliferative capacity in tendon-derived fibroblasts, restricting their ability to maintain biosynthesis of tendon extracellular matrix.

Methods: Using an *in vitro* cell culture system, rat Achilles tendon fibroblasts were treated with glycolaldehyde-derived advanced glycation end-products (0, 50, 100, and 200μg/ml) for 48 hours in normal glucose (5.5mM) and high glucose (25mM) conditions.

Results: We demonstrate that tendon fibroblasts treated with advanced glycation end-products display reduced ATP production, electron transport efficiency, and proliferative capacity. These impairments were coupled with alterations in mitochondrial DNA content and expression of genes associated with extracellular matrix remodeling, mitochondrial energy metabolism, and apoptosis.

Conclusion: Our findings suggest that advanced glycation end-products disrupt tendon fibroblast homeostasis and may be involved in the development and progression of diabetic tendinopathy.

3.2 Introduction

Over 30 million Americans suffer from diabetes. Evaluation of this patient population suggests that tendon abnormalities (i.e., tendinopathies) such as collagen fibril disorganization, increased tissue stiffness, and tissue degeneration are present in the majority of diabetic individuals (1-3). Notably, the high rate of tendinopathies in diabetic individuals persists even when glucose levels are well controlled (hemoglobin A1c<7) (2, 4, 5). Given the high prevalence

of diabetic tendinopathy, there is a critical need to define the molecular process underlying the diabetic tendon phenotype.

Advanced glycation end-product (AGE) formation is a non-enzymatic process in which free amine terminals are subjected to covalent modification by reactive glucose or other carbonyl containing molecules. AGEs form at an accelerated rate in diabetes and these modifications can result in non-enzymatic cross-links in long-lived extracellular proteins such as tendon collagen, which increase tissue stiffness and alter tissue mechanical properties (6-10). Furthermore, circulating AGE adducts are able to interact with the receptor for advanced glycation end-products (RAGE) to initiate a noxious feed forward cycle of sustained inflammation and tissue damage (11). In other tissues, endogenous AGE formation as result of chronic hyperglycemia and spontaneous oxidation of glycolytic intermediates contribute significantly to elevated levels of bound and circulating AGE adducts in diabetic patients (12, 13). Additionally, AGE-rich diets can increase serum AGE levels and result in accumulation of AGEs in tendon of mice (14). It is not known, however, what role circulating AGEs play in the development and progression of diabetic tendinopathy.

In non-tendon models, AGEs have been shown to activate RAGE-mediated cellular pathways leading to impairments in mitochondrial function and apoptosis (15-18). To our knowledge, the impact of AGEs on tenocyte mitochondrial function (e.g., ATP production and electron transport efficiency) has not previously been evaluated. Specifically, ATP has been shown to promote biosynthesis of the extracellular matrix (ECM) in intervertebral cells (19). In diabetic tendinopathy, degeneration and loss of organization in the ECM may be driven, in part, by the imbalance of energy (ATP) demand and supply. We propose that AGEs contribute to the diabetic tendon phenotype by activating cellular pathways that limit mitochondrial function, thereby interfering with the capacity of tendon fibroblasts to maintain biosynthesis of tendon ECM.

In effort to better understand diabetes associated tendon pathology, we sought to characterize impairments to energy producing systems and proliferative capacity of tendon-derived fibroblasts in response to AGE treatment and high glucose containing medium. We hypothesized that AGEs, *in vitro*, would impair mitochondrial function and proliferative capacity, independent of glucose concentrations. Further, we hypothesized that AGEs and high glucose medium would reduce mitochondrial DNA (mtDNA) content and further contribute to

limitations imposed to energy producing pathways in tendon fibroblasts. To advance our understanding of mitochondrial biogenesis and energy production during AGE exposure, analyses of mitochondrial apoptotic regulators, as well as regulators of mitochondrial energy metabolism were completed. This study provides new functional and descriptive perspective of the AGE insult on tendon fibroblast homeostasis.

3.3 Materials and Methods

3.3.1 AGE Preparation

Glycolaldehyde-derived AGEs were generated under sterile conditions as described by Valencia *et al.* (20). Briefly, sterile filtered 30% BSA solution (Sigma, St. Louis, MO) was incubated with 70mM glycolaldehyde dimer (Sigma) in sterile PBS without calcium chloride and magnesium chloride for three days at 37°C. After incubation, the AGE product was dialyzed against sterile PBS for 24 hours at 4°C using gamma-irradiated 10kDa cut-off cassettes (Thermo Scientific, Waltham, MA) to remove unreacted glycolaldehyde. Unmodified control BSA was prepared similarly, without the addition of glycolaldehyde dimer. Protein concentration was determined by BCA assay (Thermo Scientific) and absence of endotoxin (<0.25Eu/ml) was confirmed via the LAL gel-clot assay (GenScript, Piscataway, NJ).

3.3.2 Extent of AGE Modification

Extent of BSA modification was confirmed by fluorescence, absorbance, and loss of primary amines (20-23). AGE-BSA and Control-BSA were diluted to 1mg/ml in PBS and fluorescent spectra and absorbance were recorded at 335nm excitation/420nm emission and 340nm, respectively (Molecular Devices, San Jose, CA). For determination of loss of primary amines AGE-BSA and Control-BSA were diluted to 0.2mg/ml in PBS. An equal volume of ortho-phthalaldehyde solution (Sigma) was added and fluorescent spectrum was recorded at 340nm excitation/455nm emission (Molecular Devices). A standard curve of 0 to 0.2mg/ml of BSA that did not undergo 37°C incubation was used to generate a standard curve of free amine content and data was normalized to represent percentage of amine terminals remaining with reference to the standard curve (20). Respective fluorescence or absorbance values of PBS alone were subtracted from all data. AGE-BSA fluorescence was increased to 697.78 arbitrary units

(AU) compared to -0.72 AU for control BSA. Absorbance readings were completed to determine the extent of glycation. AGE-BSA showed increased glycation with absorbance readings of 0.682 AU compared to 0.01 AU for control BSA. Finally, AGE-BSA primary amine terminals underwent complete modification (-0.03% accessible amine terminals remaining), while control BSA retained 81.48% of accessible amine terminals. Spectra and absorbance values of PBS alone were subtracted from data but resulted in negative values for fluorescent-based detection because the spectra of PBS alone was greater than the obtained sample values. Negative values were interpreted as zero, and extent of modification was similar to previous reports (20).

3.3.3 Animal Protocol

This study was approved by the Purdue University Institutional Animal Care and Use Committee and all animals were cared for in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (24). Eight-week-old female Sprague-Dawley rats (n=8) were purchased from Charles River Laboratories (Wilmington, MA) and maintained for an additional eight weeks. Rats were housed on a 12-hour light-dark cycle and provided access to standard rat chow and water ad libitum. At sixteen weeks (Final Weight: 256.43±5.19g), rats were euthanized by decapitation after CO_2 inhalation.

3.3.4 Tendon Fibroblast Isolation and Cell Culture

Tendon-derived fibroblasts were isolated from the Achilles tendons of eight rats. After euthanasia, Achilles tendons were carefully excised and trimmed of remaining muscle and fascia. The tendon was rinsed with sterile PBS, finely minced, placed in DMEM containing 0.2% type I collagenase, and incubated in a 37°C shaking water bath for four hours. After tissue digestion, the cell suspension was filtered through a 100μm mesh filter, pelleted by centrifugation, resuspended in 5.5mM glucose DMEM containing 10% FBS, 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin (Thermo Scientific) and plated in 100mm collagen coated dishes. After reaching ~75-80% confluence, tendon fibroblasts were subcultured with either normal glucose (NG, 5.5mM) DMEM (Sigma) or high glucose (HG, 25mM) DMEM (Sigma) for a minimum of one passage to allow for glucose condition acclimation. Tendon fibroblasts were then seeded into 6-well, 24-well, or 96-well collagen coated culture plates and treated for 48

hours with 0, 50, 100, and 200µg/ml of AGEs within both glucose conditions. The 0µg/ml AGE group was treated with 200µg/ml of unmodified control BSA. Tendon fibroblasts from passage 2-4 were used for all experiments.

3.3.5 Proliferative Capacity

Proliferative capacity was assessed by cell counts, cytostatic MTT, and incorporation of synthetic nucleoside 5-ethynyl-2´-deoxyuridine (EdU). After AGE treatment in 6-well plates, cells were enzymatically released and manually counted with a hemocytometer. Cell counts were completed in duplicate and normalized to total volume in which cells were resuspended. For cytostatic MTT analysis, cells cultured in a 96-well plate were incubated with MTT reagent (5mg/ml, VWR, Radnor, PA) for the final 90 minutes of AGE treatment. Formazan crystals were solubilized with DMSO and absorbance was read at 550nm (Multiskan, Thermo Scientific). Each assay was completed in duplicate and data was represented as absolute arbitrary absorbance units. Cells were labeled with 2.5µM EdU (Carbosynth, Newbury, UK) during the final 12 hours of AGE treatment. EdU is a thymidine analog that incorporates during active DNA synthesis and is used to mark proliferating cells. Fixed cells (4% PFA) were stained with DAPI, and EdU was visualized via the Click-iT method (100mM Tris-HCl pH8.5, 1mM $CuSO_4$, 2.5µM red-fluorescent tetramethylrhodamine azide, and 100mM ascorbic acid; Thermo Scientific) (25). Fluorescent images were captured using a DMI 6000B microscope (Leica, Wetzlar, Germany) with a 10x objective and MetaMorph software (Molecular Devices). EdU data was analyzed using the ImageJ Multi-Point Tool (National Institutes of Health, Bethesda, Maryland) and is reported as a ratio of EdU^+ nuclei to total nuclei from two fields-of-view within each treatment.

3.3.6 Mitochondrial Stress Tests

Tendon fibroblasts were treated for 48 hours prior to being plated (8,000 cells) into collagen coated XFp cell culture miniplates (Seahorse Bioscience, Agilent, Santa Clara, CA). Cultured cells from each treatment condition were reseeded in duplicate, allowed to attach to the miniplates, and washed twice in pre-warmed XF base medium (Agilent). Miniplates were then incubated in XF base medium (1mM sodium pyruvate, 2mM glutamine, and either 5.5mM or 25mM glucose) for one hour in a 37°C incubator without CO_2 to allow plate outgassing.

Mitochondrial stress experiments were completed on an XFp extracellular flux analyzer (Agilent) with the Mito Stress Test Kit (Agilent). Chemicals (1μM oligomycin, 0.5μM carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP), 0.5μM rotenone, and 0.5μM antimycinA) were preloaded into cartridge ports and injected in succession during measurement of oxygen consumption rate (OCR).

3.3.7 Mitochondrial Membrane Potential

Mitochondrial membrane potential (Δψm) was determined using a commercially available tetramethylrhodamine (TMRE) assay (113852, Abcam, Cambridge, MA). Briefly, after 48 hours of AGE treatment, cultured cells were seeded in duplicate (8,000 cells) into collagen coated black 96-well microplates with clear bottoms, allowed to attach, and treated with 1 μM of TMRE for 30 minutes at 37°C. After incubation, wells were washed with 0.2% BSA in PBS and fluorescence was recorded at 549nm excitation/575nm emission (Molecular Devices). Depolarization controls were treated with 20 μM FCCP for 15 minutes prior to addition of TMRE.

3.3.8 Gene Expression Analysis

Total RNA for gene expression analysis was isolated after AGE treatment using the Direct-zol RNA Miniprep kit (Zymo Research). On-column DNase digestion was completed on all samples prior to elution of RNA. RNA concentration was determined using a NanoDrop 2000 (Thermo Scientific). Quality of RNA was assessed using the 260/280 and 260/230 ratios. Reverse transcription (iScript, BioRad) was completed to produce complementary DNA from 100ng of RNA. Absolute quantification of mRNA target transcripts was completed using the ddPCR platform (BioRad) with validated probe-based assays (BioRad) as previously described (26). For optimal detection, Col1a1 and Col3a1 reactions contained 0.55ng of cDNA, while MMP2, MMP9, and TIMP1 contained 4.4ng of cDNA (26). All other target PCR reactions contained 2.2ng of cDNA. A list of measured gene targets is provided in Table 3.

3.3.9 Mitochondrial DNA Content

Mitochondrial DNA (mtDNA) content was quantified by ratio of a target mitochondrial gene, NADH dehydrogenase subunit 1 (ND1), to nuclear reference gene, protein argonaute-1 (EIF2C1) (27, 28). Total DNA was isolated using the Quick-DNA Microprep kit (Zymo Research, Irvine, CA). DNA concentration was determined using a NanoDrop 2000 (Thermo Scientific) and serial diluted to 1ng/µl. A droplet digital PCR (ddPCR) method was used to complete quantification of absolute copy number of both ND1 and EIF2C1. Reactions were prepared in duplex format, in a final volume of 20µl with 2x ddPCR Supermix for Probes (No dUTP) (BioRad, Hercules, CA), 20x reference probe ND1 labeled with FAM (BioRad), 20x reference probe EIF2C1 labeled with HEX (BioRad), 5ng of DNA, 1µl of HindIII enzyme (FastDigest, Thermo Scientific), and nuclease-free water. The assembled reaction was incubated at room temperature for 20 minutes to permit digestion with the HindIII restriction enzyme. After digestion, droplets were generated from prepared PCR reactions using Droplet Generation Oil for Probes (BioRad) on a QX200 Droplet Generator (BioRad), transferred to a deep-well 96-well plate, and amplified (95°C-10 minutes, 1 cycle; 94°C-30 seconds and 60°C-90 seconds, 40 cycles; 98°C-10 minutes, 1 cycle) on a C1000 thermal cycler (BioRad). At completion of end-point PCR, absolute quantification of PCR products was completed on a QX200 Droplet Reader (BioRad) with QuantaSoft Software Version 1.7 (BioRad) and reported as a ratio of positive ND1/EIF2C1 counts per 20µl reaction.

3.3.10 Protein Expression

Cultured cells were lysed in RIPA buffer containing 50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50mM NaF, 0.2mM Na_3VO_4, and 0.2% protease inhibitor cocktail (Sigma). Samples were prepared as described previously (29). Equal amounts of protein (15µg) were separated in duplicate on an Any-kD TGX polyacrylamide gel (BioRad). Protein was transferred to a PVDF membrane (TransBlot Turbo, BioRad) and blocked for 24 hours at 4°C in 5% blotting-grade blocker (BioRad). Blots were incubated in primary OXPHOS antibody (1:1000, 110413, Abcam) and then HRP-conjugated goat anti-mouse antibody (1:5000, 31430, Invitrogen), each for 2 hours at room temperature. Bands of interest were compared against positive control (Rat Heart

Mitochondria, Abcam) and all targets were probed on the same membrane, but required different exposure times using signal accumulation mode (ChemiDoc, BioRad). Volume intensity analysis of matched exposure duration for each target was completed using Image Lab Version 6.0.1 (BioRad). Total protein volume intensity obtained by UV activated Stain-Free imaging was used for data normalization.

3.3.11 Superoxide Production

Mitochondrial ROS/superoxide production was determined using a commercially available fluorometric assay (219943, Abcam). Briefly, after 48 hours of AGE treatment, cultured cells were seeded in duplicate (8,000 cells) into collagen coated black 96-well microplates with clear bottoms, allowed to attach, and treated with MitoROS 580 stain for 30 minutes at 37°C. Fluorescence was recorded at 540nm excitation/590nm emission (Molecular Devices). Total ROS/superoxide production was determined by dihydroethidium (DHE) derived fluorescence. Cultured cells were detached, pelleted, and resuspended in Hank's Balanced Salt Solution. An aliquot was saved for determination of cell concentration. DHE (Thermo Scientific) was added at final concentration of 10μM and samples were incubated with shaking at 37°C for 30 minutes (30-32). Fluorescent spectrum was recorded at 530nm excitation/590nm emission (Molecular Devices) and fluorescence units were normalized to cell concentration.

3.3.12 Statistical Analysis

Statistical analyses on the four main effect contrasts of interest (50μg/ml AGE vs. 0μg/ml AGE, 100μg/ml AGE vs. 0μg/ml AGE, 200μg/ml AGE vs. 0μg/ml AGE, and HG vs. NG) for each outcome variable proceeded via a mixed effects regression model with consideration of technical data replicates and random effects to account for the tendon fibroblast donor rats. Regression models were fit to the data using the lmer function in R. The Kenward-Roger F-test was performed to determine whether the main effects regression model or the "full" model containing both main effects and two-factor interactions between the AGE and glucose contrasts should be fitted. Validity of the assumptions for the models were assessed via standard regression diagnostics. Specifically, the standardized residuals were examined to assess whether they were approximately Normally distributed, centered at zero, and did not exhibit any patterns

with respect to the predictor variables. If assumptions appeared invalid for the original scale, the logarithmic and square root transformations were considered. After identifying a model that provided a good fit to the outcome data, hypothesis tests were performed for the contrasts using Satterthwaite's method, and confidence intervals were created using the bootstrap. Hypothesis tests for the contrasts use a Bonferroni-adjusted significance level of $\alpha=0.05/4=0.0125$ (Figures 7, 8, 10-12, and 15) or $\alpha=0.05/2=0.025$ (Figures 9 and 16) or $\alpha=0.05/3=0.017$ (Figure 13) separately across the different outcome variables to account for the multiple comparisons of the specified final models. Certain specified, direct comparisons between two specific groups were performed via the paired t-test in R with $\alpha=0.05$ to test conditional main effects, where HG was conditioned for testing the AGE contrast of interest. Summary of applied contrasts and exact p-values for significant findings are provided in Table 4. Figures were generated in Prism 7.0 (GraphPad, La Jolla, CA) and are represented as mean ± standard error.

3.4 Results

3.4.1 Proliferative Capacity

Cell proliferation (EdU), cell counts, Mybl2 mRNA, Pcna mRNA, and cytostatic MTT were significantly reduced at AGE-50µg/ml, AGE-100µg/ml, and AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 7b-f, respectively). The HG condition had no effect on cell proliferation (EdU), cell counts, and Mybl2 mRNA ($p>0.0125$, Figure 7b-d, respectively). However, the HG condition reduced Pcna mRNA transcript counts ($p<0.0125$, Figure 7e) and increased absorbance values of cytostatic MTT data ($p<0.0125$, Figure 7f).

3.4.2 Mitochondrial Stress Tests

ATP production-coupled respiration was significantly reduced at AGE-100µg/ml and AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 8a). ATP production-coupled respiration was also significantly reduced in the HG condition ($p<0.0125$, Figure 8a). Basal respiration was significantly reduced at AGE-100µg/ml and AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 8b), but no glucose effect was observed ($p>0.0125$, Figure 8b). Neither AGE treatment nor glucose condition had a significant effect on maximal respiration ($p>0.0125$, Figure 8c). AGE treatment had no effect on proton leak ($p>0.0125$, Figure 8d), but

the HG condition increased proton leak across the inner mitochondrial membrane ($p<0.0125$, Figure 8d). Coupling efficiency was significantly reduced at AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 8e). Coupling efficiency was also significantly reduced in the HG condition ($p<0.0125$, Figure 8e). Spare respiratory capacity was significantly increased at AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 8f), but no glucose effect was observed ($p>0.0125$, Figure 8f).

3.4.3 Mitochondrial Membrane Potential

AGE treatment had no effect on mitochondrial membrane potential ($p>0.025$, Figure 9), but mitochondrial membrane potential was decreased in the HG condition ($p<0.025$, Figure 9).

3.4.4 Transcriptional Analysis of Extracellular Matrix Remodeling

Col1a1 and MMP9 mRNA transcript counts were significantly reduced in the AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 10a&d). Col3a1 mRNA transcript counts were significantly increased in the AGE-50μg/ml and AGE-100μg/ml groups compared to AGE-0μg/ml ($p<0.0125$, Figure 10b). MMP2 mRNA transcript counts were significantly increased in the AGE-100μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 10c). AGEs had no effect on TIMP1 mRNA transcript counts ($p<0.0125$, Figure 10e). No glucose effect was observed for Col1a1, Col3a1, MMP2, MMP9, or TIMP1 ($0<0.0125$, Figure 10a-e).

3.4.5 Mitochondrial DNA Content

Mitochondrial DNA (mtDNA) content was significantly increased at AGE-50μg/ml, AGE-100μg/ml, and AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 11a), but no effect of glucose condition was observed ($p>0.0125$, Figure 11a). Neither AGE treatment nor glucose condition had a significant effect on DNA content when normalized to cell counts ($p>0.0125$, Figure 11b).

3.4.6 Transcriptional Analysis of Mitochondrial Complexes

Ndufa1 mRNA transcript counts were significantly increased at AGE-50μg/ml, AGE-100μg/ml, and AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 12a), but no effect

of glucose condition was observed ($p>0.0125$, Figure 12a). Sdha mRNA transcript counts were significantly reduced at AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 12b), but no effect of glucose was observed ($p>0.0125$, Figure 12b). A secondary direct comparison to test the conditional main effect revealed that Sdha transcript counts were significantly reduced at HG AGE-200µg/ml compared to HG AGE-0µg/ml ($p<0.05$, Figure 12b). Bcs1l mRNA was significantly reduced at AGE-50µg/ml, AGE-100µg/ml, and AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 12c). Bcs1l mRNA was also significantly reduced in the HG condition ($p<0.0125$, Figure 12c). Neither AGE treatment nor glucose condition altered Cox4i1 mRNA transcript counts ($p>0.0125$, Figure 12d). MT-ATP6 mRNA transcript counts were significantly reduced at HG AGE-200µg/ml compared to HG AGE-0µg/ml ($p<0.05$, Figure 12e), but no effect of glucose was observed ($p>0.0125$, Figure 12e).

3.4.7 Protein Analysis of Mitochondrial Complexes

Expression of complex III (UQCRC2) was significantly increased at AGE-200µg/ml compared to AGE-0µg/ml ($p<0.017$, Figure 13c), but no glucose effect was observed ($p>0.017$, Figure 13c). Expression of complex I (NDUFB8), II (SDHB), IV (MTCO1), or V (ATP5A) were not altered by either AGE treatment or glucose condition ($p>0.017$, Figure 13a, b, d, & e, respectively). However, a significant interaction between AGE treatment and glucose condition was observed for complex I (NDUFB8) and complex IV (MTCO1) ($p<0.017$, Figure 13a&d). All five targets were probed on the same membrane, but required different exposure times using signal accumulation mode (ChemiDoc, BioRad). Two full and unedited blot images are shown in order of exposure time (Figure 14). Bands that were cropped and used for representative purposes in Figure 13f are indicated (Figure 14).

3.4.8 Transcriptional Analysis of Mitochondrial Apoptosis

Bak1 mRNA transcript counts were significantly reduced at AGE-100µg/ml and AGE-200µg/ml compared to AGE-0µg/ml ($p<0.0125$, Figure 15a), but no effect of glucose condition was observed ($p>0.0125$, Figure 15a). Bax and Casp8 mRNA transcript counts were significantly reduced at HG AGE-200µg/ml compared to HG AGE-0µg/ml ($p<0.05$, Figure 15b&e, respectively), but no effect of glucose was observed ($p>0.0125$, Figure 15b&e, respectively).

Bcl2 and Tgfbr3 mRNA transcript counts were significantly reduced at AGE-200μg/ml compared to AGE-0μg/ml ($p<0.0125$, Figure 15c&f, respectively), but no effect of glucose condition was observed ($p>0.0125$, Figure 15c&f, respectively). AGE treatment had no effect on Casp3 mRNA ($p>0.0125$, Figure 15d), but the HG condition reduced Casp3 mRNA transcript counts ($p<0.0125$, Figure 15d).

3.4.9 Superoxide Production

Mitochondrial and total ROS/superoxide production were significantly increased at AGE-200μg/ml compared to AGE-0μg/ml ($p<0.025$, Figure 16a&b, respectively), but no glucose effect was observed in either ($p>0.025$, Figure 16).

3.5 Discussion

AGE-induced non-enzymatic collagen cross-link formation in the ECM has been proposed to increase tendon stiffness in diabetic individuals (6, 7, 10). However, factors contributing to tendon degeneration and collagen fibril disorganization in diabetic tendon have not been extensively characterized. The detrimental effects of AGE associated pathology are well described in non-tendon tissues and have been linked to several diabetic complications such as cardiomyopathy, retinopathy, nephropathy, and endothelial dysfunction (10, 33). In this study, we sought to address the effect of AGEs at the cellular level to better understand factors that may contribute to the disorganization and degeneration of tendon ECM noted in diabetic individuals. Using an *in vitro* primary cell culture system, we demonstrate dose-dependent AGE-induced reductions in proliferative capacity and mitochondrial ATP production of tendon-derived fibroblasts. Additionally, we demonstrate increased mtDNA content and modifications to mitochondrial complexes and markers of apoptosis after AGE treatment. While previous research has established AGE dependent mitochondrial and proliferative limitations (17, 18, 34), these data, to the best of our knowledge, are the first to show these impairments in tendon-derived fibroblasts.

Tendon injuries and degenerative pathology are a common and debilitating clinical problem in diabetic individuals (2, 3, 35-39). Diabetic tendons are thicker (37, 40), and more commonly present with fibril disorganization (2). Despite convincing epidemiological data, the

molecular factors contributing to the development and degenerative process of tendinopathy in diabetic individuals are not well characterized. Much focus has been directed to the influence of elevated glucose on cellular and structural tendon parameters. *In vitro* and *in vivo* data have indicated that elevated glucose availability can alter cell signaling dynamics and structural properties in tendon (26, 41, 42). These data suggest that glucose may contribute to tendon pathology, however conflicting reports exist and more conclusive human data is needed to confirm hyperglycemia-associated tendon degeneration in diabetes.

As evidenced, glucose does seem to be implicated in modulation of some tendon cellular and structural properties (26, 41, 42), however, the underlying mechanisms influencing tendon degeneration in diabetic patients remain inconclusive. Couppé *et al.* (4) have demonstrated that Achilles tendon Young's modulus (stiffness) is greater in diabetic subjects compared to control subjects, but no difference exists between subjects with well-controlled and poorly controlled diabetes. These data highlight that hyperglycemia is unlikely to be the sole contributor of diabetic tendon pathology. In support of this notion, we explored the role of AGEs on modulation of tendon cellular properties that may consequently interfere with regulation and maintenance of the ECM.

Tendon fibroblast proliferation is a vital component to tendon development, adaptation, and healing (43, 44). An inability of tenocytes to proliferate in the presence of AGEs would significantly precipitate the development of tendinopathy (45). Tendinopathy is thought to develop, in part, from a failed healing response to minor tendon damage during loading. Specifically, delayed and abnormal healing is a common complication of both type I and type II diabetes (46, 47). As evidence, tendinopathies in diabetic patients are generally more pervasive and can present with more severe degeneration, which may, in part, be driven by prolonged injury status (2, 48, 49). We note dramatic impairments to tendon fibroblast proliferative capacity in the presence of AGEs. Our *in vitro* data demonstrate, in a dose dependent fashion, reduced cell proliferation (EdU) and cell counts after 48 hours of AGE treatment (Figure 7b&c, respectively). In concert, we demonstrate a reduction in proliferative gene markers, Mybl2 and Pcna, and reduced absorbance values of cytostatic MTT with AGE treatment (Figure 7d-f, respectively). These data are in agreement with data from Hu *et al*. (34), which also demonstrates a lack of proliferative capacity in the presence of AGEs. While the HG condition did impact Pcna mRNA and cytostatic MTT (Figure 7e-f), the primary insult to proliferative capacity

appears to be driven by AGE treatment. Impaired proliferation is likely mediated by RAGE signaling (50), but further work is needed to confirm AGE-mediated impairments to tendon fibroblast proliferation and tendon healing.

AGEs have been linked to numerous diabetes related complications and their detrimental effects are well documented in several tissues (10, 33). The primary consequences of AGE-mediated RAGE activation are chronic inflammation, as result of the NF-κB mediated cascade, and oxidative stress, due to NADPH oxidase stimulation. Among resulting complications, reports in other cell types have identified AGE-mediated impairments to mitochondrial function and dynamics (15-18). Similarly, we report impaired mitochondrial parameters and reduced ATP production-coupled respiration in tendon fibroblasts treated with AGEs (Figure 8a). The role of ATP is diverse and essential for a multitude of cellular processes including cell proliferation, contraction, and wound healing. Specifically, ATP has been shown to promote biosynthesis of the ECM in intervertebral disk cells (19). In tendon, the resident fibroblast population maintains the ECM, which is primarily composed of collagen. Due to the energy demanding nature of ECM maintenance, it is possible, that in the presence of AGEs, limited ATP production contributes to loss of organization in the ECM, which is commonly noted in diabetic tendon (1-3). While maximal respiration of tendon fibroblasts was unchanged due to identical XFp seeding density, basal respiration was reduced after AGE treatment (Figure 8b&c, respectively). In addition to loss of ATP production and reduced basal respiration, we demonstrate that AGEs also impair electron transport coupling efficiency; thereby increasing spare respiratory capacity of AGE treated tendon fibroblasts and indicating overall reduction to electron transport efficiency (Figure 8e&f, respectively). Interestingly, the HG condition reduced ATP production and coupling efficiency (Figure 8a&e) and increased proton leak across the inner mitochondrial membrane (Figure 8d), suggesting that glucose alone can also impact electron transport efficiency. Proton leak across the inner membrane is known to influence mitochondrial membrane potential (51, 52). In agreement with our proton leak data, our analysis revealed that the HG condition reduces mitochondrial membrane potential, however, no effect of AGE is observed (Figure 9). It is important to note that AGE and/or glucose-mediated mitochondrial impairments are likely not the sole contributor of tendon ECM disorganization. For example, AGEs increase matrix metalloproteinases (MMP) expression and activity, which may contribute in an additive manner to enhanced degeneration in tendon ECM (53).

To address our hypothesis that the presence of AGEs and resulting limited ATP production contribute to loss of organization in the ECM, we completed gene analysis of targets associated with ECM maintenance and remodeling. We note that AGE treatment reduces Col1a1 mRNA expression (Figure 10a), consistent with previous work which has demonstrated reduced collagen synthesis during AGE treatment in fibroblasts (54). Col3a1 mRNA expression was however increased with AGE 50µg/ml and 100µg/ml, but not at 200µg/ml (Figure 10b). Col3a1 expression is upregulated after injury and during the early stages of wound healing (55, 56). It is possible that in our cell culture model, increased Col3a1 mRNA expression in the AGE 50µg/ml and 100µg/ml groups was an attempt to overcome the AGE insult to tendon fibroblast collagen production. However, it appears that the potential compensatory response was unsuccessful in the AGE 200µg/ml group (Figure 10b). Further, MMPs and tissue inhibitors of metalloproteinases (TIMPs) work to tightly regulate ECM remodeling. Previous work in chondrocyte cell culture has demonstrated an increase to MMP mRNA expression with AGE treatment (53, 57). While we did observe increased MMP2 mRNA expression in the AGE 100µg/ml group, MMP9 mRNA expression was reduced in the AGE 200µg/ml group (Figure 10c&d). Lastly, we did not observe an AGE or glucose effect of TIMP1 mRNA (Figure 10e). Reduced collagen expression and altered MMP modulation may favor an environment that promotes loss of ECM organization. Future work evaluating post-translational MMP activation and collagen fibril organization is needed to determine the role of AGEs in modulation of tendon ECM.

We also noted increased content of mtDNA after 48 hours of AGE treatment, despite striking reductions to ATP production. mtDNA content can be used as an estimate of mitochondrial volume (58), however, contrary to our hypothesis, 48 hours of AGE treatment resulted in increased mtDNA content in a dose dependent manner independent of glucose condition (Figure 11a). Based on our findings, it is plausible that the increase in mtDNA in AGE-treated tendon fibroblasts was indicative of a compensatory response to overcome the AGE insult and meet energy demands (59, 60). In support of this theory, cells under oxidative stress have been shown to increase mitochondria and mtDNA (61). Additionally, Kim *et al*. (60) have demonstrated a relationship between mtDNA content and severity of histopathology in cancerous lesions, suggesting that increased mtDNA content may be used as a measure of DNA injury and pathology. Despite this compensatory response, functional mitochondrial limitations were still

evident after 48 hours of AGE treatment. Alternatively, it is possible that AGE-induced limitations to mitochondrial biogenesis may result in failure of tendon fibroblasts to meet energy demands. For example, mtDNA is more susceptible to mutation than nuclear DNA and AGEs have been shown to induce damage to mtDNA, which could ultimately impact mitochondrial biogenesis (62, 63). However, further molecular work is needed to conclusively define the pathway of AGE-mediated mitochondrial damage. To confirm that mtDNA content measurements were not falsely elevated by an increase in total DNA, despite equal DNA loading per PCR reaction, we normalized total DNA yield to cell counts and noted no difference in DNA content with either glucose condition or AGE treatment (Figure 11b).

Reactive oxygen species (ROS), produced primarily by mitochondria, have been implicated in AGE-mediated cell apoptosis and mitochondrial damage (18, 64, 65). To further explore and identify specific targets of the AGE insult, we assessed regulation of electron transport complexes and apoptosis. Analysis of gene transcripts associated with complex I-V (Figure 12a-e, respectively) of the electron transport chain revealed increased expression of Ndufa1 mRNA (Figure 12a) and marked reduction of Bcs1l mRNA (Figure 12c) with AGE treatment. Additionally, the HG condition further reduced Bcs1l mRNA expression (Figure 12c). Increased Ndufa1 mRNA after AGE treatment may be interpreted as a compensatory response to increase mitochondrial complex I in effort to meet energy demands after the AGE insult. However, Bcs1l mRNA, which encodes mitochondrial complex III, is dramatically reduced in a dose dependent manner after AGE treatment, suggesting that the AGE insult may be targeted to complex III. Lastly, Sdha and MT-ATP6 mRNA, coding for mitochondrial complex II and complex V (ATP synthase), respectively, were reduced in the HG AGE-200µg/ml group compared to HG AGE-0µg/ml (Figure 12b&e, respectively). A direct comparison was made between HG AGE-0µg/ml and HG AGE-200µg/ml to test the conditional main effect and reveal a significant reduction to Sdha and MT-ATP6 mRNA, suggesting that both AGE treatment and HG condition were contributing to this reduction. Cox4i1 (complex IV, Figure 12d) was not impacted by either AGE treatment or HG condition. While these gene data provide indication that AGEs may indeed have targeted effects to the electron transport chain, protein analysis revealed a significant AGE main effect for only complex III (UQCRC2) (Figure 13c). Additionally, complex III protein expression was increased with AGE exposure, while complex III mRNA expression was reduced. Lack of response to remaining complexes and discrepancies

between gene and protein analysis may be attributed to the acute duration of AGE exposure. Further, an interaction between AGEs and glucose condition was observed in complex I (NDUFB8, Figure 13a) and complex IV (MTCO1, Figure 13d). These results merit further work to discover mechanistic pathways by which AGEs limit mitochondrial function during long-term AGE exposure.

To assess the impact of AGEs on mitochondrial-related tendon fibroblast apoptosis, we completed analysis of associated gene transcripts and measurement of superoxide production. Transcript counts of pro-apoptotic gene Bak1 were reduced with AGE treatment (Figure 15a). Additionally, pro-apoptotic Bax mRNA expression was reduced only in the HG AGE-200µg/ml group compared to HG AGE-0µg/ml (Figure 15b). Although these findings are contrary to previous work (34), it is possible that these may be compensatory responses to AGE-mediated apoptotic signals. In agreement with previous work by Hu *et al*. (34), expression of Bcl2 mRNA, an anti-apoptotic mediator, was reduced with AGE treatment (Figure 15c). Previous reports have indicated an inverse relationship between Bcl2 and ROS, where ROS may act to reduce expression of Bcl2 and sensitize cells to apoptosis (66). In agreement, our analysis of superoxide, the primary mitochondrial- derived reactive oxygen species (67), demonstrates increased production of both mitochondrial derived and total superoxide after AGE treatment (Figure 16). Further, cytochrome c release is suppressed by Bcl2, but is promoted by Bak1 and Bax. Cytochrome c release will ultimately result in caspase activation (68). Previous reports indicate AGE treatment induces caspase activation (34, 69), however we did not note any AGE-induced changes to Casp3 mRNA (Figure 15d) but did note reduced Casp8 mRNA in the HG AGE-200µg/ml group compared to HG AGE-0µg/ml (Figure 15e). Finally, transcript counts of Tgfbr3 were reduced with AGE treatment (Figure 15f). Tgfbr3 overexpression has been shown to increase Bax and Bcl2 expression, as well as promote activation of caspase 3 (70). Casp3 mRNA expression was reduced in the HG condition and was the only target associated with apoptosis to be affected by glucose (Figure 15d). From these data, it is evident that AGEs alter transcriptional regulation of gene transcripts associated with apoptosis, however, we are limited in our interpretation and further post-translational and activity assays are needed to define the precise mechanisms by which AGEs may induce apoptosis in diabetic tendinopathy.

In summary (Figure 17), we demonstrate that AGEs, which are elevated in serum of diabetic individuals, impair proliferative capacity, ATP production, and electron transport chain

efficiency. Additionally, we demonstrate that AGEs alter regulation of mitochondrial complex expression and gene transcripts associated with apoptosis. While the HG condition did impact some mitochondrial parameters, AGEs appear to be the primary insult and may be responsible for the development of the diabetic tendon phenotype. This work provides new insights to the pathophysiology of tendon ECM in diabetic patients. However, future *in vivo* and mechanistic work is needed to determine whether controlling serum AGEs in diabetic patients can reduce risk of degenerative tendinopathy.

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Table 3: ddPCR Gene Targets

Gene Symbol	Description	Gene ID
Bak1	BCL2-antagonist/killer 1	116502
Bax	BCL2 associated X, apoptosis regulator	24887
Bcl2	B-cell lymphoma 2, apoptosis regulator	24224
Bcs1l	BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone	301514
Casp3	Caspase 3	25402
Casp8	Caspase 8	64044
Col1a1	Collagen alpha-1(I) chain	29393
Col3a1	Collagen alpha-1(III) chain	84032
Cox4i1	Cytochrome c oxidase subunit 4i1	29445
MMP2	Matrix metallopeptidase 2	81686
MMP9	Matrix metalloproteinase 9	84687
MT-ATP6	ATP synthase 6, mitochondrial	26197
Mybl2	MYB proto-oncogene like 2	296344
Ndufa1	NADH:ubiquinone oxidoreductase subunit A1	363441
Pcna	Proliferating cell nuclear antigen	25737
Sdha	Succinate dehydrogenase complex flavoprotein subunit A	157074
Tfgbr3	Transforming growth factor beta receptor 3	29610
TIMP1	Tissue inhibitor of matrix metalloproteinase 1	116510

Table 4: Summary of p-values for Statistical Findings

Figure	AGE-0µg/ml vs. AGE-50µg/ml	AGE-0µg/ml vs. AGE-100µg/ml	AGE-0µg/ml vs. AGE-200µg/ml	Normal vs. High Glucose	Interaction
7B	$4x10^{-5}$	$3x10^{-14}$	$< 2x10^{-16}$	NS	N/A
7C	$1x10^{-8}$	$5x10^{-15}$	$< 2x10^{-16}$	NS	N/A
7D	$1.5x10^{-10}$	$< 2x10^{-16}$	$< 2x10^{-16}$	NS	N/A
7E	$4x10^{-9}$	$2x10^{-15}$	$< 2x10^{-16}$	$7x10^{-3}$	N/A
7F	$5x10^{-9}$	$1.5x10^{-14}$	$< 2x10^{-16}$	$8x10^{-4}$	N/A
8A	NS	$3x10^{-7}$	$2x10^{-14}$	$1x10^{-3}$	N/A
8B	NS	$9x10^{-6}$	$5x10^{-10}$	NS	N/A
8C	NS	NS	NS	NS	N/A
8D	NS	NS	NS	$2x10^{-3}$	N/A
8E	NS	NS	$1x10^{-14}$	$1x10^{-7}$	N/A
8F	NS	NS	$3x10^{-4}$	NS	N/A
9	N/A	N/A	NS	$2.8x10^{-7}$	N/A
10A	NS	NS	$5x10^{-11}$	NS	N/A
10B	$5x10^{-4}$	$1.4x10^{-5}$	NS	NS	N/A
10C	NS	$3x10^{-4}$	NS	NS	N/A
10D	NS	NS	$7x10^{-3}$	NS	N/A
10E	NS	NS	NS	NS	N/A
11A	$6x10^{-7}$	$1x10^{-12}$	$2x10^{-15}$	NS	N/A
11B	NS	NS	NS	NS	N/A
12A	$6x10^{-6}$	$8x10^{-7}$	7x10-5	NS	N/A
12B	NS	NS	5x10-3	NS	N/A
12C	$2x10^{-5}$	$1x10^{-11}$	$< 2x10^{-16}$	$1x10^{-2}$	N/A
12D	NS	NS	NS	NS	N/A
12E	NS	NS	NS	NS	N/A
13A	N/A	N/A	NS	NS	$1.2x10^{-2}$
13B	N/A	N/A	NS	NS	NS
13C	N/A	N/A	$1.3x10^{-2}$	NS	NS
13D	N/A	N/A	NS	NS	$2.8x10^{-3}$
13E	N/A	N/A	NS	NS	NS
15A	NS	$9x10^{-7}$	$< 2x10^{-16}$	NS	N/A
15B	NS	NS	NS	NS	N/A
15C	NS	NS	$7x10^{-10}$	NS	N/A
15D	NS	NS	NS	$1.1x10^{-2}$	N/A
15E	NS	NS	NS	NS	N/A
15F	NS	NS	$1x10^{-4}$	NS	N/A
16A	N/A	N/A	$5.26x10^{-16}$	NS	N/A
16B	N/A	N/A	$8.6x10^{-3}$	NS	N/A

NS = Not Significant. N/A = Not Tested.

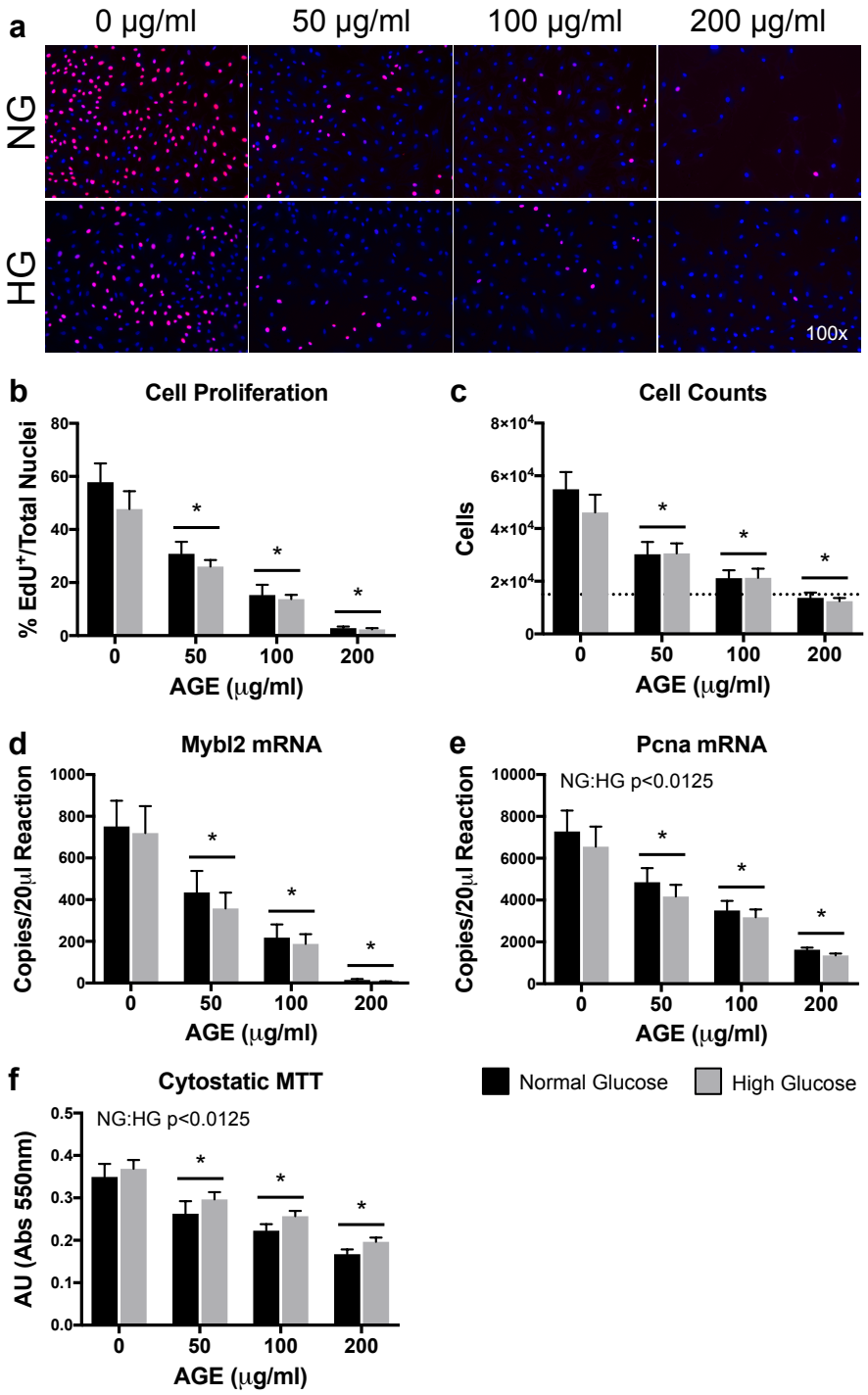

Figure 7: Proliferative Capacity

a. Representative images of EdU^+/DAPI **b.** Ratio of EdU^+ nuclei to total nuclei. **c.** Cell counts. **d&e.** ddPCR gene transcript counts for Myb-related protein B (Mybl2) and proliferating cell nuclear antigen (Pcna). **f.** Cytostatic MTT. n=8. *$p<0.0125$ main effect for AGE vs. AGE-0µg/ml. NG:HG $p<0.0125$ main effect for glucose condition.

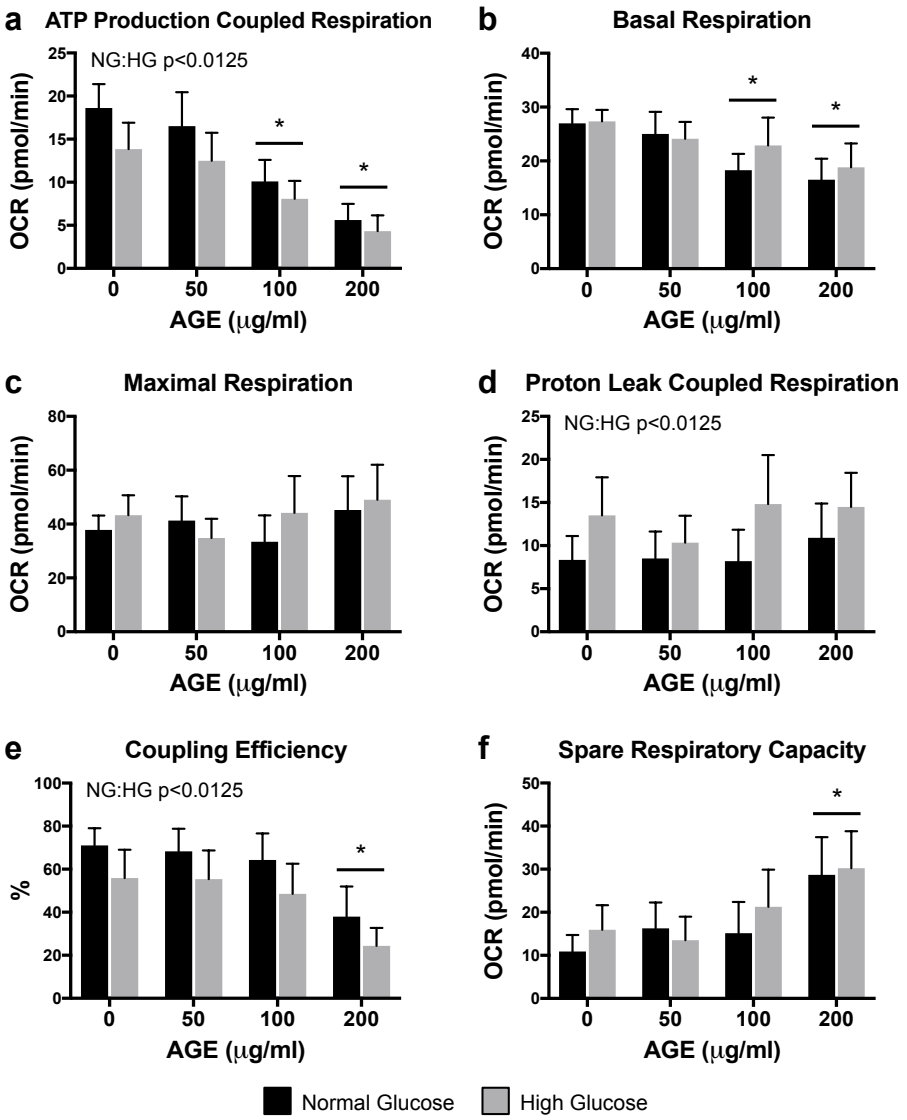

Figure 8: Mitochondrial Stress Tests

a-f. Mitochondrial parameters as a function of oxygen consumption rate (OCR). *p<0.0125 main effect for AGE vs. AGE-0μg/ml. n=8. NG:HG p<0.0125 main effect for glucose condition.

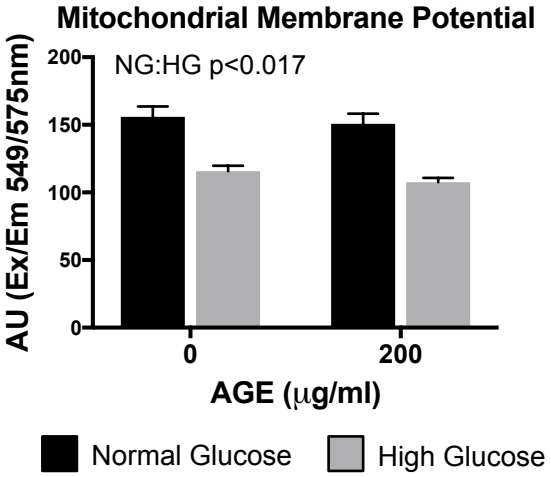

Figure 9: Mitochondrial Membrane Potential

NG:HG $p<0.025$ main effect for glucose condition (n=4).

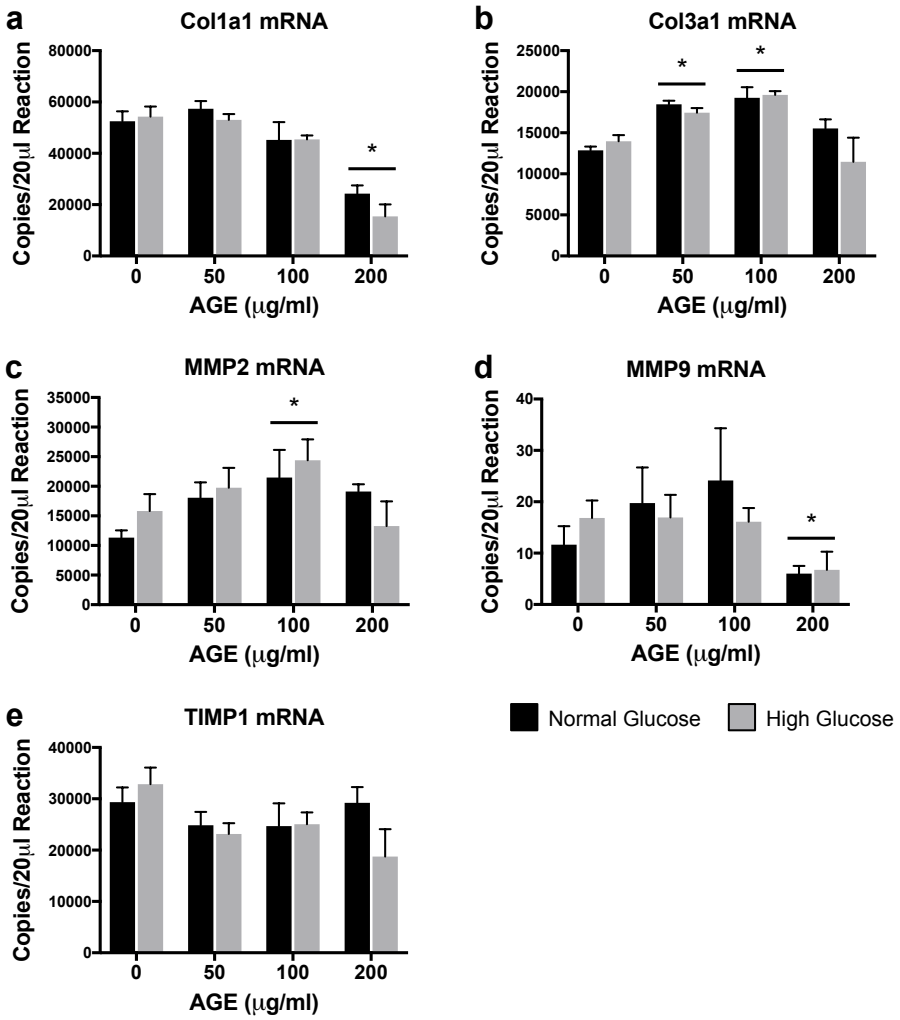

Figure 10: Transcriptional Analysis of Extracellular Matrix Remodeling

a-e. ddPCR gene transcript counts for Collagen alpha-1(I) chain (Col1a1), Collagen alpha-1(III) chain (Col3a1), Matrix metallopeptidase 2 (MMP2), Matrix metalloproteinase 9 (Col3a1), and Tissue inhibitor of matrix metalloproteinase 1 (TIMP1). n=4. *$p<0.0125$ main effect for AGE vs. AGE-0μg/ml.

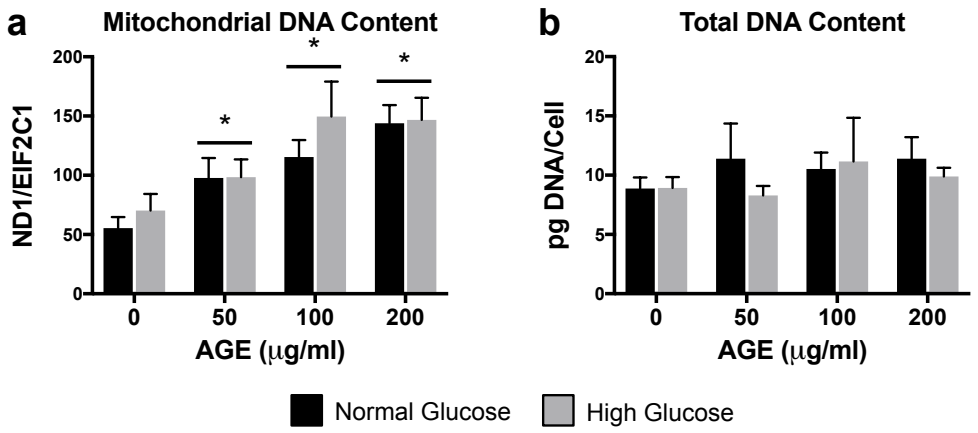

Figure 11: Mitochondrial DNA Content

a. mtDNA content quantified by ratio of mitochondrial gene, NADH dehydrogenase subunit 1 (ND1), to nuclear reference gene, protein argonaute-1 (EIF2C1). **b.** Total DNA content normalized to cell counts. n=8. *$p<0.0125$ main effect for AGE vs. AGE-0μg/ml.

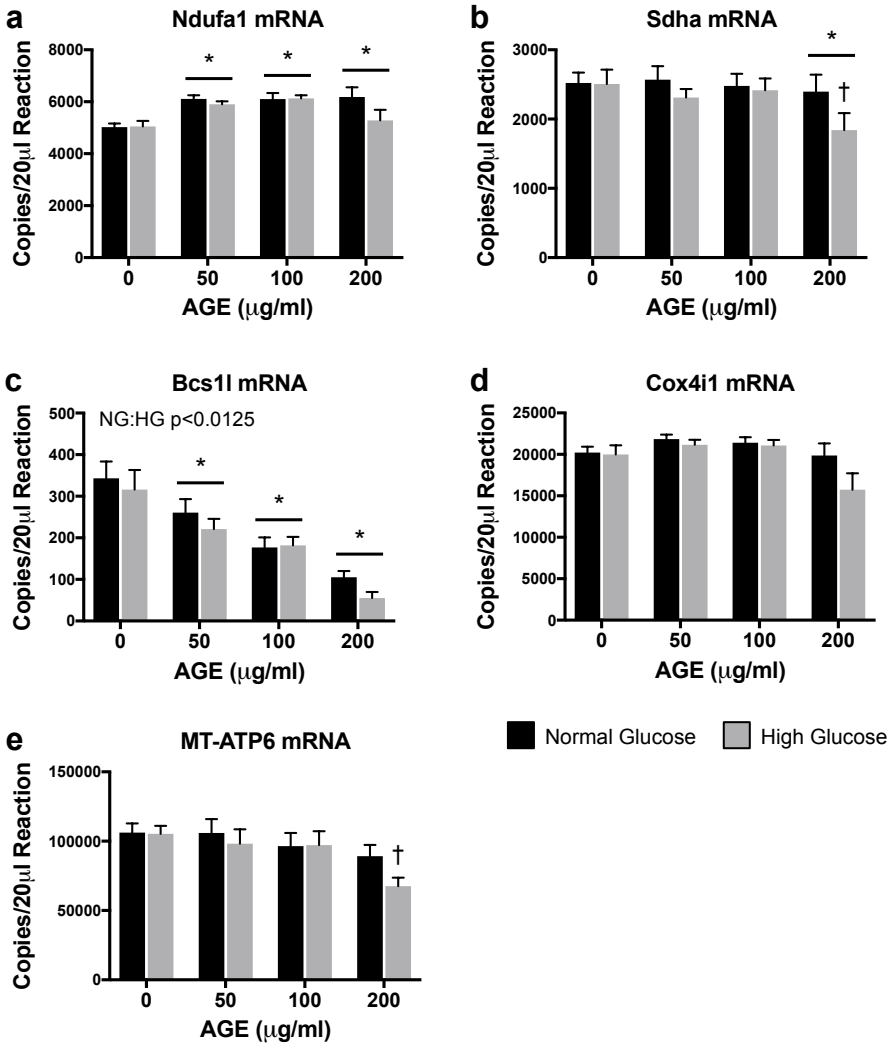

Figure 12: Transcriptional Analysis of Mitochondrial Complexes

a-e. ddPCR gene transcript counts for NADH:ubiquinone oxidoreductase subunit A1 (Ndufa1), Succinate dehydrogenase complex flavoprotein subunit A (Sdha), BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone (Bcs1l), Cytochrome c oxidase subunit 4i1 (Cox4i1), and ATP synthase 6, mitochondrial (MT-ATP6). n=8. *$p<0.0125$ main effect for AGE vs. AGE-0µg/ml. †$p<0.05$ vs. HG AGE-0µg/ml. NG:HG $p<0.0125$ main effect for glucose condition.

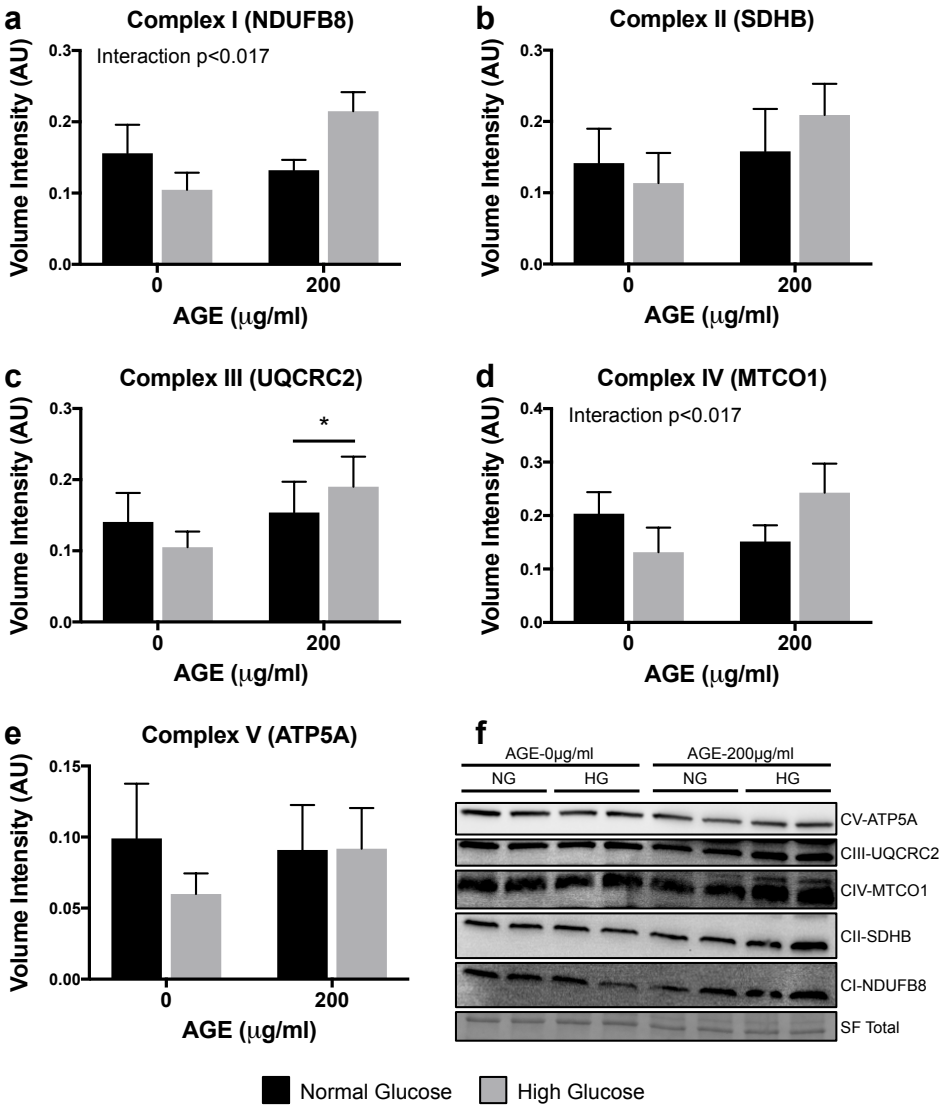

Figure 13: Protein Analysis of Mitochondrial Complexes

a-e. OXPHOS protein expression for mitochondrial complex I (NDUFB8), complex II (SDHB), complex III (UQCRC2), complex IV (MTCO1), and complex V (ATP5A). F. Representative cropped blot images (displayed in order of molecular weight) including Stain Free (SF) UV visualized total protein. For each sample, all five targets were probed on the same blot. Individual representative images of targets from different parts of the same blot are cropped due to different exposure times required for each target (Signal Accumulation Mode). Representative images were chosen from two of the four blots. n=4. *p<0.017 main effect for AGE vs. AGE-0μg/ml. Interaction p<0.017, interaction between AGE and glucose condition.

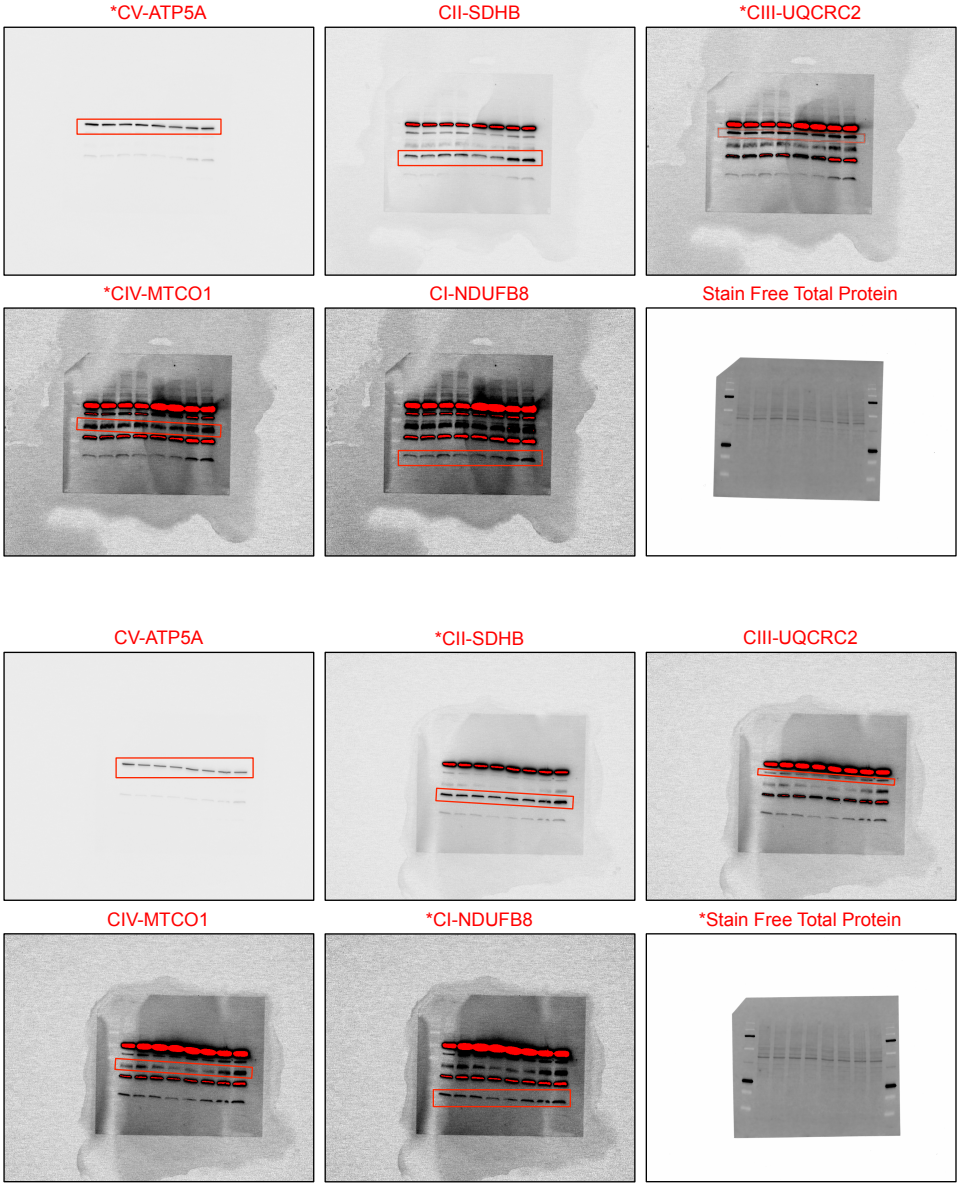

Figure 14: Two Full and Unedited Blots for Figure 13

All five targets were probed on the same membrane, but required different exposure times using signal accumulation mode (ChemiDoc, BioRad). Blot images are shown in order of exposure time and (*) indicates the image that was cropped and used for representative purposes in Figure 13f.

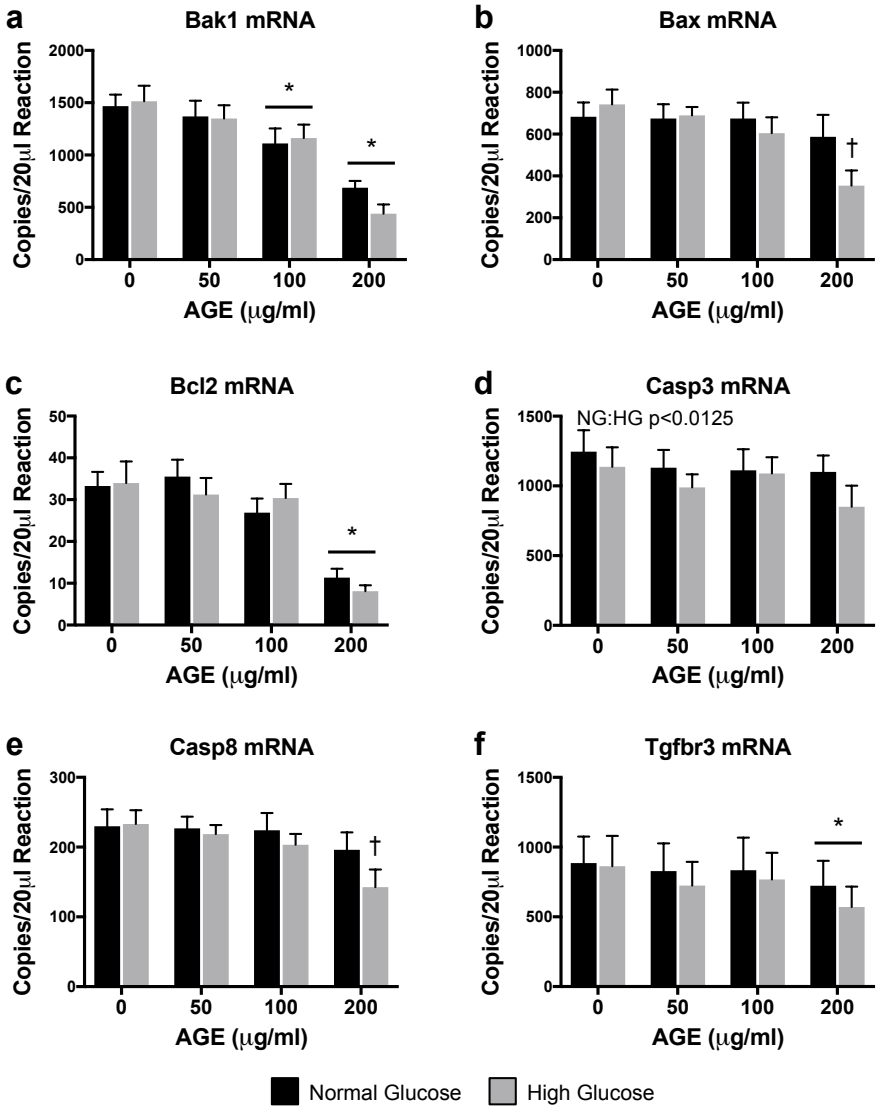

Figure 15: Transcriptional Analysis of Mitochondrial Apoptosis

a-f. ddPCR gene transcript counts for BCL2-antagonist/killer 1 (Bak1), BCL2 associated X, apoptosis regulator (Bax), B-cell lymphoma 2, apoptosis regulator (Bcl2), Caspase 3 (Casp3), Caspase 8 (Casp8), and Transforming growth factor beta receptor 3 (Tgfbr3). n=8. *$p<0.0125$ main effect for AGE vs. AGE-0µg/ml. †$p<0.05$ vs. HG AGE-0µg/ml. NG:HG $p<0.0125$ main effect for glucose condition.

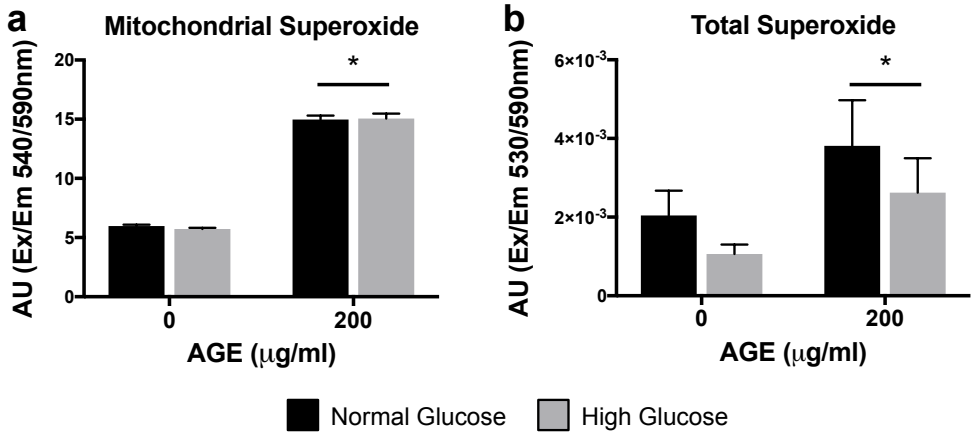

Figure 16: ROS/Superoxide Production

a. Mitochondrial Superoxide. **b.** Total Superoxide. n=4. *p<0.025 main effect for AGE vs. AGE-0μg/ml.

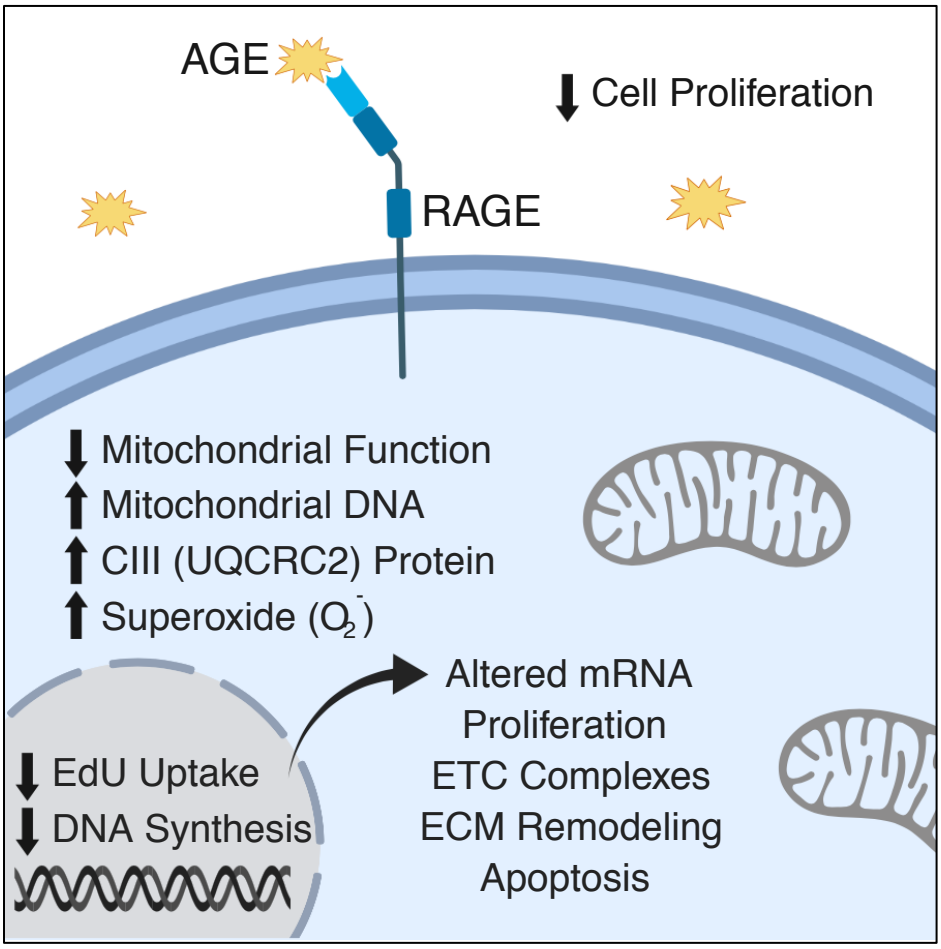

Figure 17: Summary of Major AGE-Mediated Findings

Figure created with BioRender.

CHAPTER 4. DESCRIPTIVE TRANSCRIPTOME ANALYSIS OF TENDON DERIVED FIBROBLASTS FOLLOWING IN-VITRO EXPOSURE TO ADVANCED GLYCATION END PRODUCTS

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Running Head: AGEs and RNA sequencing in tendon fibroblasts

Keywords: tendon, diabetes, advanced glycation end-products, transcriptome, RNA Sequencing

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4.1 Abstract

Introduction: Tendon pathologies affect a large portion of diabetic patients. This high rate of tendon pain, injury, and disease appears to manifest independent of well-controlled HbA1c and fasting blood glucose. Previous work has indicated that advanced glycation end products (AGEs) severely impact tendon fibroblast proliferation and mitochondrial function. The purpose of this study was to investigate transcriptome wide changes that occur to tendon-derived fibroblasts following treatment with AGEs. We propose to complete a descriptive approach to pathway profiling to broaden our mechanistic understanding of cell signaling events that may contribute to the development of tendon pathology.

Methods: Rat Achilles tendon fibroblasts were treated with glycolaldehyde-derived AGEs (200µg/ml) for 48 hours in normal glucose (5.5mM) conditions. Total RNA was isolated and the PolyA$^+$ library was sequenced.

Results: We demonstrate that tendon fibroblasts treated with 200µg/ml of AGEs differentially express 2,159 gene targets compared to fibroblasts treated with an equal amount of BSA-Control. Additionally, we report in a descriptive and ranked fashion, 21 implicated cell-signaling pathways.

Conclusion: Our findings suggest that AGEs disrupt the tendon fibroblast transcriptome on a large scale and that these pathways may contribute to the development and progression of diabetic tendinopathy. Specifically, pathways related to cell cycle progression and extracellular matrix remodeling were largely affected in our data set and these changes may play a contributing role in the development of diabetic tendon pathology.

4.2 Introduction

Debilitating tendon pathologies, collectively referred to as tendinopathies, affect the majority of patients with type 1 and 2 diabetes and are responsible for a considerable reduction in the quality of life (1, 2). It is well documented that diabetes is associated with changes to tendon tensile properties and extensive tendon degeneration (1-8). Interestingly, changes in tendon

mechanical (e.g. stiffness) and structural (reduced fibril diameter, fibril disorganization) properties noted with diabetes persist even in patients with superior glucose control (HbA1c<6.5). Simply controlling blood glucose does not improve tendon properties in diabetic individuals nor does it reduce the risk of developing tendinopathies (9). Therefore, it is highly likely that other unexplored mechanisms contribute to the development of diabetic tendinopathies.

Tendons of diabetic patients are thicker than non-diabetic controls (1, 9-12). Advanced glycation end product (AGE) formation due to chronic hyperglycemia has been shown to increase tendon stiffness (13-19). Under normal physiological conditions, oxidation of lysine and hydroxylysine by lysyl oxidase (LOX) forms cross-links between adjacent collagen fibrils (20), which increases the tensile strength of tendon and stabilizes the collagen fibril assembly (21, 22). AGEs represent a second type of collagen cross-link that is normally very low in young healthy tendons (23). Non-enzymatic AGE cross-links accumulate in long-lived extracellular matrix proteins, such as collagen, resulting in excessive tissue stiffness (23). This process is associated with normal aging but is accelerated in diabetes (14, 18, 23, 24).

In addition to forming non-enzymatic cross-links, unbound AGEs are ligands for the receptor for AGEs (RAGE). Interaction of AGE with RAGE mediates a multitude of cell-signaling events such as increased formation of reactive oxygen species and pro-inflammatory pathways (25, 26). AGE formation as a result of chronic uncontrolled hyperglycemia and spontaneous oxidation of glycolytic intermediates contributes significantly to elevated serum levels of circulating AGE adducts in diabetic patients (27, 28). Additionally, AGE-rich diets can increase levels of unbound serum AGEs (29). Our laboratory has established that *in vitro* exposure of AGEs disrupts several genes responsible for cell proliferation, extracellular matrix (ECM) regulation, and mitochondrial function in tendon-derived fibroblasts, all of which are areas of interest to tendon pathology and healing (5, 30-35).

Diabetes affects nearly 30 million Americans and many of these individuals suffer from painful diabetic tendinopathies and conditions aggravated by tendinopathies (2, 36). Although diabetic tendinopathies are pervasive and disabling there are few effective therapies for these conditions. Additionally, the paucity of knowledge regarding the pathogenesis of diabetic tendinopathies further contributes to the lack of successful therapeutic options. A thorough understanding of the cell signaling events contributing to the development of AGE-mediated diabetic tendinopathies will assist in exploring alternative areas of thought and develop

therapeutic options to target this large patient population. Therefore, we sought to characterize the alterations to the tendon fibroblast transcriptome following exposure to AGEs. Although many of these pathways have already been implicated with AGEs, the primary goal of this study was to establish a descriptive and ranked evaluation of pathway disruptions that occur to tendon fibroblasts following an AGE insult.

4.3 Materials and Methods

4.3.1 Animal Protocol

Animals utilized in this study were from a previous investigation (37). The study was approved by the Purdue University Institutional Animal Care and Use Committee and all animals were cared for in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Eight-week-old female Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained for an additional eight weeks. Rats were housed on a 12-hour light-dark cycle and provided access to standard rat chow and water ad libitum. At sixteen weeks (Final Weights: 256.43±5.19g), rats were euthanized by decapitation after CO_2 inhalation.

4.3.2 Tendon Fibroblast Isolation and Cell Culture

Briefly, Achilles tendons were rinsed with sterile PBS, minced, placed in DMEM containing 0.2% type I collagenase, and incubated in a 37°C shaking water bath for four hours. After tissue digestion, the cell suspension was filtered through a 100µm mesh filter, pelleted by centrifugation, resuspended in 5.5mM glucose DMEM containing 10% FBS, 1% sodium pyruvate (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (Thermo Scientific, Waltham, MA) and plated in 100mm collagen coated dishes. After reaching confluency, tendon fibroblasts were split and seeded (100,000 cells) in 100mm collagen coated culture plates. Each donor animal's tendon fibroblasts were treated separately with 200µg/ml of BSA-Control or AGE-BSA for 48 hours for downstream paired DESeq2 analysis. Tendon fibroblasts treated at passages 2-4 were used for RNA isolation and RNA-sequencing (RNAseq).

4.3.3 RNAseq

Total RNA was isolated as previously described (37). Briefly, RNA was isolated after BSA-Control or AGE-BSA treatment using the Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA). On-column DNase digestion was completed on all samples prior to elution of RNA. Total RNA from BSA-Control (n=5) and AGE-BSA (n=5) treated tendon fibroblasts was submitted to the Purdue University Genomics Core Facility (West Lafayette, IN) for PolyA$^+$ library construction. Integrity of input total RNA was assessed using a Bioanalyzer RNA Nano chip (Agilent 2100, Santa Clara, CA). Libraries from 500ng of input total RNA were constructed largely as directed by the Nugen Universal Plus mRNA-Seq + UDI kit (PN#9144-96), but the RNA fragmentation time was decreased from 8 minutes to 4 minutes. Final library products were subjected to a 0.7 Ampure:1 Sample ratio purification to reduce lower molecular weight amplicons. The resulting libraries were assessed with an Agilent DNA High Sensitivity Chip for yield and quality and sequenced by Novogene (Sacramento, CA). Ten libraries were pooled and evenly distributed across a single HiSeq lane to generate ~40,000,000 2X150bp reads on the HiSeq 4000 platform (Illumina, San Diego, CA).

4.3.4 Bioinformatics

RNAseq raw data set quality and analysis was completed using Basepair software (New York, NY) pipelines. Reads were first aligned to the transcriptome derived from rn6 genome assembly using STAR with default parameters (38). Read counts for each transcript were measured using featureCounts and differentially expressed genes were determined using DESeq2 using a paired analysis (39, 40). An adjusted p-value cut-off of 0.05 (corrected for multiple hypotheses testing) was used. GSEA was performed on normalized gene expression counts, using gene permutations for calculating p-value. A $\log_2$ fold change cut-off of 1.5 was enforced.

4.3.5 Descriptive Pathway Profiling

To preserve unbiased gene target selection, while maintaining hypothesis-driven pathway selection, GeneGlobe (Qiagen, Hilden, Germany) pathway database was utilized to complete a descriptive approach to pathway analysis. Heat maps were generated based on GeneGlobe RT^2 profiler arrays independent of whether those gene targets were significantly altered in our dataset.

Gene targets that existed in the RT^2 profilers but not in our dataset were excluded from heat maps. To rank the most implicated pathways, the percentage of significantly altered genes, both increased and decreased, was calculated based on the number of total genes included in each pathway's respective heat map. This systematic approach was employed to maintain an objective view of the global data.

4.3.6 Pathway Analysis

RNAseq data was imported into Ingenuity Pathway Analysis (IPA, Qiagen) to determine select pathways and biological functions that were altered in response to AGE-BSA treatment.

4.4 Results

4.4.1 Overview

A total of 2,159 genes within our data set met the criteria of $q<0.05$ and fold change of greater than or less than 1.5 ($\log_2$ fold change greater than or less than 0.584). There were 1,046 genes that were significantly increased, and 1,113 genes that were significantly decreased (Figure 18).

4.4.2 Most Affected Gene Targets

The top ten increased and the top ten decreased gene targets within our data set were identified based on our $\log_2$ fold change and adjusted p-value thresholds. The top ten increased gene targets in order of highest to lowest positive $\log_2$ fold change were Cyp1a1, Pipox, Btc, Slc22a14, Tbxas1, Itgb2, Slc13a3, Cldn1, Ncf1, and Tnfrsf17 (Table 5). The top ten decreased gene targets in order of highest to lowest negative $\log_2$ fold change were Pimreg, Pmch, E2f7, Pbk, Parpbp, Ube2c, Troap, Cenpf, Cldn23, and Ccnb2 (Table 5).

4.4.3 Descriptive Pathway Profiling

A total of 21 GeneGlobe (Qiagen) pathways were explored. Pathway selection was based on the literature, hypotheses that we have explored previously, and hypotheses we plan to explore in future studies. Select pathways that have strong association to AGE/RAGE biology

were also included. Pathways were ranked strictly based on the percentage of significantly altered genes within that respective pathway. Pathways explored, in order from most to least implicated, were cell cycle (51.2 %, Fig 19), ECM and tenogenic markers (48.4%, Fig 20), DNA damage (40.3%, Fig 21), cellular senescence (39.2%, Fig 22), p53 signaling (38.7%, Fig 23), TGF-β signaling (32.4%, Fig 24), fibrosis (29.2%, Fig 25), oxidative stress (28.1%, Fig 26), wound healing (23.8%, Fig 27), growth factors (21.9%, Fig 28), transcription factors (20.6%, Fig 29), cytoskeleton (16%, Fig 30), cytokines (14.9%, Fig 31), innate and adaptive immunity (13.2%, Fig 32), NF-κB signaling (11.3%, Fig 33), cellular stress responses (10%, Fig 34), mitochondria (9.5%, Fig 35), apoptosis (8.5%, Fig 36), glycosylation (8.2%, Fig 37), inflammasomes (7.8%, Fig 38), and mitochondrial energy metabolism (2.6%, Fig 39). Pathways, listed in order of most implicated along with respective figure numbers for heat maps, are summarized in Table 6.

4.4.4 Pathway Analysis

Using the IPA disease and function tool, ten pathways or biological functions were selected. Apoptosis (Z Score: 4.70), morbidity and mortality (Z Score: 4.53), organismal death (Z Score: 4.47), DNA damage (Z Score: 3.36), and diabetes mellitus (Z Score: 2.24) were selected as activated pathways. Cell survival (Z Score: -4.91), cell viability (Z Score: -4.62), repair of DNA (Z Score: -3.85), cell proliferation (Z Score: -3.67), and growth of connective tissue (Z Score: -3.02) were selected as inhibited pathways. IPA pathways are summarized in Table 7 with respective p-values and activation Z-scores.

4.5 Discussion

Diabetes is a significant burden to the healthcare system and affects the quality of life for ~30 million Americans. Diabetes-related complications, such as those implicating connective tissue, further impact quality of life. Our knowledge of diabetes-related tendon pathology has primarily been limited to macroscopic and structural changes and thus, very limited molecular insight exists. In this study, we report for the first time the use of RNAseq methodology to complete a descriptive transcriptome profile of Achilles tendon-derived fibroblasts following AGE exposure. The primary goal of this study was to identify and rank pathways that appear to

be most implicated following AGE exposure to allow for more precise mechanistic exploration of AGE-mediated development of diabetic tendon pathology.

Tendinopathies can be devastating often requiring surgery and lengthy physical therapy with a high incidence of post-injury complications (41). Unfortunately, the mechanisms responsible for noted tendon degeneration and alterations to mechanical properties during diabetes are not well understood. Recent evidence has implicated the AGEs in development of tendon pathology (37). AGE formation as result of chronic hyperglycemia and spontaneous oxidation of glycolytic intermediates contributes significantly to elevated levels of circulating AGE adducts in diabetic patients (27, 28). AGEs form at an accelerated rate in diabetes and these modifications can result in non-enzymatic cross-links in long-lived extracellular proteins such as tendon collagen, which increase tissue stiffness and alter tissue mechanical properties (16, 17, 42-44). More importantly, circulating AGE adducts are able to interact with RAGE to initiate a noxious feed forward cycle of sustained inflammation and tissue damage (45). Previous work from our laboratory has demonstrated that AGEs induce severe limitations to tendon fibroblast proliferative capacity and mitochondrial function, while increasing mitochondrial DNA content, all of which are variables that indicate histopathology (37, 46). In order to provide more effective therapeutic options to reduce tendon degeneration and associate sequela in diabetic patients, it is important that we further develop and expand our understanding of the basic molecular mechanisms contributing to tendinopathies in this large patient population. For the purposes of this discussion, we focused on gene targets associated with cell cycle (Figure 19), ECM remodeling (Figure 20), and wound healing (Figure 27). While we demonstrate in Table 6 several other implicated pathways, these pathways were selected to retain relevance to development and progression of tendon pathology.

Our transcriptome analysis revealed that genes associated with the cell cycle are the most implicated (Figure 19). Tendon fibroblast proliferation is a vital component to tendon development, adaptation, and healing (33, 34). Inability of tenocytes to proliferate in the presence of AGEs would significantly precipitate the development of tendinopathy (47). Tendinopathy is thought to develop, in part, from a failed healing response to minor tendon damage during loading. Tendinopathies in diabetic patients are generally more pervasive and can present with more severe degeneration, which may, in part, be driven by prolonged injury status (2, 48, 49). We have previously demonstrated incorporation of synthetic nucleoside 5-ethynyl-2´-

deoxyuridine (EdU), in tendon-derived fibroblasts to be ~3% following AGE-BSA (200µg/ml) exposure as compared to ~53% in the BSA-Control exposed group, which proliferate normally (37). Reduced proliferative capacity occurred in conjunction with reductions to proliferative gene markers, Mybl2 and Pcna, and reduced absorbance values of cytostatic MTT with AGE-BSA treatment in tendon fibroblasts. Our RNAseq data corroborate our previous findings of reduced Mybl2 and Pcna gene expression, and revealed several additional genes responsible for cell cycle progression to be impacted (Figure 19).

Gene targets associated with ECM maintenance and remodeling were also dramatically affected in our dataset. The ECM is vital to tendon tissue health and serves several important functions including cell adhesion, communication, and differentiation. Additionally, the ECM provides structural and biochemical support to the surrounding resident cell population. The tendon ECM consists primarily of type I and type III collagen fibers surrounded by proteoglycans which assist with the assembly and stability of collagen fibrils (50). Precise and linear arrangement of collagen fibrils is vital to tissue integrity, and therefore mechanical function (51). Inclusion of multiple collagen isoforms allows the ECM to specialize and adapt to specific types of mechanical loading and functional responses (52). For instance, type I collagen (Col1a1) is a stronger isoform of collagen, whereas type III collagen (Col3a1) is weaker and generally upregulated in the early stages of tissue remodeling following exercise or during initial stages of healing (53, 54). Col3a1 is able to provide temporary tensile strength to the tissue assembly until it is later replaced by stronger Col1a1 (55). Although Col1a1 mRNA was unaffected in our RNAseq data set, Col3a1 mRNA expression was significantly increased (Figure20). Similarly, our previous report indicated Col3a1 mRNA expression to be increased with 50µg/ml and 100µg/ml AGE exposure when compared to an equal dose of BSA-Control (37). It is likely that this increase in Col3a1 mRNA expression is in response to the AGE insult and an attempt to maintain the ECM environment. Further, gene expression of decorin (Dcn), the most abundant tendon proteoglycan, was increased in our RNAseq data set (Figure 20). Dcn aids in maintenance and regulation of collagen fibril structure as well as regulation of resident fibroblast proliferation (51). As a key regulator in matrix assembly, loss of Dcn would likely prove to be unfavorable to the strength of the tendon assembly, which would decrease the tissue's ability to withstand sudden strain (51). Our observed increased in Dcn gene expression

may be a compensatory response to the AGE insult. However, the effect of a loss to Dcn protein or gene expression would need to be externally validated in whole diabetic tendon.

Lysine and hydroxylysine are found within the collagen amino acid sequence and play an important role in cross-link formation. Oxidation of lysine and hydroxylysine, by lysyl oxidase (Lox), forms cross-links within collagen fibrils, which contributes to tissue integrity by increasing tensile strength and stabilizing the collagen fibril assembly. Strength and stability of the tissue assembly are important, especially given the high contractile forces transmitted from muscle to bone during skeletal muscle contraction. Our dataset revealed Lox gene expression to be significantly reduced following AGE exposure (Figure 20). If in fact reduced mRNA expression of Lox coincides with reduced enzymatic cross-link formation, AGEs may contribute to a weakened tendon assembly due to loss of enzymatic cross-links between adjacent collagen fibrils. While tendons of diabetic animals are shown to be stiffer, likely a result of non-enzymatic AGE cross-linking, they generally have a reduced load to failure capacity which may be a result of greater tissue degeneration at the macroscopic level (5, 56, 57). More work is needed to determine the impact of AGEs on the whole tendon fibril assembly.

Remodeling of the ECM is largely regulated by enzymes known as matrix metalloproteinases (MMPs), which contribute to the degradation portion of ECM remodeling. Specifically, collagenases such as MMP-1 and MMP-13 are able to cleave type I collagen molecules in the ECM once they are secreted from the cell. Similarly, gelatinases, such as MMP-2 and MMP-9 are released from cells and degrade collagen isoforms in the ECM. MMPs are transcribed and translated as proenzymes and then secreted into the ECM and later activated through proteolytic cleavage of the N-terminal. Although MMP activity is degenerative, it is essential in facilitating ECM remodeling and adaptation of tendon tissue. MMP activity can be reversibly inhibited by tissue inhibitors of metalloproteinases (TIMPs). Degenerative MMP activity is inhibited when a TIMP molecule binds to the active site of an MMP enzyme. TIMPs play an important role to ECM remodeling by limiting MMP activity and preventing excessive degradation. Counter-regulation via TIMP activity tightly regulates the breakdown and synthesis of collagen in response to external stresses, such as mechanical loading. Loss of ECM regulation, such as favoring degradation over synthesis, could alter the ECM responses resulting in damage to the tissue assembly. It is thus no surprise that the dysregulation of degenerative enzymes, such as MMPs, have been thought to play an important role in the development of tendon pathology

in diabetes as overexpression of MMPs may favor ECM degradation (58). Similarly, if inhibitory TIMPs are less expressed, the environment will favor degradation by allowing MMPs to act on the ECM for a longer period of time.

Previous reports have indicated that AGEs increase MMP -2, -3, -9, and -13 secretion and expression in chondrocytes with 100μg/ml of AGEs (59, 60). Further, mRNA expression of MMP -1, -3, and -13 in porcine chondrocytes was increased with 100μg/ml of AGE exposure (61). Our RNAseq analysis confirmed MMP -2 and -3 to be elevated, along with MMP -15 and -17. However, we did not observe any changes to TIMP -1, -2, -3, or -4 in our RNAseq dataset, suggesting that MMPs may be exerting their function in an unorganized fashion that would favor a degenerative ECM environment. MMP gene expression data is limited in scope as it does not account for ECM secretion and N-terminal cleavage. However, the large impact that AGE exposure has to ECM-related gene expression is further evidence that elevated serum AGEs may be contributing to the development of connective tissue pathology in diabetic populations (Figure 20).

Delayed and abnormal healing is a common complication of both type I and type II diabetes (62, 63). Not only does it appear that diabetic patients are at risk of developing tendon tears, but healing post-repair is also impaired (1, 64, 65). Interestingly, transforming growth factor (TGF)β1 expression was reduced in our RNAseq data (Figure 24 and 27). In addition to TGFβ1 being one of the affected genes in the wound-healing pathway (Figure 27), the GeneGlobe TGFβ signaling pathway was also strongly affected (Table 6 and Figure 24). TGFβ is a key factor in the regulation of fibrosis and ECM homeostasis (66). It has previously been demonstrated that TGFβ levels are significantly reduced in diseased human rotator cuff tendon samples (66). Further, TGFβ can modulate inflammation by influencing fibroblast recruitment and stimulating collagen production (67, 68). Inconsistent with this, Col1a1 was unchanged in our RNAseq dataset and Col3a1 was increased (Figure 20). However, expression of Col5a1, Col5a2, and Col5a3 mRNA was reduced in our RNAseq dataset. Type V is a fibrillar collagen isoform that is found less abundantly in tendon, but exists to provide support to other tissues that rely more heavily on type V collagen isoforms (69). While the wound-healing GeneGlobe pathway was not as affected as other pathways, it is still likely that these gene targets contribute in some manner to the delayed healing response that is commonly observed following tendon injury in diabetic patients.

Several studies have shown that risk of developing tendinopathy is greater in those with diabetes mellitus (1, 8, 64, 65). Our new data highlights several cell-signaling pathways affected by AGE exposure, thus expanding our understanding of the diabetic tendon phenotype. While our discussion to only transcriptome data, the purpose of this study was to complete a descriptive profile of the AGE insult to tendon fibroblasts. This is the first data set to utilize RNAseq methodology to study the tendon fibroblast transcriptome following AGE exposure and these data will prove to be useful for further elucidation of the diabetic tendon disease process.

4.6 References

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Table 5: Most Affected Gene Targets

Gene	$\log_2$ Fold Change	q Value
Cyp1a1	7.07	6.77E-07
Pipox	4.78	7.59E-04
Btc	4.70	1.87E-05
Slc22a14	4.66	2.49E-04
Tbxas1	4.08	1.46E-02
Itgb2	4.06	3.42E-02
Slc13a3	4.05	4.78E-03
Cldn1	4.03	3.80E-06
Ncf1	4.01	1.19E-03
Tnfrsf17	3.94	9.65E-03
Pimreg	-4.94	1.01E-12
Pmch	-4.83	1.49E-33
E2f7	-4.45	8.80E-08
Pbk	-4.29	3.99E-05
Parpbp	-4.24	3.47E-23
Ube2c	-4.19	7.41E-24
Troap	-4.13	3.23E-07
Cenpf	-4.12	8.15E-11
Cldn23	-4.10	4.98E-03
Ccnb2	-4.08	5.44E-25

Table 6: Descriptive Pathway Profiling

Figure	GeneGlobe Pathway	Altered Genes in Pathway	Total Genes in Pathway	Percent Affected
19	Cell Cycle	42	82	51.2
20	ECM and Tenogenic Markers	31	64	48.4
21	DNA Damage	29	72	40.3
22	Cellular Senescence	31	79	39.2
23	p53 Signaling	29	75	38.7
24	TGF-β Signaling	24	74	32.4
25	Fibrosis	19	65	29.2
26	Oxidative Stress	18	64	28.1
27	Wound Healing	15	63	23.8
28	Growth Factors	14	64	21.9
29	Transcription Factors	14	68	20.6
30	Cytoskeleton	13	81	16
31	Cytokines	7	47	14.9
32	Innate and Adaptive Immunity	7	53	13.2
33	NF-κB Signaling	8	71	11.3
34	Cellular Stress Responses	7	70	10
35	Mitochondria	7	74	9.5
36	Apoptosis	6	71	8.5
37	Glycosylation	6	73	8.2
38	Inflammasomes	5	64	7.8
39	Mitochondrial Energy Metabolism	2	77	2.6

Table 7: Select IPA Pathway Analysis

Pathway	p Value	Activation Z Score
Apoptosis	1.45E-33	4.70
Morbidity or Mortality	4.62E-34	4.53
Organismal Death	2.06E-33	4.47
DNA Damage	7.32E-09	3.36
Diabetes Mellitus	1.27E-13	2.24
Cell Survival	4.74E-25	-4.91
Cell Viability	5.59E-23	-4.62
Repair of DNA	7.15E-15	-3.85
Cell Proliferation (Fibroblast)	7.59E-12	-3.67
Growth of Connective Tissue	5.10E-23	-3.02

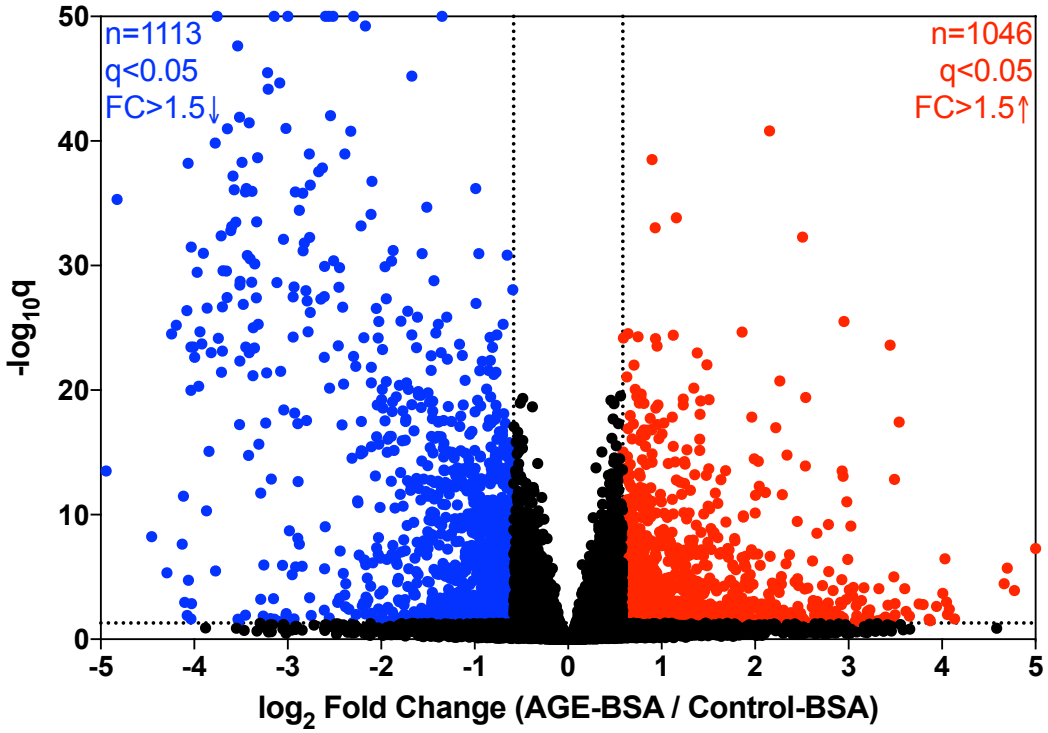

Figure 18: Volcano Plot Overview of RNA Sequencing Results

Each point represents a single gene target. Red (n=1046) indicates significant increase in gene expression. Blue (n=1113) indicates significant decrease in gene expression. Black (n=10,648) indicates gene targets that were either unaltered or did not meet our thresholds of $q<0.05$ and fold change of greater that 1.5 or less than -1.5.

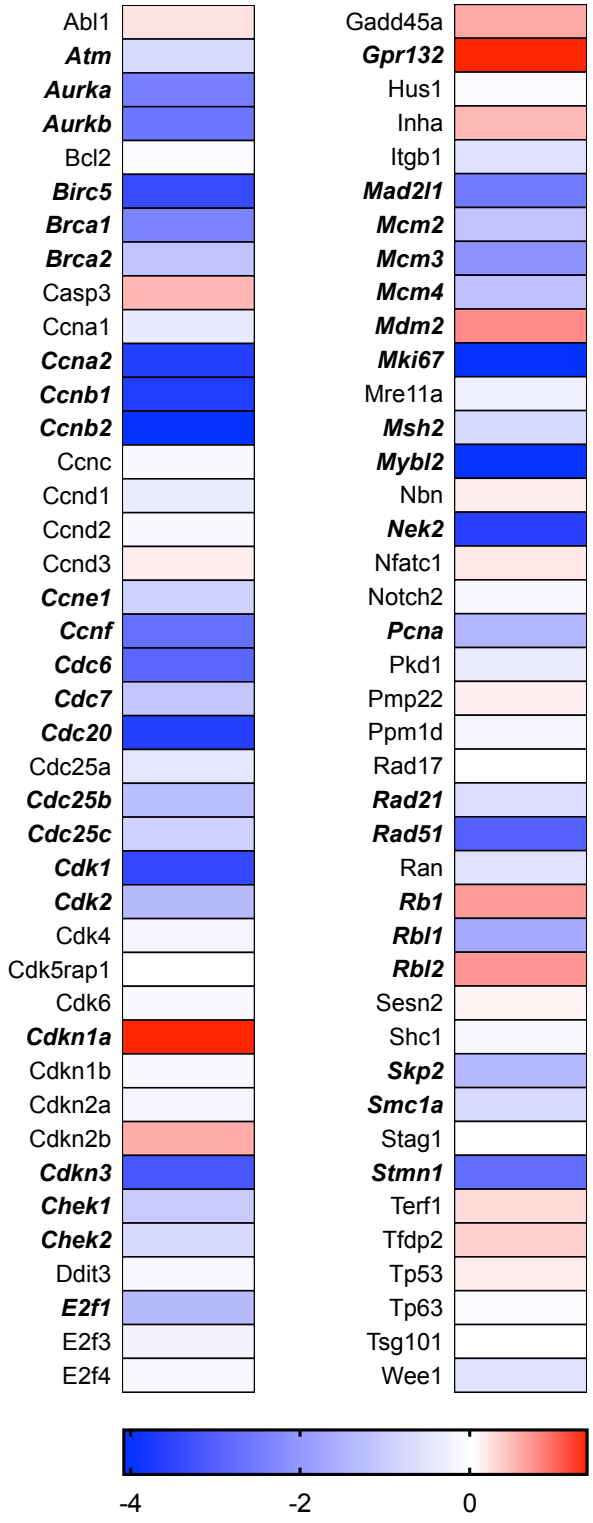

Figure 19: Cell Cycle Heat Map

Bold text indicates significantly altered gene targets.

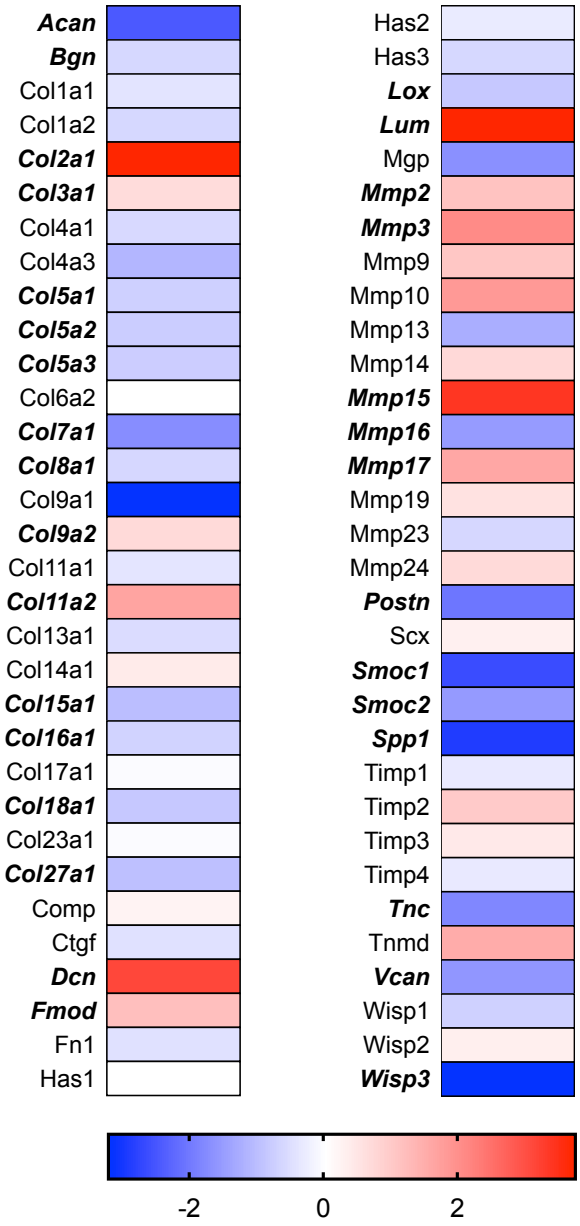

Figure 20: ECM and Tenogenic Markers Heat Map

Bold text indicates significantly altered gene targets.

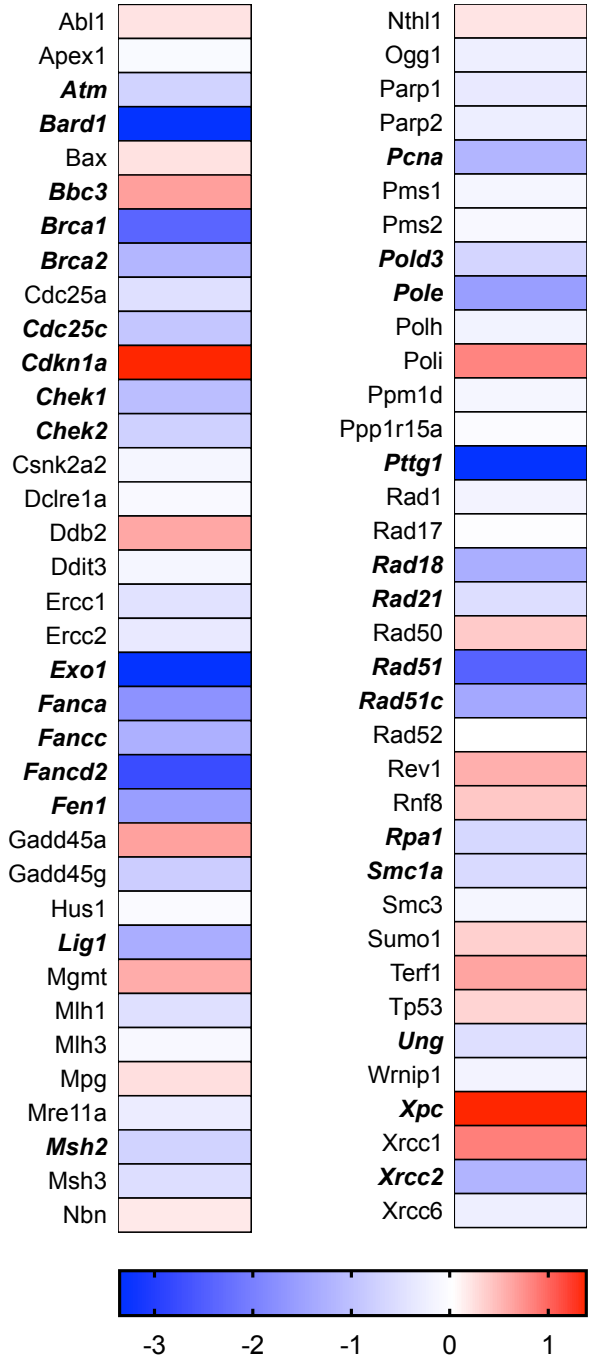

Figure 21: DNA Damage Heat Map

Bold text indicates significantly altered gene targets.

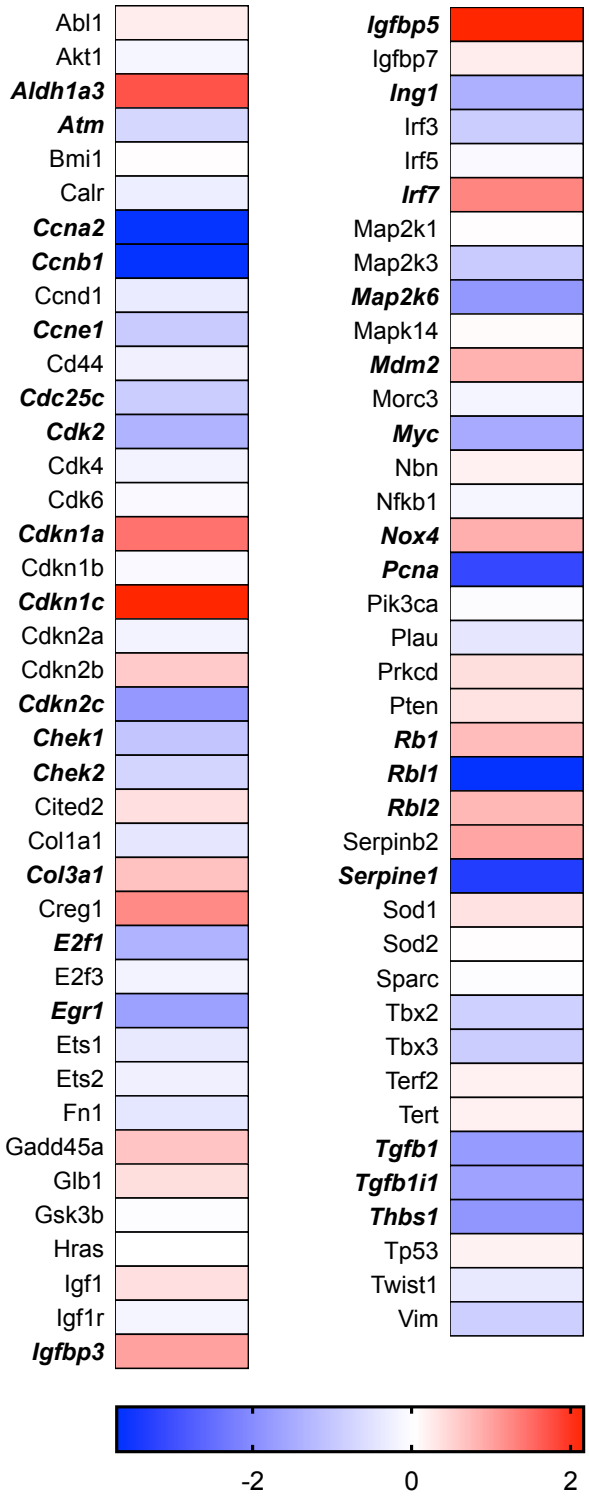

Figure 22: Cellular Senescence Heat Map

Bold text indicates significantly altered gene targets.

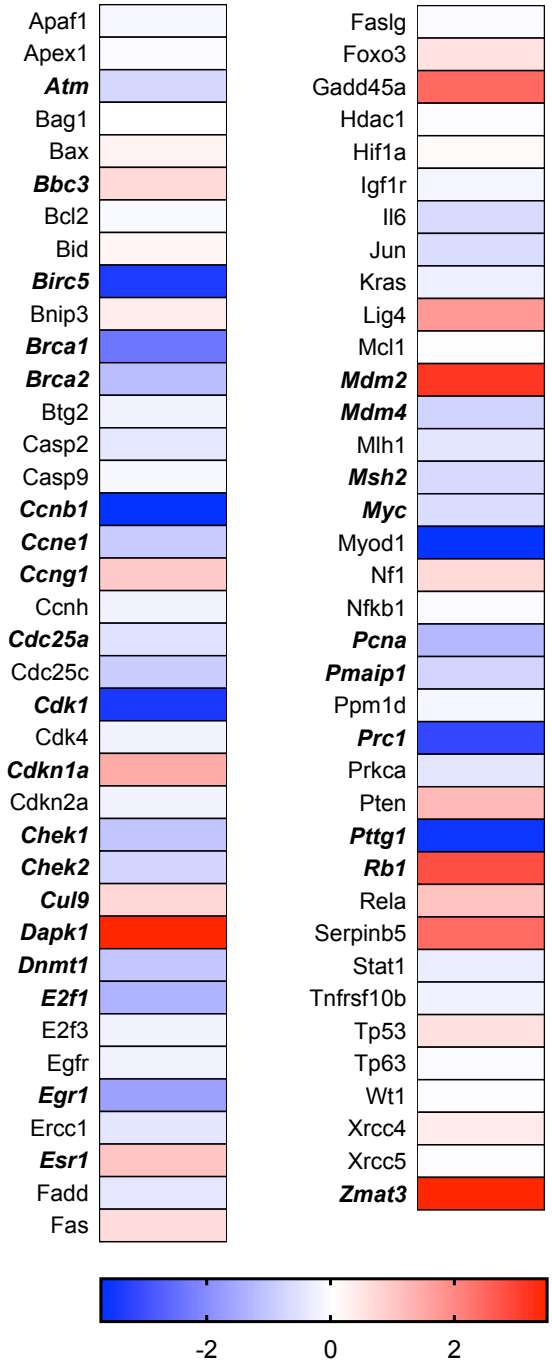

Figure 23: p53 Signaling Heat Map

Bold text indicates significantly altered gene targets.

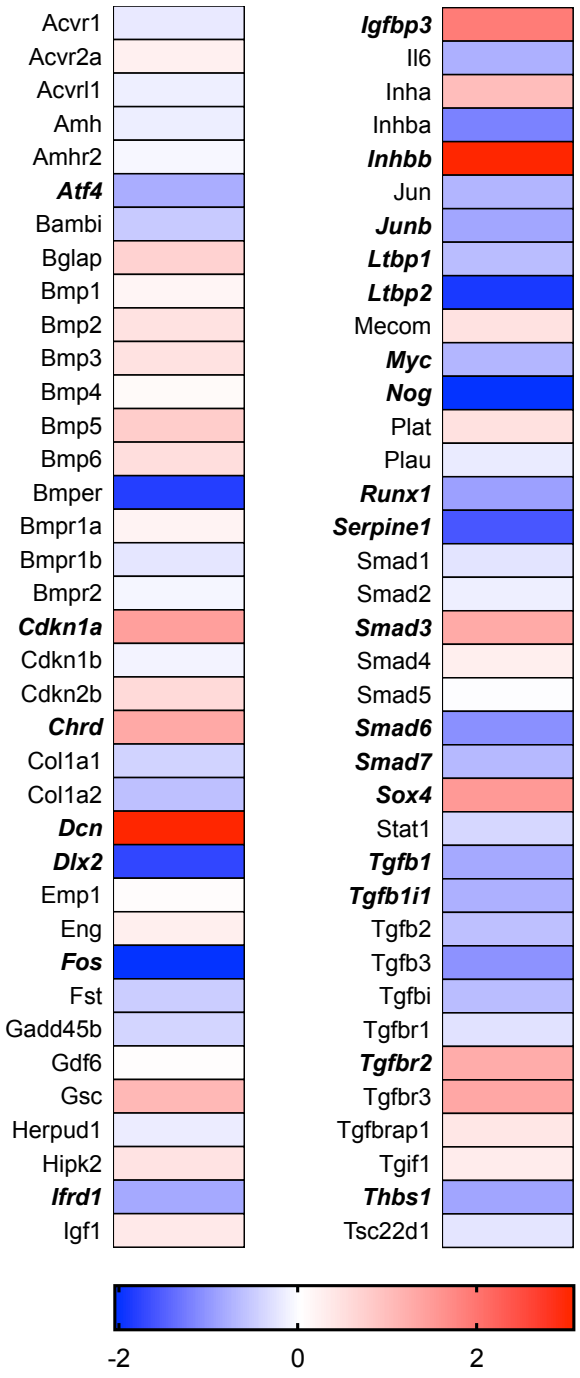

Figure 24: TGF-β Signaling Heat Map

Bold text indicates significantly altered gene targets.

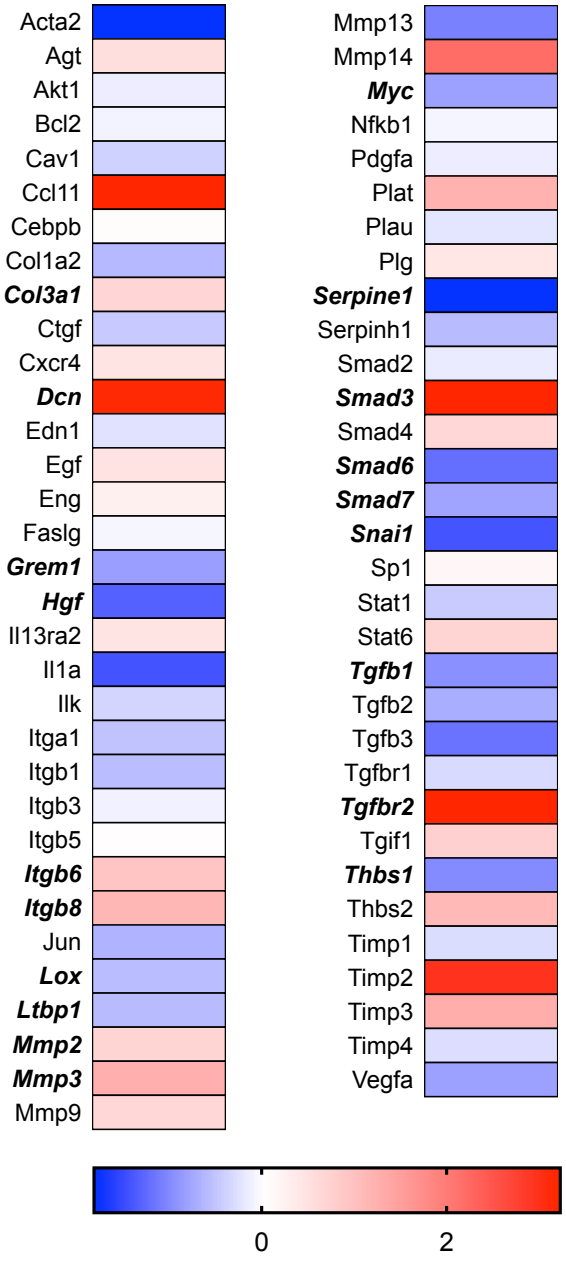

Figure 25: Fibrosis Heat Map

Bold text indicates significantly altered gene targets.

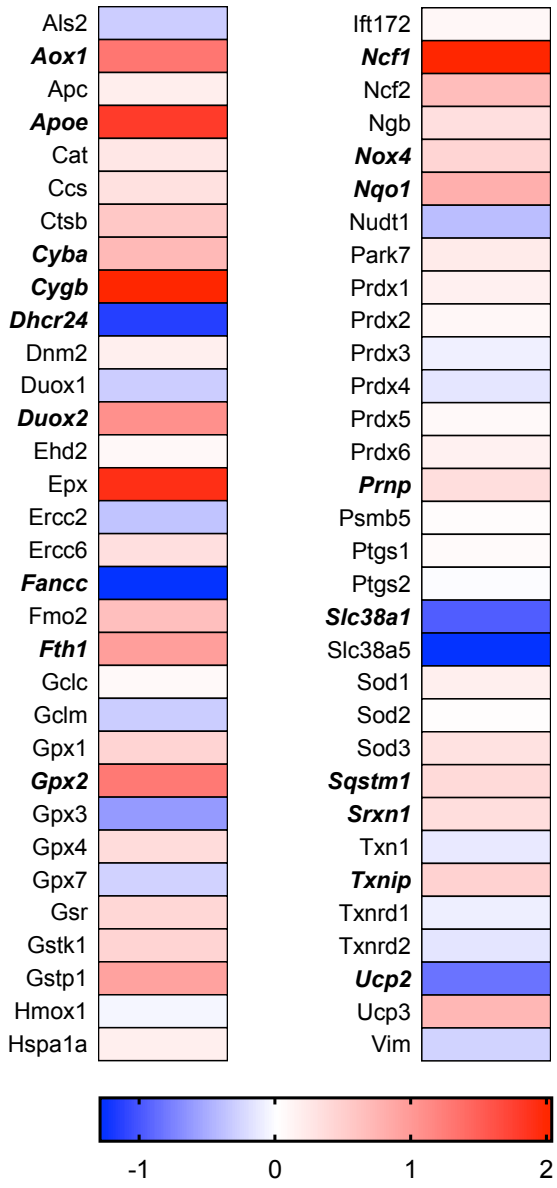

Figure 26: Oxidative Stress Heat Map

Bold text indicates significantly altered gene targets.

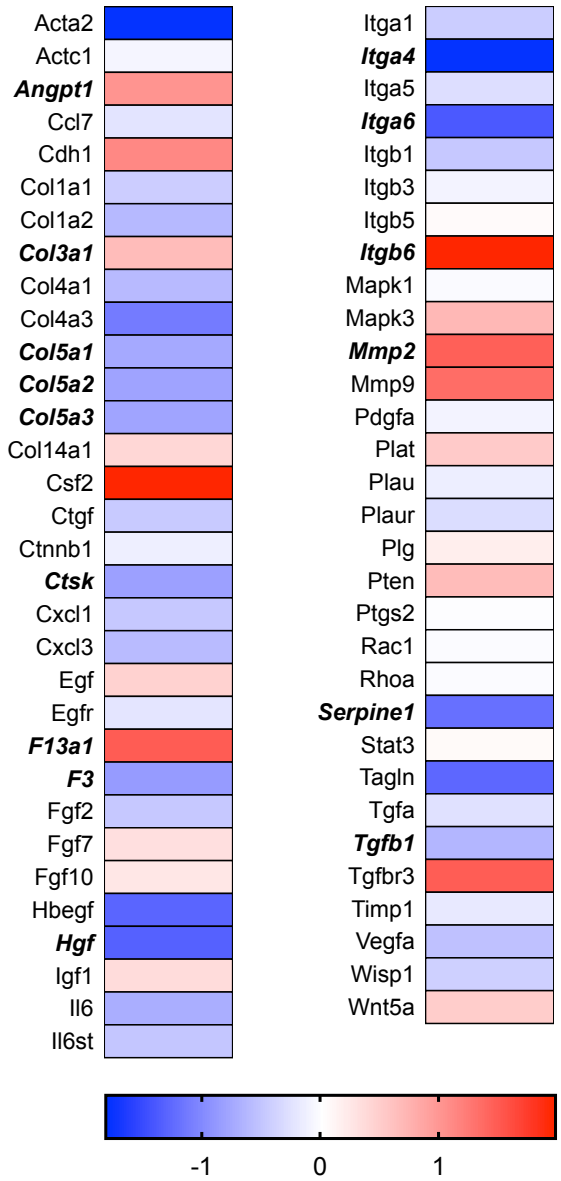

Figure 27: Wound Healing Heat Map

Bold text indicates significantly altered gene targets.

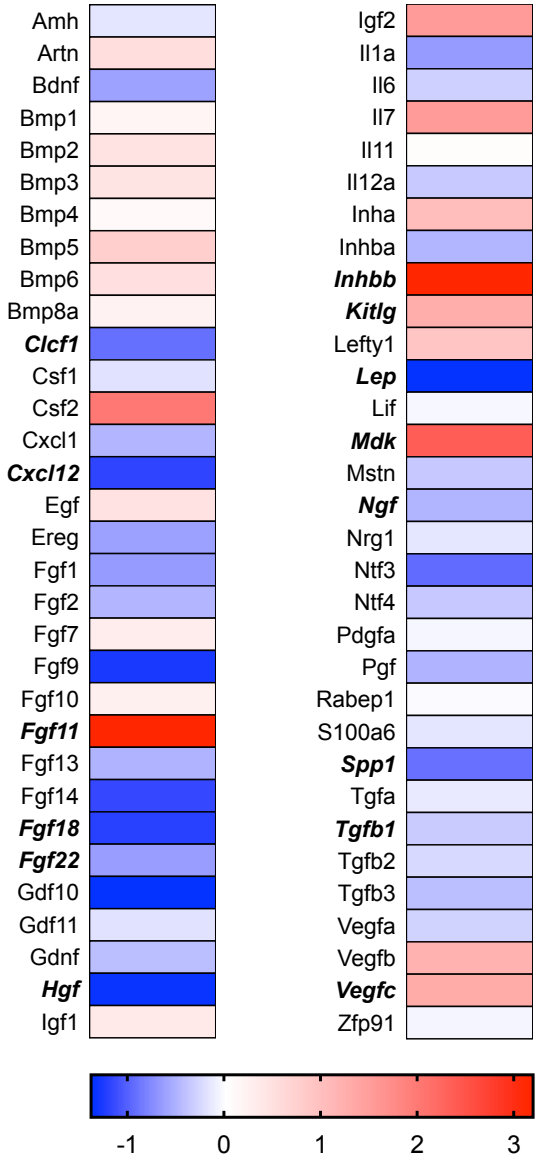

Figure 28: Growth Factors Heat Map

Bold text indicates significantly altered gene targets.

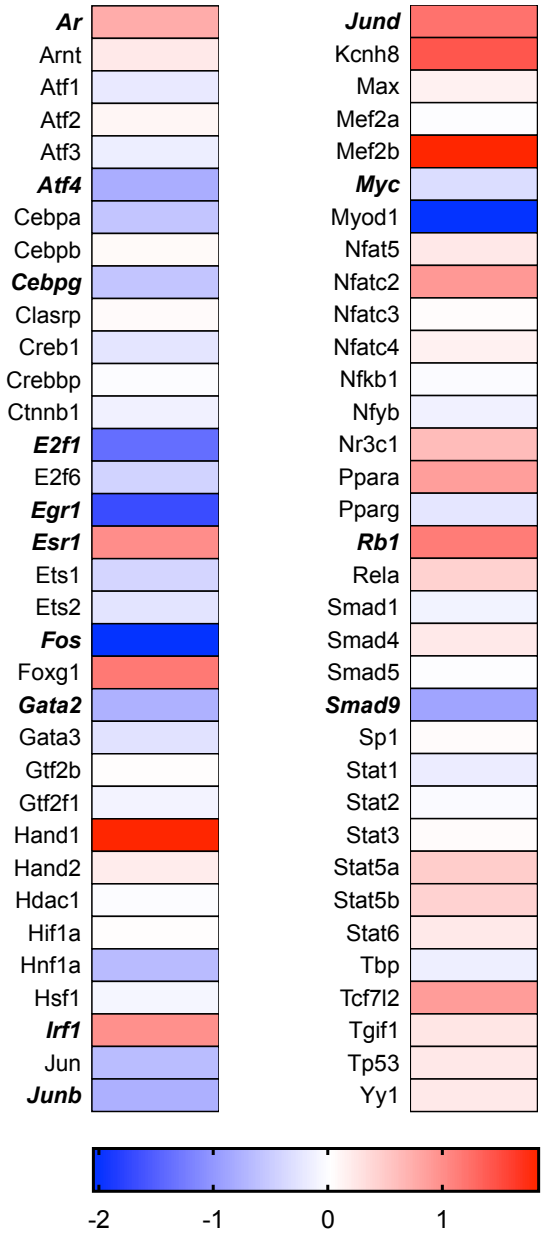

Figure 29: Transcription Factors Heat Map

Bold text indicates significantly altered gene targets.

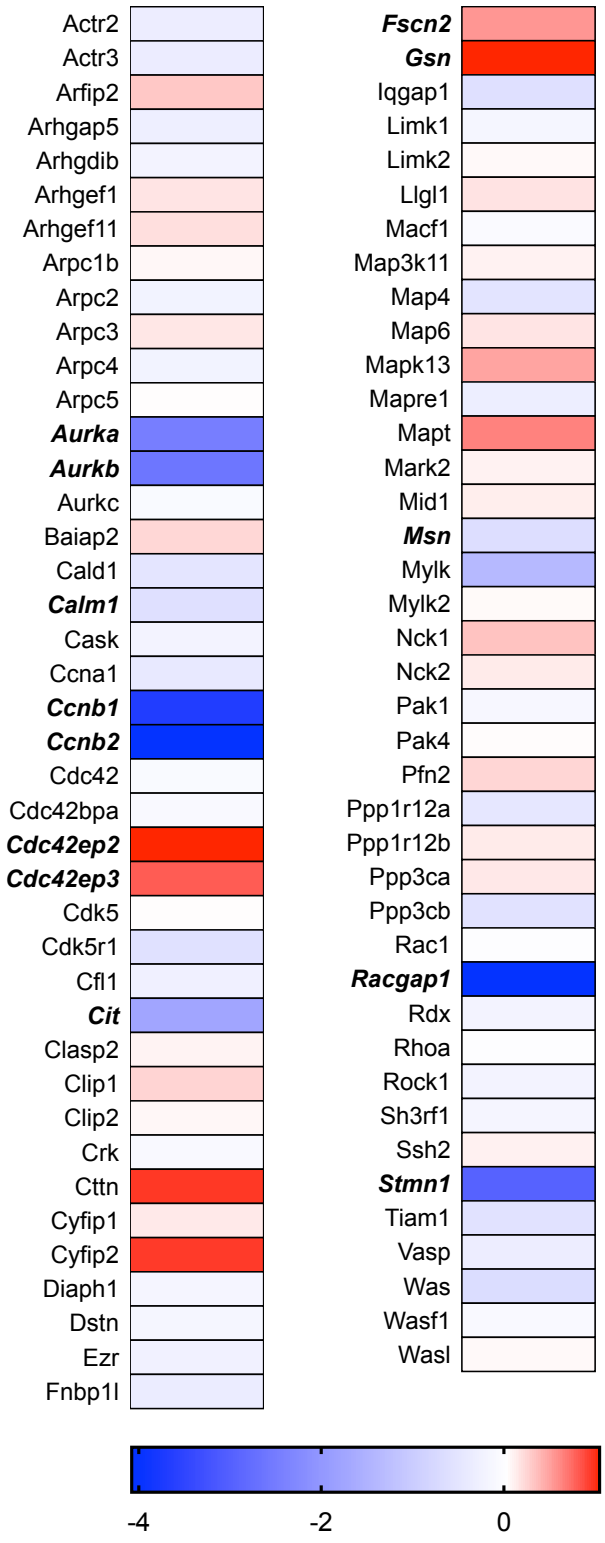

Figure 30: Cytoskeleton Heat Map

Bold text indicates significantly altered gene targets.

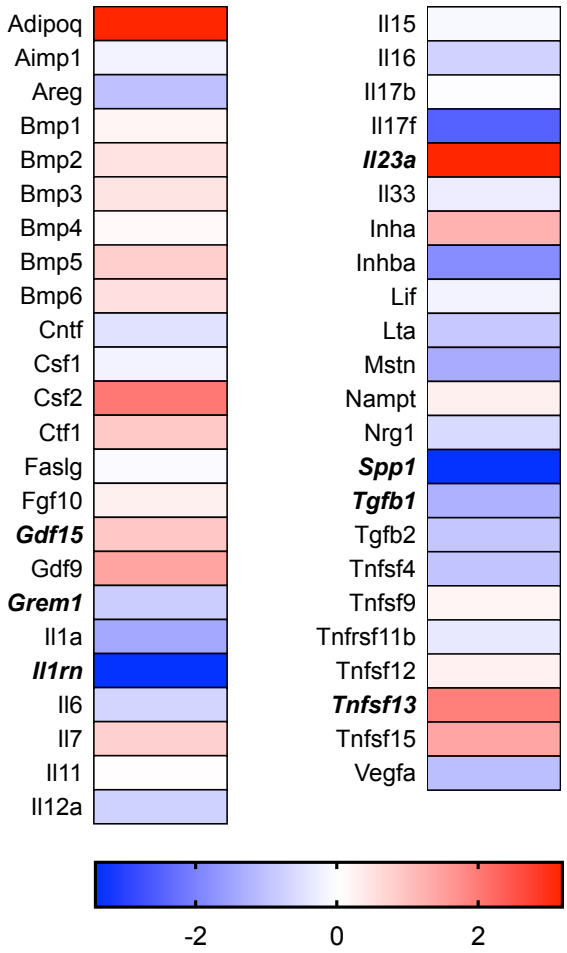

Figure 31: Cytokines Heat Map

Bold text indicates significantly altered gene targets.

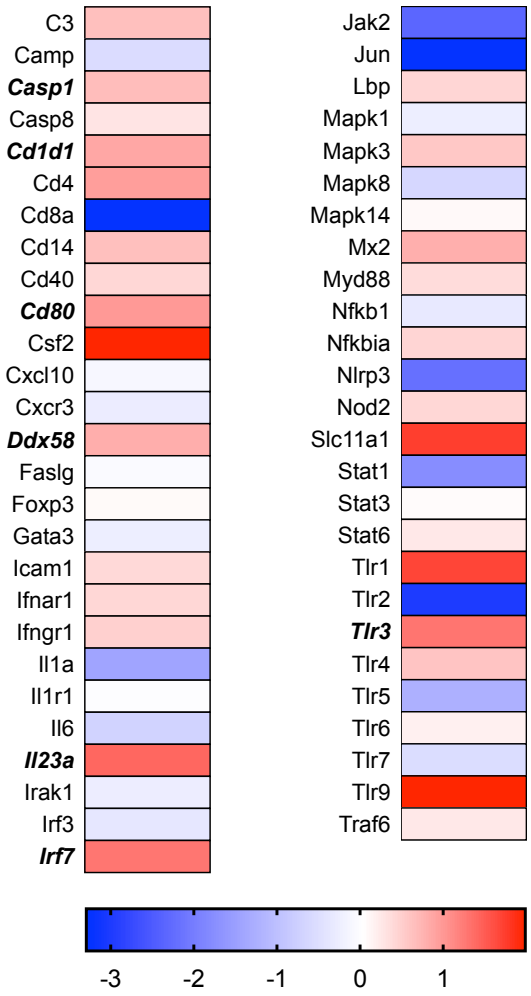

Figure 32: Innate and Adaptive Immunity Heat Map

Bold text indicates significantly altered gene targets.

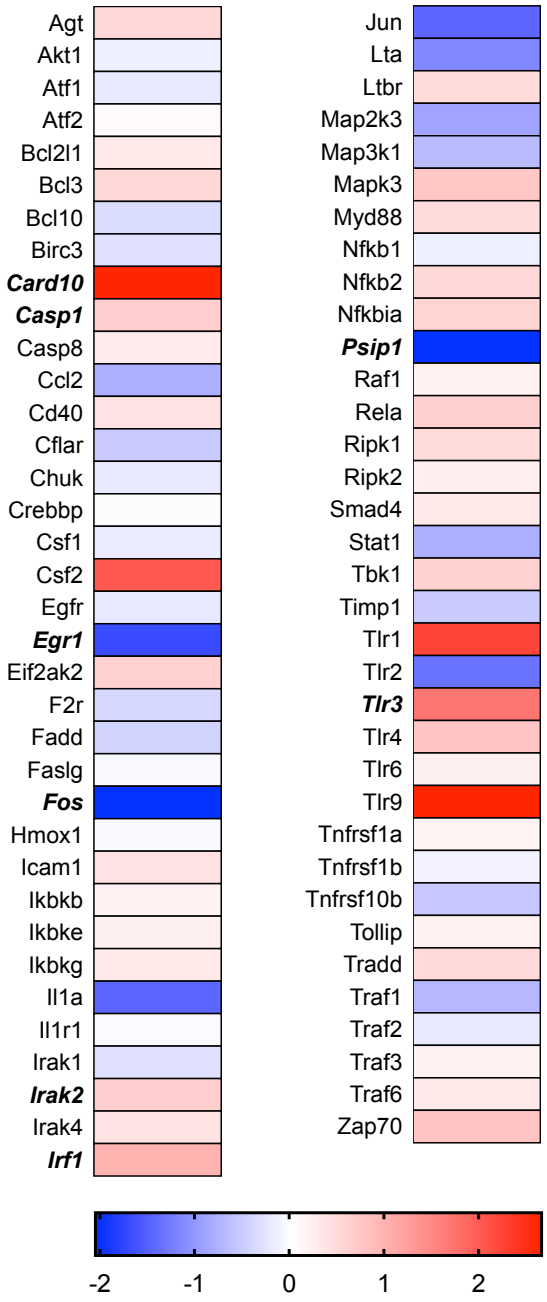

Figure 33: NF-κB Signaling Heat Map

Bold text indicates significantly altered gene targets.

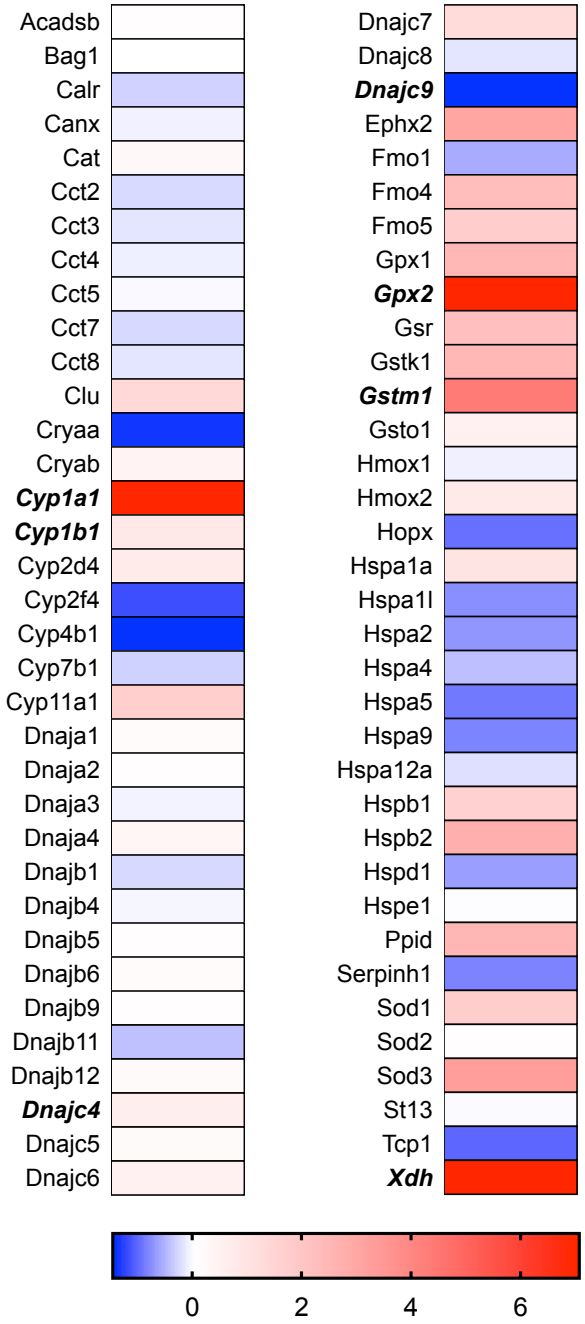

Figure 34: Cellular Stress Responses Heat Map

Bold text indicates significantly altered gene targets.

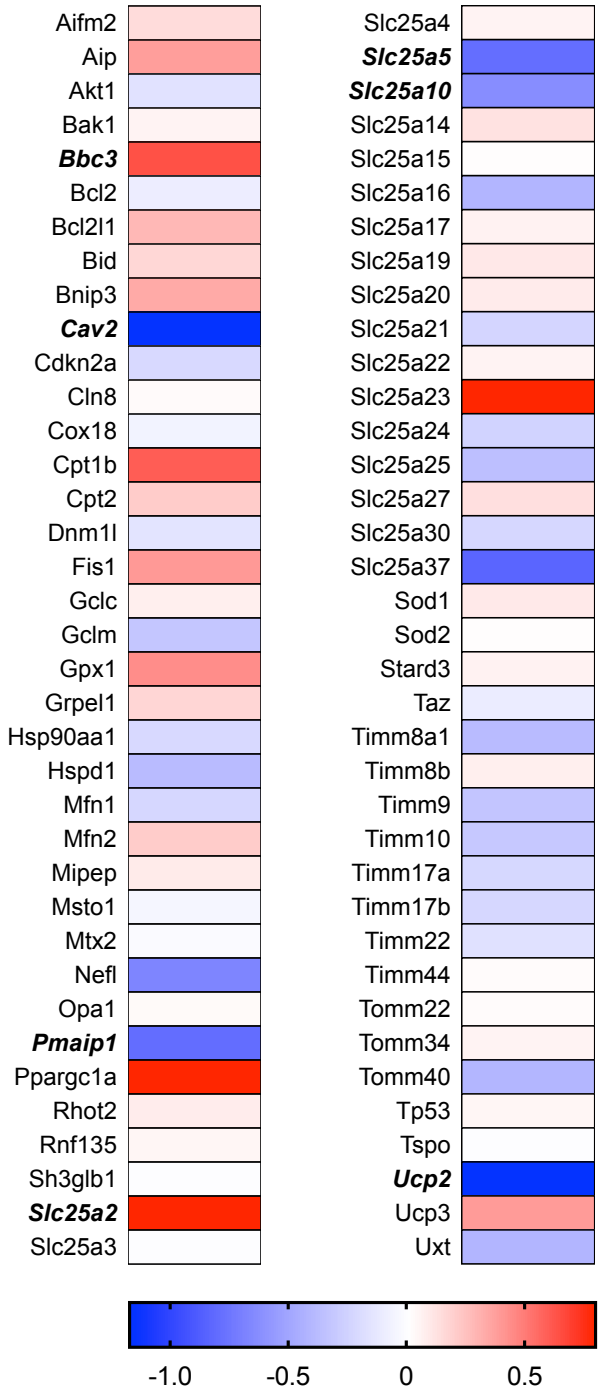

Figure 35: Mitochondria Heat Map

Bold text indicates significantly altered gene targets.

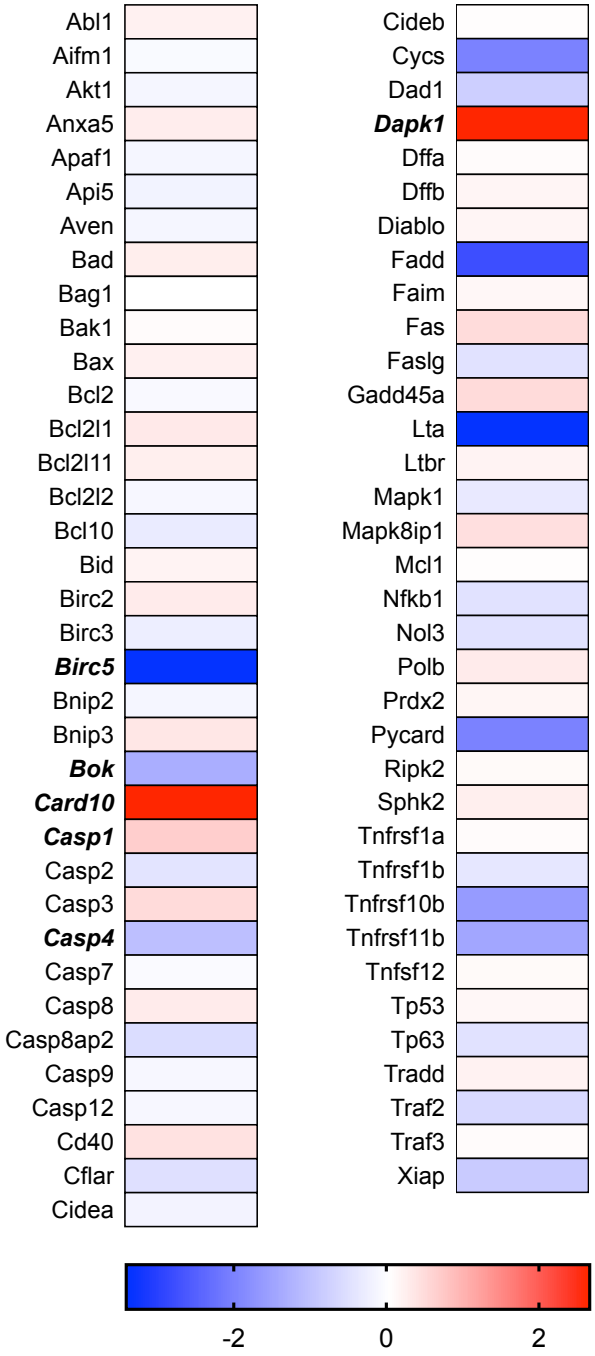

Figure 36: Apoptosis Heat Map

Bold text indicates significantly altered gene targets.

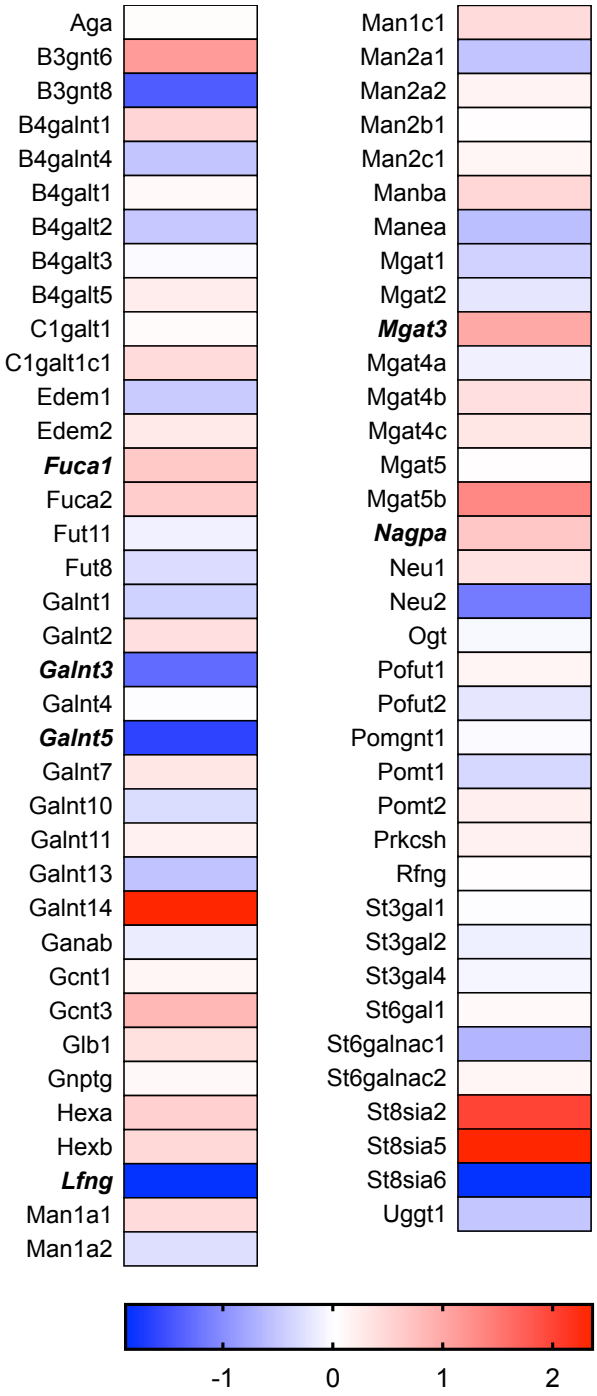

Figure 37: Glycosylation Heat Map

Bold text indicates significantly altered gene targets.

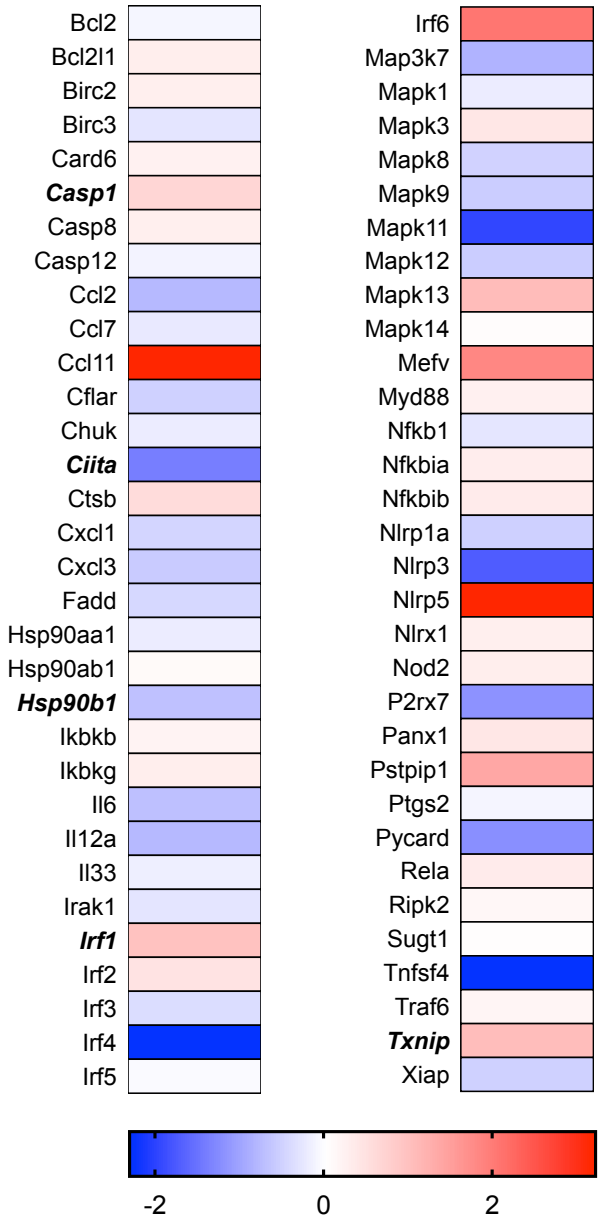

Figure 38: Inflammasomes Heat Map

Bold text indicates significantly altered gene targets.

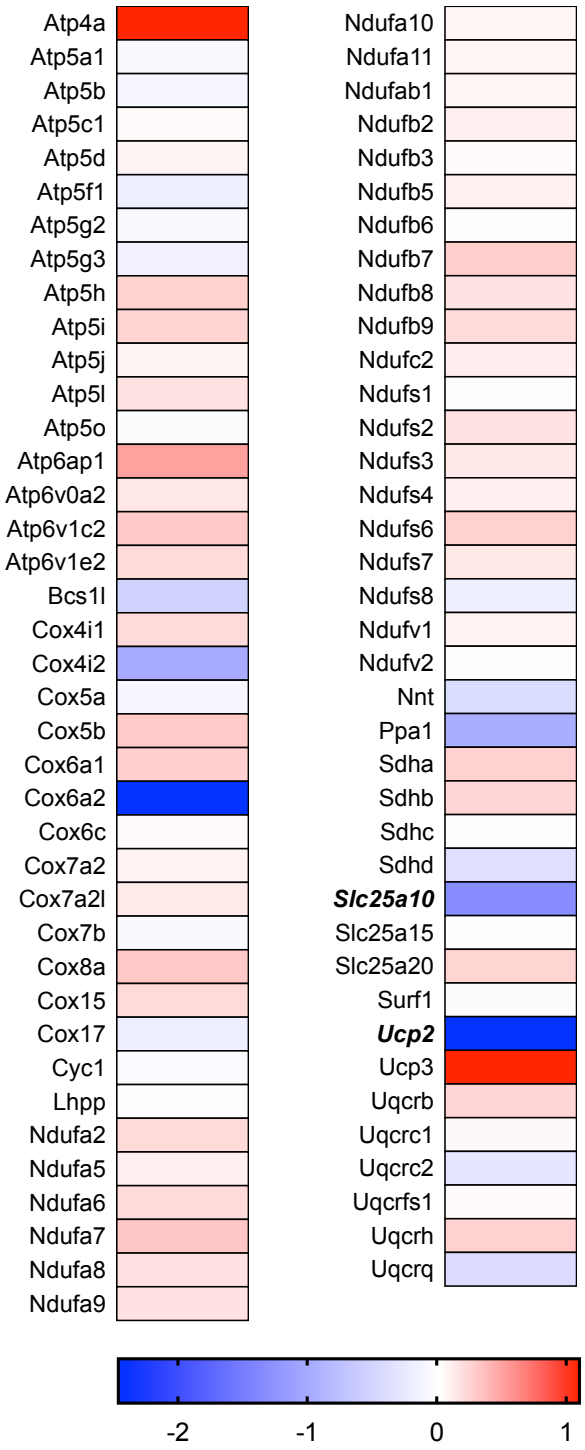

Figure 39: Mitochondrial Energy Metabolism Heat Map

Bold text indicates significantly altered gene targets.

CHAPTER 5. EVALUATION OF TENDON HEALING IN A MOUSE MODEL OF ELEVATED SERUM ADVANCED GLYCATION END PRODUCTS FOLLOWING TENDON INJURY

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Running Head: AGEs and tendon healing

Keywords: tendon, diabetes, advanced glycation end-products, healing

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5.1 Abstract

Introduction: Delayed tendon healing following injury is a significant problem among diabetic patients. Data describing this problem is largely based on reported clinical outcomes, with very little mechanistic insight. The purpose of this study was to explore the role of advanced glycation end products (AGEs), a protein modification present at elevated levels in serum of diabetic individuals, on tendon healing following injury. Cell proliferation following tissue injury is a vital component of healing. Based on our preliminary work demonstrating that AGEs limit cell proliferation, we proposed that AGEs are responsible for the delayed healing process that is commonly observed in diabetic patients. Further, in pursuit of interventional strategies, we proposed that moderate treadmill exercise may support a healing environment in the presence of AGEs as exercise has been shown to stimulate cell proliferation in tendon.

Methods: Mice began receiving daily BSA-Control or AGE-BSA injections (200µg/ml) at 16-weeks of age. After the mouse patellar tendon was fully developed at 18-weeks, a tendon injury was created in the central third of both patellar tendons. Animals assigned to an exercise group began a moderate treadmill protocol (13 meters/min, five days/week, five weeks) one week following injury. Mice were euthanized at 24-weeks and serum and tissue was collected.

Results: Results: Our injection dosing and schedule lead to an increase in serum AGEs (~200µg/ml, $p<0.05$), levels typically seen in type II diabetic patients. Additionally, a main effect ($p<0.05$) for AGEs was observed in genes related to cell proliferation (Mybl2), mitochondrial function (Bcs1l), and growth factors (Fgf2). However, moderate treadmill exercise did not alter gene markers that are typically indicative of improved tendon healing, such as Ctgf and Fgf2 ($p>0.05$).

Conclusion: Our findings suggest that AGEs modulate tendon gene expression following patellar tendon injury. Additionally, patellar tendon gene transcripts were not affected by the exercise intervention. Further, we have developed a mouse model of elevated serum AGEs that can be utilized in future studies related to AGE biology.

5.2 Introduction

Diabetes mellitus is a major risk factor for tendon pain and injury. Tendons, which connect muscle to bone, are critical for optimal musculoskeletal function. Tendinopathies affect the majority of diabetic patients, are chronic, and respond poorly to therapy (1, 2). Patients with type II diabetes are nearly four times more likely to live with a tendinopathy and the duration of diabetes further increases the risk of developing tendinopathies (3). Tendon syndromes, including shoulder tendinopathy, diabetic cheiropathy, calcification, Achilles tendon shortening, rupture, and general tendon pain are responsible for considerable reduction in the quality of life for many diabetic patients. Surprisingly, controlling blood glucose and HbA1c does not reduce tendon stiffness to levels of euglycemic controls, suggesting that other mechanisms contribute to tendon abnormalities in diabetes (4, 5). Surgical complications following tendon injury are greater in diabetic patients and repaired tendons are weaker compared to repairs in non-diabetic patients. This impairment in tendon healing for diabetic individuals may be one factor that contributes to the prolonged injury status that is commonly observed in diabetic patients (6, 7). Diabetic tendinopathies are painful and disabling and a significant need exists for a comprehensive understanding of diabetic tendon healing and the development of promising therapeutic strategies to improve healing outcomes in diabetic patients.

Advanced glycation end-products (AGEs) form at an accelerated rate in diabetes and have been implicated in several non-tendon diabetes-related complications (8, 9). AGE formation is a non-enzymatic process in which free amine terminals are subjected to covalent modification by reactive glucose or other carbonyl containing molecules. Accelerated AGE formation can result in non-enzymatic cross-links in long-lived extracellular proteins such as tendon collagen, which alters tissue stiffness and tissue mechanical properties (8, 10-13). More importantly, circulating AGE adducts are able to interact with the receptor for advanced glycation end-products (RAGE) to initiate a noxious feed forward cycle of sustained inflammation and tissue damage (14). AGE-rich diets have been shown to increase serum AGE levels and result in accumulation of AGE products in tendon of mice (15). We have demonstrated that AGEs severely impair tendon fibroblast proliferative capacity and mitochondrial function and these factors may be contributing to delayed healing with diabetes (16).

Tendon fibroblast proliferation is a vital component of tendon development, adaptation, and healing (17). Specifically, delayed and abnormal healing is a common complication of both type I and type II diabetes (6, 7). As evidence, tendinopathies in diabetic patients are generally more pervasive and can present with more severe degeneration, which may, in part, be driven by prolonged injury status (18). Following tendon injury, a process of healing and scar formation is initiated. This process occurs over three overlapping phases of inflammation, proliferation, and remodeling (19). Our previous *in vitro* data strongly suggest that after tendon injury, diabetic patients with high levels of circulating AGEs may be unable to progress through the healing process due to an impaired tendon fibroblast proliferative response (16).

In an effort to better characterize the healing process following diabetic tendon injury, we developed a mouse model of elevated serum AGEs that would be used for creation of a surgical tendon injury. As this is a new model of elevated serum AGEs, we decided to use the uninjured Achilles tendons and soleus muscles as reference tissues to compare tissue-specific changes to gene expression in response elevated serum AGEs. We hypothesized that injured patellar tendons would display gene expression characteristics of poor healing in the presence of AGEs. We further hypothesized that a moderate treadmill running intervention, which is a known stimulus of tendon cell proliferation, would help improve tendon healing in the presence of elevated serum AGEs.

5.3 Materials and Methods

5.3.1 AGE Preparation

Glycolaldehyde-derived AGEs were generated under strict sterile conditions as described previously (16) and as established by Valencia *et al.* (20). Briefly, sterile filtered 30% BSA solution (Sigma, St. Louis, MO) was incubated with 70mM glycolaldehyde dimer (Sigma) in sterile PBS without calcium chloride and magnesium chloride for three days at 37°C. The AGE product was dialyzed against sterile PBS for 24 hours at 4°C using gamma-irradiated 10kDa cut-off cassettes (Thermo Scientific, Waltham, MA) to remove unreacted glycolaldehyde dimer. Unmodified control BSA was prepared similarly, without the addition of glycolaldehyde dimer. Protein concentration was determined by BCA assay (Thermo Scientific) and extent of BSA modification was confirmed by fluorescence, absorbance, and loss of primary amines (20-23).

5.3.2 Animals

C57BL/6J mice (n=40, Strain 000664, Jackson Labs, Bar Harbor, ME) were purchased at 16-weeks of age. Animals were randomly assigned to one of four AGE/moderate treadmill running (MTR) groups (n=10/group): 1) AGE(-)/MTR(-), 2) AGE(-)/MTR(+), 3) AGE(+)/MTR(-), and 4) AGE(+)/MTR(+). At 16-weeks, animals began receiving daily intraperitoneal injections of either AGE-BSA or unmodified control BSA at a dose of 3μM (200μg/mL), similar to that of an uncontrolled type II diabetic patient (24, 25). Injection volume was based on estimated blood volume determined by body weight. Animal weights were recorded weekly for the duration of the study.

5.3.3 Surgical Injury

At 18-weeks of age, all four groups underwent a surgical procedure to create a full-thickness, partial width defect in the central third of both patellar tendons (26). After 120 days, the mouse tendon is considered "mature" (26). One author (SHP) completed all surgeries and a sterile field was maintained. Preoperative analgesia (Carprofen, 5mg/kg) in 0.9% saline was administered subcutaneously. Under general anesthesia (1.5% Isoflurane, 1.0L/min O_2), using established methodology, the patellar tendon was exposed with a single incision, and two incisions were made in the retinaculum on either side parallel to the patellar tendon to allow a sterile custom plastic backing to pass underneath the patellar tendon (27). The plastic backing provided support to prevent damage to underlying tissue from the 0.75mm punch biopsy blade (Robbins Instruments, Chatham, NJ) used to create the injury. Skin incisions were closed with simple interrupted sutures and animals were allowed to resume normal cage activity for 1-week prior to beginning MTR.

5.3.4 Treadmill Exercise

Animals assigned to the exercise group ran on a flat treadmill (Columbus Exer3/6) set to 13 meters/min for 30 minutes. Animals completed the exercise protocol five times per week for 5-weeks. This exercise protocol has previously been shown to enhance would healing in patellar tendon and is considered a moderate training intervention (28). Training with higher intensities following a tendon injury would not be externally valid for translation to a human population due

to associated pain and reduced compliance (29). Therefore, to maintain validity to the target population, a moderate intervention was selected.

5.3.5 Tissue Collection

At 24-weeks of age, under deep anesthesia, animals were euthanized by terminal cardiac puncture followed by cervical dislocation. Whole blood was processed for serum and stored at -80°C until analysis. The patellar tendons, Achilles tendons, and soleus muscles were carefully removed, flash frozen in liquid nitrogen, and transferred to -80°C. A full timeline of events is shown in Figure 40.

5.3.6 Serum Analysis

Level of serum AGEs at termination were determined by a total AGE ELISA (Cell Biolabs, San Diego, CA). Glycolaldehyde modified BSA, the same AGE modification used in the present study, was used as the antigen to prepare the polyclonal anti-AGE antibody provided with the AGE ELISA kit. The assay was completed largely as directed by the manufacturer, but the standard curve was extended to 400µg/mL. Commercially available assay kits (Crystal Chem, Elk Grove Village, IL) were used to determine non-fasted levels of serum glucose, insulin, HDL, and LDL.

5.3.7 RNA Isolation and Gene Expression

RNA from tendon was isolated as previously described (30). Briefly, both patellar tendons from the same animal were pooled and pulverized to a fine powder under cryogenic conditions (OPS Diagnostics, Lebanon, NJ). Pulverized tissue was transferred to a pre-chilled 2.0 ml reinforced tube containing 2.8 mm ceramic beads (Omni, Kennesaw, GA) and. A bead mill homogenizer (Omni) was used to disrupt samples in TRIzol (Thermo Scientific) and the Direct-zol RNA Miniprep kit (Zymo Research) was used to extract RNA. An identical process was completed for the pooled Achilles tendons. Only one soleus muscle was used for RNA isolation, but tissue was not pulverized prior to bead homogenization. On-column DNase digestion was completed on all samples prior to elution of RNA. RNA concentration was determined using a NanoDrop 2000 (Thermo Scientific). Quality of RNA was assessed using the

260/280 and 260/230 ratios. Reverse transcription (iScript, BioRad) was completed to produce complementary DNA from 25ng of RNA from all three tissues to permit comparison of transcript counts across tissues. Absolute quantification of mRNA target transcripts was completed using the ddPCR platform (BioRad) with validated probe-based assays (BioRad) as previously described (16, 30). For optimal detection, Col1a1 and Col3a1 reactions contained 0.625ng of resultant cDNA. Reactions for all other gene targets contained 5ng of resultant cDNA. A list of measured gene targets is provided in Table 8.

5.3.8 Statistical Analysis

Data was evaluated with a two-way ANOVA (AGE and MTR). The Bonferroni multiple corrections test was used to explore differences when a significant interaction was detected. Values were considered significant at an α level of $p<0.05$. All data are expressed as mean ± SE and data was analyzed using in Prism 8.0 (GraphPad, La Jolla, CA).

5.4 Results

5.4.1 Serum Measures

Serum AGEs were greater in the mice administered daily AGE injections (AGE(-): 96.1 ±29.6 μg/ml, AGE(+): 202.0 ± 37.6 μg/ml, main effect, $p<0.05$, Figure 41a). Serum glucose levels were lower in mice that completed MTR (MTR(-): 245.3 ± 6.0 mg/dL, MTR(+): 208.5 ± 8.8 mg/dL, main effect $p<0.05$, Figure 41b). Serum insulin, HDL, and LDL were unaffected by AGE or MTR ($p>0.05$, Figure 41 c, d, & e).

5.4.2 Patellar Tendon Gene Expression

Mybl2 (AGE(-): 22.2 ± 6.1 counts, AGE(+): 170.1 ± 56.9 counts), Bcs1l (AGE(-): 20.1 ± 4.0 counts, AGE(+): 123.7 ± 34.9 counts), Ager (AGE(-): 11.3 ± 3.1 counts, AGE(+): 85.7 ± 21.4 counts), and Fgf2 (AGE(-): 34.7 ± 8.2 counts, AGE(+): 106.9 ± 34.9 counts) gene expression was greater in mice that received daily AGE injections (main effect, $p<0.05$, Figure 42 c, d, e, & h). Gene expression for Col1a1, Col3a1, Ctgf, and Tgfb1 was unaffected by AGEs and MTR ($p>0.05$, Figure 42 a, b, f, & g).

5.4.3 Achilles Tendon Gene Expression

Col1a1 (MTR(-): 683.8 ± 206.1 counts, MTR(+): 1852.4 ± 468.0 counts), Col3a1 (MTR(-): 91.2 ± 27.4 counts, MTR(+): 234.0 ± 54.8 counts), and Ctgf (MTR(-): 552.9 ± 170.9 counts, MTR(+): 1340.8 ± 279.3 counts) gene expression was greater in mice that completed MTR (main effect, $p<0.05$, Figure 43 a, b, & f). Col3a1 gene expression was greater in mice that received daily AGE injections (AGE(-): 80.5 ± 25.4 counts, AGE(+): 211.8 ± 46.8 counts, main effect, $p<0.05$, Figure 43b). Col3a1 gene expression was greater in the AGE(+)/MTR(+) (339.5 ± 37.7 counts) group compared to the AGE(-)/MTR(-) (72.8 ± 39.0 counts), AGE(-)/MTR(+) (109.7 ± 34.2 counts), and AGE(+)/MTR(-) (93.3 ± 28.9 counts) groups ($p<0.05$, Figure 43b). Neither AGEs nor MTR significantly affected gene expression for Mybl2, Bcs1l, Ager, Tgfb1, and Fgf2 ($p>0.05$, Figure 43 c, d, e, g, & h).

5.4.4 Soleus Tendon Gene Expression

Col3a1 gene expression was greater in mice that received daily AGE injections (AGE(-): 311.4 ± 32.2 counts, AGE(+): 585.4 ± 135.3 counts, main effect, $p<0.05$, Figure 44b). Neither AGEs nor MTR significantly affected the gene expression of Col1a1, Mybl2, Bcs1l, Ager, Ctgf, Tgfb1, and Fgf2 ($p>0.05$, Figure 44 a, c, d, e, f, g, & h).

5.5 Discussion

The detrimental effects of AGE associated pathology are well described in non-tendon tissues and have been linked to several diabetic complications such as cardiomyopathy, retinopathy, nephropathy, and endothelial dysfunction (8, 9). The contributing role of circulating AGEs on tendon healing is not well described or understood. Treatment of diabetic tendinopathies is costly, often requiring surgical intervention and lengthy physical therapy. While previous data has established contributing effects of AGEs on diabetes-related complications, there has not been a thorough evaluation of AGEs and tendon healing (8, 9). In order to provide effective therapeutic options to reduce tendon degeneration and associate sequela in diabetic patients, it is important that we expand our understanding of the mechanisms contributing to delayed healing in this large patient population and explore promising non-surgical interventions, such as exercise.

In this study, we sought to establish a mouse model of elevated serum AGEs and use this model to better understand mRNA transcript changes that occur with tendon injury and healing. We also sought to determine whether a MTR intervention would help to improve wound-healing characteristics. Treadmill exercise has been shown to mechanically stimulate fibroblasts in tendon to promote cell proliferation and increase collagen synthesis (28, 31). We have previously shown that AGEs severely impair tendon fibroblast proliferation, however, exercise may serve to overcome the AGE-driven insult to promote a proliferative response in tendon to improve the healing process (16). Additionally, the selected exercise protocol has previously been shown to enhance wound healing in patellar tendon and is considered a moderate training intervention (28). As more intense exercise training interventions would have lower compliance, we selected this moderate protocol to maintain external validity to the diabetic population (29). To the best of our knowledge, this is the first evaluation of tendon healing using an *in vivo* model with elevated serum AGEs and MTR.

As our mouse model of elevated serum AGEs has not previously been utilized, we completed serum measures to confirm elevation of serum AGEs, as well as, determine if AGEs alter serum markers of metabolic syndrome. While we were able to confirm that the AGE(+) groups had increased levels of total AGEs (Figure 41a), no other serum markers were altered by AGE-BSA injection (Figure 41 b-e). Not surprisingly, a main effect for MTR was observed for serum glucose (Figure 41b). While no evidence of AGE-mediated metabolic syndrome was detected, we were able to confirm that our AGE injections increase serum AGE levels similar to that of an uncontrolled T2-DM patient (24, 25). The AGE product used in this investigation is a non-physiological AGE, and rather a tool used to activate RAGE. Therefore, it must be stated that the possibility of RAGE-mediated metabolic syndrome should not be completely ignored, as this was only a single AGE dose and a fixed duration of AGE administration. Longer durations and different doses of AGEs may have differing effects on RAGE activation and progression of metabolic syndrome.

Collagen isoforms I (Col1a1) and III (Col3a1) are important for tendon ECM. Col1a1 is thought to be a stronger form of collagen, whereas the weaker type III collagen is generally upregulated in the early stages of tissue remodeling following exercise or during initial stages of wound healing and tissue regeneration (32-34). Type III collagen provides tensile substance to the tissue assembly until it is later replaced by type I collagen (35). Our previous work has

indicated that AGEs reduce Col1a1 gene expression, but increase Col3a1 gene expression (16). It is highly likely that the increase in Col3a1 is a compensatory response to aid in overcoming the AGE insult (16, 36). However, our *in vivo* gene expression data are not in agreement with our previous *in vitro* findings (16). Col1a1 and Col3a1 gene expression were unchanged in the injured patellar tendon with AGEs, although a trend for MTR on Col3a1 gene expression was noted (Figure 42 a & b). While no changes to collagen gene expression were observed in the injured patellar tendon, we could not assess the impact of injury alone on tendon gene expression due to the bilateral injury design. For that reason, we assessed gene expression in the Achilles tendon as a reference uninjured tendon. We did note that overall gene counts for collagen isoforms were much higher in the injured patellar tendon when compared the uninjured Achilles tendon across all four groups (Figure 42 a & b and Figure 43 a & b).

Given that this mouse model of elevated serum AGEs has not previously been characterized, we investigated the effects of AGEs on the soleus muscle. Our rationale for this was to determine if any tissue specific effects of AGE would be found when comparing well-vascularized tissues (soleus) versus a tissue that has minimal blood flow (Achilles tendon).

A main effect for MTR was observed for both Col1a1 and Col3a1 in the Achilles tendon, while no main effect of MTR was observed in the soleus muscle (Figure 43 a & b and Figure 44 a & b). This was surprising, especially given that the soleus is primarily composed of type 1 fibers and responds well to endurance training (37). However, it is also possible that our training protocol was not strong enough to induce a remodeling response to the soleus. Interestingly, a main effect for AGE was observed for Achilles and soleus Col3a1 gene expression, which is in agreement with our previous *in vitro* data where Col3a1 was shown to increase with AGE exposure (Figure 43b and Figure 44b) (16). While we did not measure expression of MMPs and TIMPs, this additional data would provide aid in a more comprehensive understanding of the effects of tendon injury, AGEs, and MTR on ECM related gene expression.

Tendon fibroblast proliferation is a vital component to tendon development, adaptation, and healing (17, 19). Delayed and abnormal healing is a common complication of both type I and type II diabetes (6, 7). In support, tendinopathies in diabetic patients are generally more pervasive and can present with more severe degeneration, which may, in part, be driven by prolonged injury status (2, 18, 38). AGEs have been shown to impair wound healing in other tissues, but their role in tendon healing is not described (39, 40). It is plausible that lack of

proliferative capacity contributes to the diabetic tendon phenotype and this may explain the impaired rates of tendon healing in diabetic individuals, particularly after surgical repair.

Our previous work has demonstrated that AGEs severely impair tendon fibroblast proliferative capacity, an important component of tissue healing (41). Specifically, our *in vitro* data demonstrated an inability of tendon fibroblasts to uptake EdU, a strong indicator of cell proliferation, in the presence of 200µg/ml AGEs (16). Further, we have shown Mybl2 and Pcna gene expression to be significantly reduced in the presence of AGEs (16). Both Mybl2 and Pcna are important for cell cycle progression and cell proliferation (16). We examined Mybl2 gene expression in all three tissues, but it was only impacted by AGEs in the injured patellar tendon (Figure 42c). Mybl2 gene expression was increased in the presence of AGEs, but this data is not in agreement with our previous *in vitro* findings or our RNA sequencing results (Chapter 4) (16). This is surprising, especially given that our previous observations very strongly indicated loss of Mybl2 gene expression. However, the observed differences may be due to *in vitro* versus *in vivo* specific characteristics, such as local AGE clearance from the tissue of interest or systemic elimination via the renal system (42).

ATP has been shown to promote biosynthesis of the ECM in intervertebral disk cells (41). In tendon, the resident fibroblast population maintains the ECM, which is primarily composed of collagen. Due to the energy demanding nature of ECM maintenance, it is possible, that in the presence of AGEs, limited ATP production contributes to loss of organization in the ECM, which is commonly noted in diabetic tendon (2, 43, 44). Our work has indicated limitations to mitochondrial function in tendon-derived fibroblasts following AGE exposure (16). Not only did we observe dramatic reductions to ATP-coupled respiration, we also noted Bcs1l gene expression to be reduced in a dose-dependent manner following AGE exposure (16). Bcs1l is a gene transcript that encodes a chaperone protein for complex III of the electron transport chain. As Bcs1l was the most dramatically impaired ATP-related gene transcript in our *in vitro* data, it's expression was also measured in the patellar tendon, Achilles tendon and soleus. While Bcs1l mRNA expression was unaffected in the uninjured Achilles tendon and soleus (Figure 43d & 44d), we noted that the AGE(+) group had greater Bcs1l gene counts. Similar to the Mybl2 data, we were surprised to find that this gene also responded in a manner that was contradictory to our *in vitro* data. However, these contrary findings may again be due to the inherent differences between experimental models.

Circulating AGE adducts are able to interact with the cell surface receptor for advanced glycation end-products (RAGE) to initiate a noxious feed forward cycle of sustained inflammation and tissue damage (14). RAGE is encoded by the Ager mRNA transcript and expression of Ager is known to increase in response to AGE-RAGE interaction (14). Congruent with the literature, Ager gene expression was greater in the injured patellar tendons of AGE treated mice (Figure 42e). It also appears that the increase in Ager expression was specific to the injured patellar tendon, as it was unaltered in the Achilles tendon and soleus muscle (Figure 43e & 44e). These data suggest that our imitation AGE product is acting on the RAGE receptor at the site of the injured tissue. The injury-specific response may be related to the increase in vascularization following injury leading to greater local AGE delivery (45). In contrast, greater vascularization could allow for more AGE clearance and subsequently less activation of RAGE. This alternative and competing argument would hold true for a highly vascularized tissue such as the soleus muscle, where no effect on Ager gene expression was observed.

Wound healing characteristics of tendon generally display increased gene expression of Ctgf and Tgfb1 (46). Both of these gene targets facilitate and regulate the expression of collagen and glycoproteins to aid in tendon healing (47-49). Ctgf and Tgfb1 were unaffected in all three tissues (Figure 42 f & g, Figure 43 f & g, Figure 44 f & g). The lack of a response to Tgfb1 is surprising, especially considering how dramatically both Tgfb1 gene expression and related pathways were implicated in our RNA sequencing dataset (Chapter 4). In our RNAseq work, we demonstrated that 200μg/ml of AGEs significantly reduce Tgfb1 gene expression and activation of Tgfb1 related cell signaling pathways. As opposed to Ctgf and Tgfb1, Fgf2 gene expression is generally reduced in the healing tendon (50). Not only is Fgf2 expressed minimally in native tendon, it is further downregulated following injury (50). This expression is contrary to how growth factors respond to the repair process (51). Our data demonstrated that AGEs significantly increase Fgf2 mRNA expression in the injured patellar tendon (Figure 42h). This interesting finding suggests that AGEs impair the growth factor response that is typically observed in a non-diabetic healing tendon (50). While the role of Fgf2 in diabetic tendon healing would require further validation, these data would help corroborate the long standing epidemiological data that have described delayed tendon healing in diabetic individuals (6, 7). Further, these data aid in implicating AGEs in delayed tendon healing rather than the metabolic syndrome as a whole.

In summary, we demonstrate that our AGE dose and injection frequency elevate serum AGEs similar to that observed in an uncontrolled type II diabetic patient (3μM or 200μg/ml AGE-BSA). While we are limited in our interpretation due to only measuring gene transcripts, we provide rationale for continued investigation of AGEs and tendon healing. Specifically, future exploration of Fgf2 in diabetic tendon healing may enlighten researchers to more specific mechanisms that delay diabetic tendon healing. Additionally, we were able to demonstrate in the patellar tendon that AGEs induce RAGE expression, which is consistent with the literature and promising for future use of our mouse model in studies related to elevated serum AGEs. Detailed work involving varying time points, an appropriate uninjured group patellar tendon group, as well as comprehensive histological assessment of the healing tendon will provide more accurate insight into precisely how AGEs affect tendon healing following injury.

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Table 8: ddPCR Gene Targets

Gene Symbol	Description	Gene ID
Col1a1	Collagen alpha-1(I) chain	12842
Col3a1	Collagen alpha-1(III) chain	12825
Mybl2	Myeloblastosis oncogene-like 2	17865
Bcs1l	BCS1 homolog, ubiquinol-cytochrome c reductase complex chaperone	66821
Ager	Advanced glycosylation end product-specific receptor	11596
Ctgf	Connective tissue growth factor	14219
Tfgb1	Transforming growth factor beta 1	21803
Fgf2	Fibroblast growth factor 2	14173

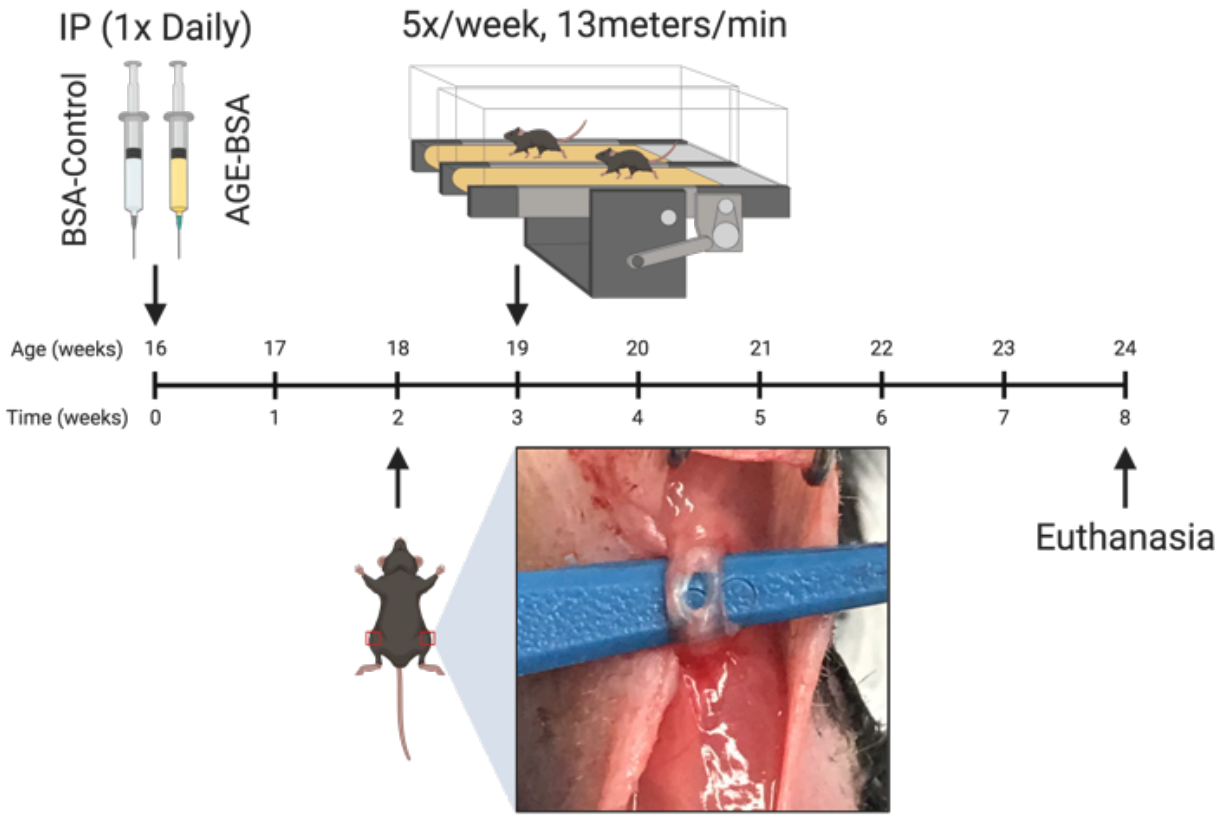

Figure 40: Timeline of Events

Animals began receiving either BSA-Control or AGE-BSA injections at 16-weeks. The 0.75mm punch biopsy injury was created in both patellar tendons at 18-weeks. Animals assigned to an exercise group began moderate treadmill running at 19-weeks. Animals were euthanized at 24-weeks.

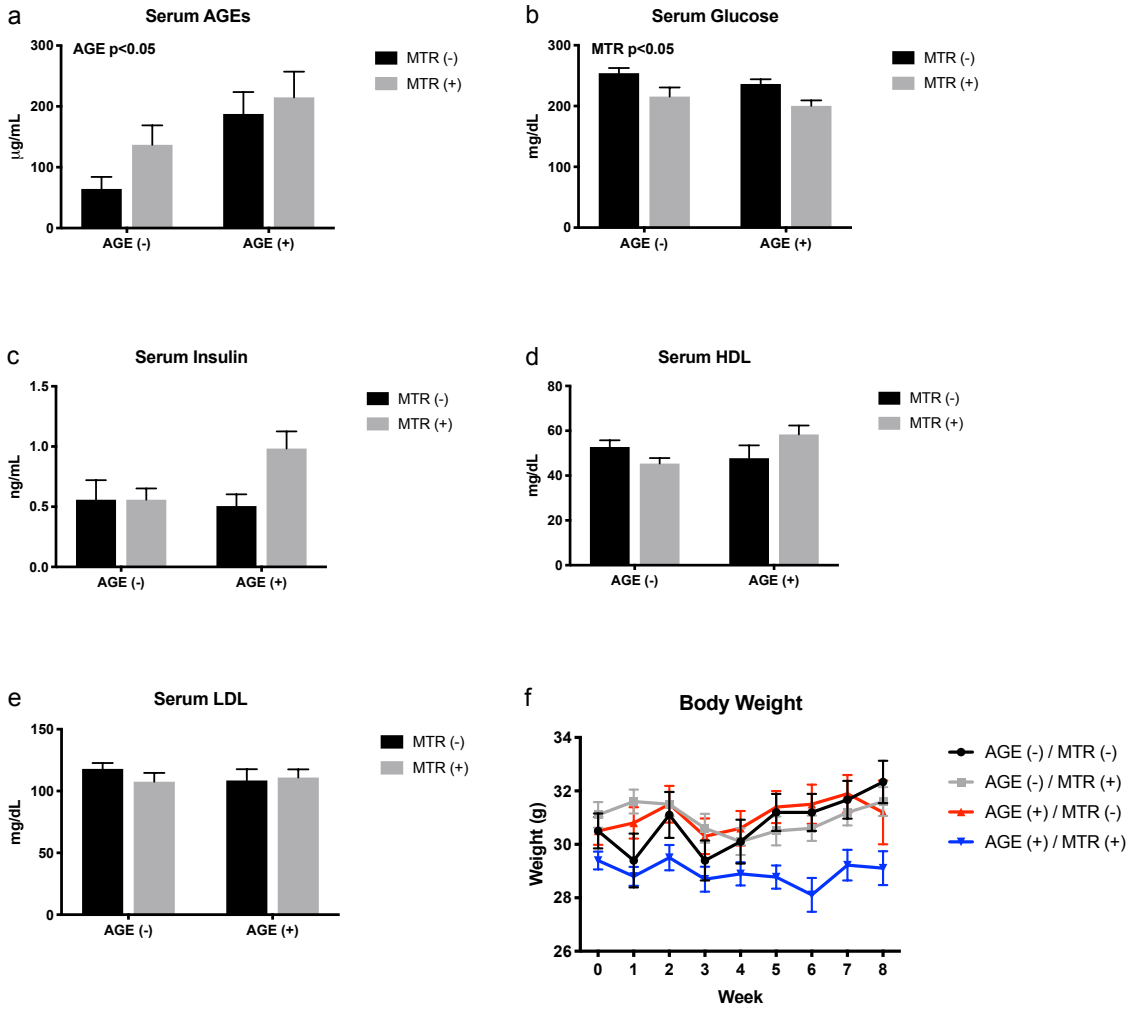

Figure 41: Serum Measures and Body Weight

a. Serum AGEs. **b.** Serum Glucose. **c.** Serum Insulin. **d.** Serum HDL. **e.** Serum LDL. **f.** Weekly Body Weights. n=8-12. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR.

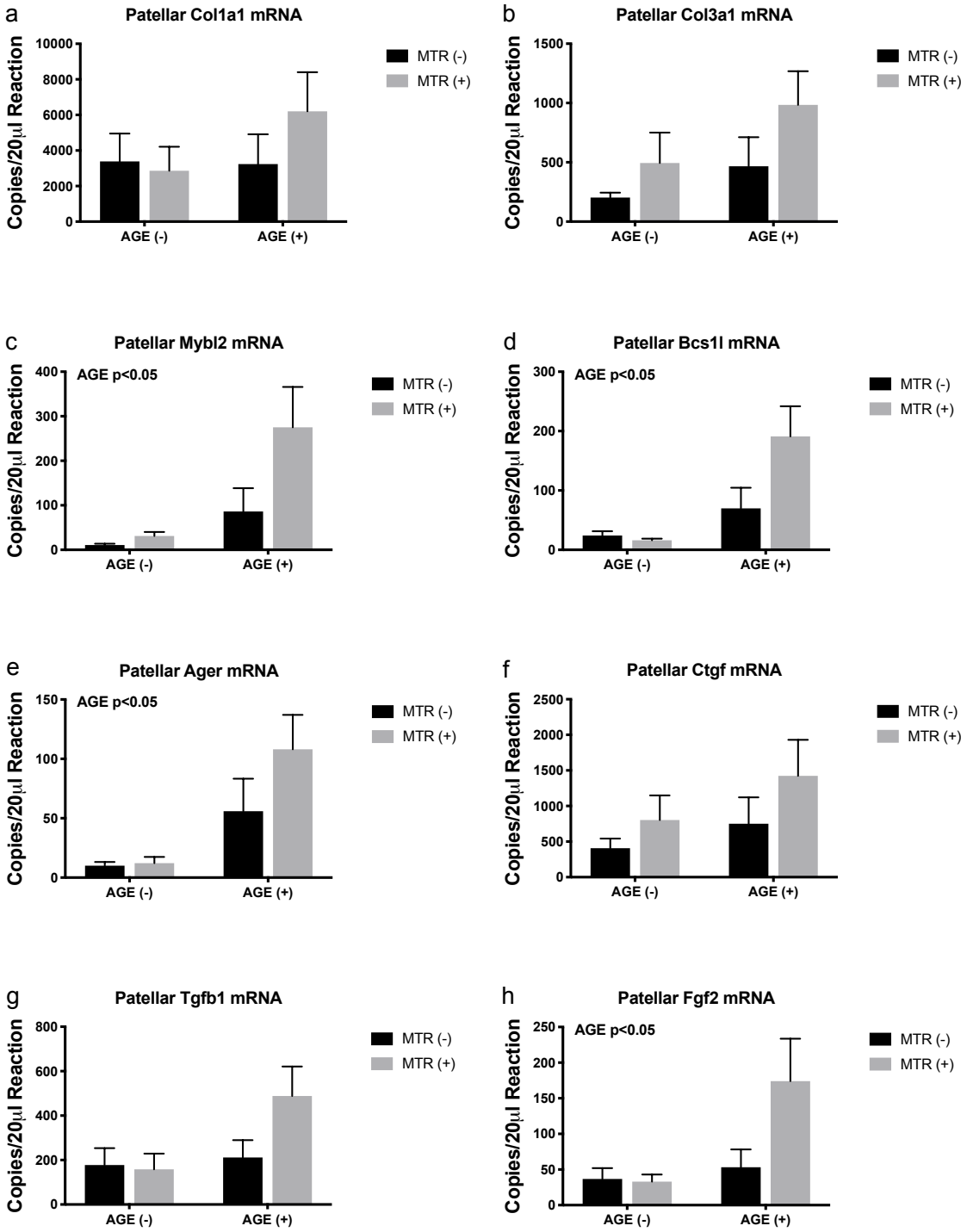

Figure 42: Patellar Tendon Gene Expression

a. Col1a1 mRNA. **b.** Col3a1 mRNA. **c.** Mybl2 mRNA. **d.** Bcs1l mRNA. **e.** Ager mRNA. **f.** Ctgf mRNA. **g.** Tgfb1 mRNA. **h.** Fgf2 mRNA. n=3-5. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR.

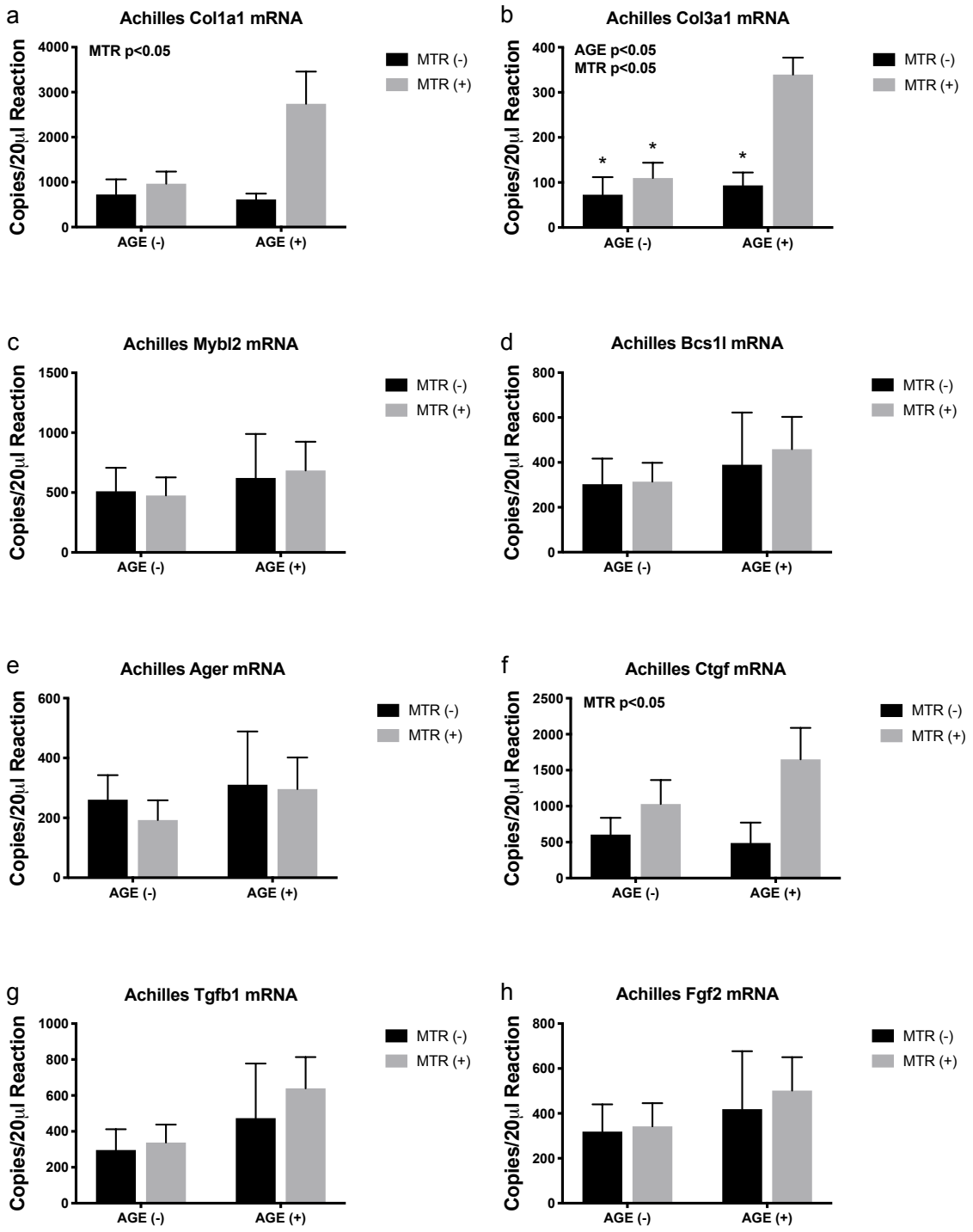

Figure 43: Achilles Tendon Gene Expression

a. Col1a1 mRNA. **b.** Col3a1 mRNA. **c.** Mybl2 mRNA. **d.** Bcs1l mRNA. **e.** Ager mRNA. **f.** Ctgf mRNA. **g.** Tgfb1 mRNA. **h.** Fgf2 mRNA. n=3-5. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR. *versus AGE(+)/MTR(+), $p<0.05$.

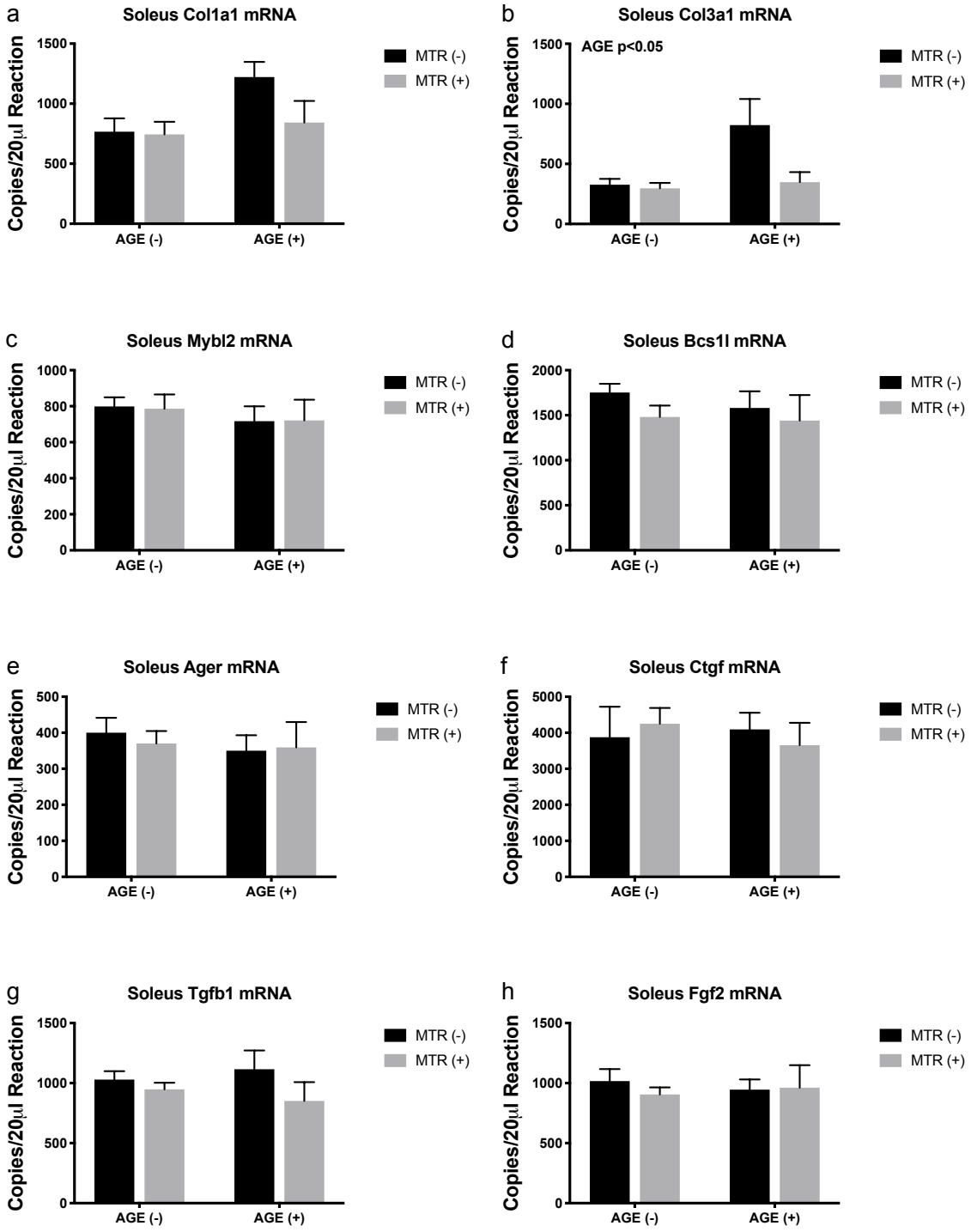

Figure 44: Soleus Muscle Gene Expression

a. Col1a1 mRNA. **b.** Col3a1 mRNA. **c.** Mybl2 mRNA. **d.** Bcs1l mRNA. **e.** Ager mRNA. **f.** Ctgf mRNA. **g.** Tgfb1 mRNA. **h.** Fgf2 mRNA. n=3-5. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR. AGE $p<0.05$ indicates main effect for AGE. MTR $p<0.05$ indicates main effect for MTR.

CHAPTER 6. SERUM LEVELS OF ADVANCED GLYCATION END-PRODUCTS AND THEIR RELATIONSHIP TO PATELLAR TENDON PROPERTIES IN DIABETES

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Running Head: AGEs and tendon healing

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6.1 Abstract

Introduction: Incidence of tendinopathy, particularly tendon thickening, is of common occurrence in diabetic patients. Data describing deterioration of normal tendon fibril morphology and functional properties appear to occur independent of blood glucose control, suggesting that other mechanisms contribute to pathology. The purpose of this study was to characterize the role of serum advanced glycation end products (AGEs) and other serum variables, such as HbA1c, on patellar tendon dimensional properties. We also sought to characterize and correlate the role of serum AGEs on indicators of tendon pathology. Based on previous work, we hypothesize that serum AGEs will be elevated in diabetic subjects and be related to patellar tendon dimensions and signal intensity.

Methods: Subjects (n=32) from a full spectrum of diabetes status, including no history of diabetes were recruited for a cross-sectional study. A fasted blood sample was collected and MRI imaging of the knee was completed. Current HbA1c and previous diagnosis were used to stratify collected data. Additionally a full correlation matrix of all measured variables was created to establish relationships that could be used to predict tendon pathology in diabetes.

Results: We demonstrate that diabetic subjects have lower body weight normalized (BWN) patellar tendon cross-sectional area (CSA) ($p<0.05$). Additionally, we demonstrate a negative correlation between homeostatic model assessment of insulin resistance (HOMA-IR) and BWN tendon CSA ($p<0.05$). Surprisingly, no difference in circulating CML was observed between groups and CML levels did not correlate to any tendon related variables ($p>0.05$).

Conclusion: Our findings demonstrate that diabetes is associated with smaller patellar tendon dimensions, which is in disagreement with the literature. Further, we show that changes to BWN tendon CSA occur independent of circulating CML levels. These new data suggest that alternative mechanisms contributing to tendon pathology in diabetes deserve attention.

6.2 Introduction

Tendinopathies (tendon pain and injury) are a common morbidity among the ~30 million Americans with diabetes (1). Tendon injury in diabetic patients is preceded by a gradual deterioration of the normal tendon fibril morphology and changes in tendon functional properties, with as many as 90% of patients exhibiting evidence of tendon structural abnormalities (2-6). Patients with type II diabetes (T2-DM) are nearly four times more likely to live with a tendinopathy compared to non-diabetics (7). Tendinopathies are responsible for a considerable reduction in the quality of life for diabetic patients and diabetic individuals often present with more severe tendon abnormalities than non-diabetic tendinopathy patients (8-11).

It is largely accepted that increased tendon stiffness with diabetes contributes to reduce mobility and aggravation of diabetic complications (5, 9, 10, 12). Many *ex vivo* studies have implied that increased tendon stiffness with diabetes is related to advanced glycation end product (AGE) cross-linking of collagen fibrils (13-18). AGEs are a protein modification that accumulates rapidly in diabetic patients, regardless of blood glucose control (5, 19). Chronic hyperglycemia and spontaneous oxidation of glycolytic intermediates contribute significantly to elevated levels of bound and circulating AGE adducts in diabetic serum (20, 21). In the case of non-enzymatic collagen cross-linking, free amine terminals, generally lysine and arginine, on tropocollagen molecules are subjected to covalent modification by reactive glucose or other carbonyl containing molecules (17). A covalent modification spanning two free amine terminals results in a collagen cross-link (17). Cross-links stabilize the collagen assembly and contribute to tissue stiffness. While an increase in collagen cross-linking among diabetics has been demonstrated in skin collagen, this has yet to be demonstrated in human tendon samples (5). Rather, the majority of data implicating the role of AGE-mediated collagen cross-links has been limited to *ex vivo* studies (13-18). While the relationship of tendon AGE accumulation in diabetics and *in vivo* tendon stiffness is unclear, common features of diabetic tendinopathies include increased tissue stiffness, disorganization of collagen fibrils, tendon thickening, and reduced tenocyte proliferation (2-4, 22).

In addition to protein bound AGEs, in the case of non-enzymatic collagen cross-links, AGEs can also exist as adducts in serum. Circulating AGE adducts are able to interact with the receptor for AGEs (RAGE) to activate cellular signaling pathways that induce sustained inflammation and tissue damage (20, 23). As a novel concept, it is possible that the increase in

serum AGEs that accompanies diabetes is a discrete mechanism contributing to the pathophysiology of diabetic tendinopathies, separate from the effects of AGE mediated collagen cross-linking (19, 24-26).

The extent to which serum AGEs alter tendon properties independent of AGE collagen cross-linking has received little attention in the literature (26). Recent reviews have advocated the potential of serum AGEs to induce tendon dysfunction by impacting cell proliferation, apoptosis, and ECM degradation in addition to AGE cross-linking of collagen (26). The effect of serum AGEs on tendon dysfunction has not been extensively studied, however, our work has demonstrated that cell proliferation and mitochondrial ATP-linked respiration are greatly reduced in tendon fibroblasts exposed to AGEs (27). In effort to better understand the role of serum AGEs on tendon properties and the transition to diabetic tendinopathy, we completed a cross-sectional study with subjects with a full spectrum of diabetes and no history of diabetes. Our goal was to correlate patellar tendon properties to serum markers of diabetes and AGE levels. We hypothesized that diabetic subjects would have greater levels of serum AGEs and that a positive correlation would exist with patellar tendon dimensional properties and pathology measured by signal intensity. This study provides a new perspective of AGEs and their relation to structural properties of tendon in diabetes.

6.3 Materials and Methods

6.3.1 Subjects

A total of 32 male (n=12) and female (n=20) subjects (aged: 23-74 years) were recruited from the West Lafayette/Lafayette, IN area. Subjects across the life span were recruited on a full spectrum of diabetes status, including no history of diabetes (HbA1c: <5.7), pre-diabetes mellitus (Pre-DM, HbA1c: 5.7-6.4) and type II diabetes mellitus (T2-DM, HbA1c: ≥6.5). Subjects were accepted into the study after voluntarily providing written consent. No subjects were experiencing any symptomatic tendinopathy, nor had any subjects previously been diagnosed with a tendinopathy of the knee. No BMI cutoff was enforced and smokers were included in this investigation. Subjects refrained from any formal exercise, both aerobic and resistance, for a period of three days prior to data collection. To ensure a thorough evaluation of the measured variables, both current HbA1c and previous diagnosis were used to stratify the data. When

stratified by diagnosis, nine T2-DM subjects were taking diabetes-related medication. Final subject characteristics and respective samples sizes for both grouping approaches are provided in Table 9. This investigation was completed with approval from the Institutional Review Board at Purdue University (Approval Number: 1710019832).

6.3.2 Blood Draw

Subjects reported to the lab after a 12-hour fast. Blood samples were collected from an antecubital venipuncture and submitted to Mid-America Clinical Laboratories (Indianapolis, IN) for measurement of fasted HbA1c, glucose, insulin, estimated average glucose (EAG), cholesterol, triglycerides, HDL, and LDL. Cholesterol to HDL ratio (CHOL/HDL) and homeostatic model assessment of insulin resistance (HOMA-IR) were also calculated (28). Heparinized plasma samples were stored at -80°C for later analysis of N^{ε}-(carboxymethyl)lysine (CML), an AGE species.

6.3.3 Magnetic Resonance Imaging

Axial and sagittal images of the patellar tendon were obtained on a 3T MAGNETOM Prisma scanner (Siemens, Munich, Germany). Each subject's dominant knee was placed into a Tx/Rx 15-Channel Knee Coil (Siemens). Slight flexion was placed on the knee joint with a foam pad. Scans were completed as previously described (29, 30). Briefly, after completing a scout scan to confirm location and ensure that the patellar tendon was straight, sagittal images were collected from the lateral to medial knee condyles in 4mm slices. Axial images were also collected in 4mm slices and extended beyond the proximal and distal ends of the patellar tendon in all cases. Tendon cross-sectional area (CSA) measurements were completed by manual planimetry using OsiriX (Version 11.0.2). Only slices of complete tendon from the distal pole of the patella to the tibial insertion were used for planimetry. Proximal and distal CSA was calculated based on an average of two most proximal or distal slices, respectively. Mid region CSA was calculated based on the middle slice or an average of the two middle slices if an even number of total slices existed. Mean CSA was calculated as an average of all available slices with complete tendon. CSA was also normalized for body size to compare patellar tendon dimensions between subjects of various size. Body weight normalized (BWN) CSA was

computed by dividing area in mm^2 by kg raised to the power of 2/3 ($mm^2/kg^{2/3}$) (31, 32). Mean gray value (MGV) was calculated in the same manner as CSA measurements. The same investigator performed all planimetry measurements and the reliability of these methods have previously been described in our laboratory (29, 30). A representation of planimetry is shown in Figure 45.

6.3.4 Serum Measurement of N^{ε}-(carboxymethyl)lysine (CML)

Content of CML in fasted plasma samples was detected by competitive ELISA (Cell Biolabs, San Diego, CA) as directed by the manufacturer. The monoclonal antibody utilized in this assay detects the CML structure, rather than the protein it is attached to. All standards and samples (50µl) were loaded in duplicate. Absorbance was recorded at 450nm (Molecular Devices, San Jose, CA) and data was fitted to a four-parameter logistics standard curve that extended to 576.0 ng/ml of CML.

6.3.5 Statistical Analysis

A correlation matrix for every pair of measured variables was completed. A two-way t-test was used to calculate p-values for the Pearson correlation coefficient. Within variable comparisons were evaluated with a one-way ANOVA. The Tukey multiple corrections test was used to explore differences when a significant AVOVA was detected. Values were considered significant at an α level of $p<0.05$. All data are expressed as mean ± SE and data was analyzed using Prism 8.0 (GraphPad, La Jolla, CA).

6.4 Results

6.4.1 Subject Characteristics

Control subjects were younger compared to Pre-DM subjects when stratified by current HbA1c (Control: 37.2 ± 4.7 years, Pre-DM: 55.9 ± 2.9 years, $p<0.05$, Figure 46a). Height, weight, and BMI were not different between groups when stratified by current HbA1c ($p>0.05$, Figure 46 c, d, and e). Age (Control: 35.9 ± 5.4 years, T2-DM: 53.4 ± 3.1 years), weight (Control: 65.1 ± 3.5 kg, T2-DM: 94.6 ± 6.9 kg), and BMI (Control: 22.8 ± 0.8, T2-DM: 33.5 ± 2.1) were

lower in control subjects compared to T2-DM subjects when stratified by previous diagnosis ($p<0.05$, Figure 46 b, f, and h).

6.4.2 Diabetes-Related Serum Measures

HbA1c (Control: 5.3 ± 0.1, Pre-DM: 5.9 ± 0.1, T2-DM: 7.6 ± 0.5), glucose (Control: 93.0 ± 3.0 mg/dL, Pre-DM: 101.4 ± 3.1 mg/dL, T2-DM: 148.8 ± 18.7 mg/dL), EAG (Control: 105.2 ± 1.9 mg/dL, Pre-DM: 123.6 ± 2.0 mg/dL, T2-DM: 170.8 ± 15.6 mg/dL), and HOMA-IR (Control: 2.0 ± 0.4, Pre-DM: 2.5 ± 0.4, T2-DM: 6.0 ± 2.1) were greater in T2-DM subjects compared to both control and Pre-DM when stratified by current HbA1c ($p<0.05$, Figure 47 a, c, g, and i). HbA1c (Control: 5.3 ± 0.1, Pre-DM: 5.9 ± 0.1) and EAG (Control: 105.2 ± 1.9 mg/dL, Pre-DM: 123.6 ± 2.0 mg/dL) were also greater in the Pre-DM subjects compared to control when stratified by current HbA1c ($p<0.05$, Figure 47 a and g). Both insulin and CML were unchanged between groups when stratified by current HbA1c ($p>0.05$, Figure 47 e and k). HbA1c (Control: 5.3 ± 0.1, T2-DM: 6.6 ± 0.3), glucose (Control: 91.9 ± 3.9 mg/dL, T2-DM: 122.6 ± 10.0 mg/dL), EAG (Control: 104.1 ± 2.5 mg/dL, T2-DM: 143.9 ± 9.4 mg/dL), and HOMA-IR (Control: 1.6 ± 0.3, T2-DM: 4.2 ± 1.0) were greater in T2-DM subjects compared to control subjects when stratified by previous diagnosis ($p<0.05$, Figure 47 b, d, h, and j). Additionally, HbA1c (Pre-DM: 5.7 ± 0.1, T2-DM: 6.6 ± 0.3) and EAG (Pre-DM: 117.0 ± 2.6 mg/dL, T2-DM: 143.9 ± 9.4 mg/dL) were greater in T2-DM subjects compared to Pre-DM when stratified by previous diagnosis ($p<0.05$, Figure 46 b and h). Neither insulin nor CML were different between groups when stratified by previous diagnosis ($p>0.05$, Figure 47 f and l).

6.4.3 Lipid Panel

Both triglycerides (Control: 88.0 ± 10.8 mg/dL, T2-DM: 249.4 ± 52.7 mg/dL) and CHOL/HDL (Control: 3.1 ± 0.3, T2-DM: 4.3 ± 0.4) ratios were greater in the T2-DM subjects compared to controls when stratified by current HbA1c ($p<0.05$, Figure 48 c and i). Triglycerides were also greater in the T2-DM subjects compared to Pre-DM when stratified by current HbA1c (Pre-DM: 107.9 ± 10.2 mg/dL, T2-DM: 249.4 ± 52.7 mg/dL, $p<0.05$, Figure 48c). Cholesterol, HDL and LDL were not different across groups when stratified by current HbA1c ($p>0.05$, Figure 48 a, e, and g). Cholesterol, triglycerides, HDL, LDL, and CHOL/HDL ratio were not

different between groups when stratified by previous diagnosis ($p>0.05$, Figure 48 b, d, f, h, and j).

6.4.4 Patellar Tendon Dimensions

No difference between groups were noted for proximal cross-sectional area (CSA), mid CSA, distal CSA, mean CSA, and mean CSA body weight normalized (BWN) when stratified by current HbA1c ($p<0.05$, Figure 49 a, c, e, g, and i). Mean CSA BWN was lower in the T2-DM subjects compared to control when stratified by previous diagnosis (Control: 7.6 ± 0.3 mm^2, T2-DM: 6.3 ± 0.4 mm^2, $p<0.05$, Figure 49j). Proximal CSA, mid CSA, distal CSA, and mean CSA were not different across groups when stratified by previous diagnosis ($p>0.05$, Figure 49 b, d, f, and h).

6.4.5 Patellar Tendon Mean Gray Value (MGV)

Proximal MGV, mid MGV, distal MGV, and MGV (total mean) were not different across groups when stratified by current HbA1c ($p>0.05$, Figure 50 a, c, e, and g). Proximal MGV, mid MGV, distal MGV, and MGV were also not different between groups when stratified by previous diagnosis ($p>0.05$, Figure 50 b, d, f, and h).

6.4.6 Correlation Matrix

A significant correlation was observed between 76 of the measured variables. Of the 76 significant correlations, 51 were positive correlations, while 25 were negative correlations. The full correlation matrix with Pearson r-values is presented in Figure 51.

6.5 Discussion

Diabetic tendinopathies are characterized by a gradual deterioration of normal tendon fibril morphology and a decline in tendon functional properties (2-6). However, many patients remain asymptomatic for years even with significant underlying pathology (33). Establishing a serum marker to assess risk of tendinopathy in diabetic patients would be useful for the implementation of interventional strategies, such as exercise, to strengthen the tendon proper or delay progression of pathology (34). Few studies have directly linked circulating AGEs to tendon

properties *in vivo*. Establishing a link between serum AGEs and *in vivo* tendon properties may provide clinical markers to estimate changes in tendon morphological or functional properties during the progression of diabetes.

Our previous *in vitro* work demonstrated a dose-dependent effect of AGEs on tendon fibroblast proliferation and mitochondrial function and established that tendon fibroblasts are sensitive to circulating AGE products. Given the dramatic cellular changes that were observed, we explored the idea that circulating AGEs contribute to tendon pathology *in vivo*. Much of the *in vivo* human work has not demonstrated that non-enzymatic cross-linking in tendon is enhanced due to diabetes (5). However, *in vivo* human work has established a higher collagen fibril density in Achilles tendon of diabetic subjects (5). Higher collagen fibril density implies that smaller collagen fibrils occupy the same space, however, it is not known why this is a feature of diabetic tendon (16, 35, 36). It has been speculated, however, that AGE induced cross-linking is the culprit for the closer packing density of fibrils, but these findings are not confirmed in diabetic tendon (5, 16, 37). Another mechanism to explain this phenomenon could be the activation of RAGE related tissue degeneration that results in smaller collagen fibrils. Previous work has demonstrated that activation of the cell surface receptor RAGE by free AGE adducts results in degeneration of intervertebral discs and macular degeneration (38, 39).

With a great degree of confidence, it can be stated that presence of pathology in diabetic tendon is common (1-6). However, the hypothesis that AGEs contribute to collagen cross-linking in diabetic tendon is ambiguous with almost no human *in vivo* data available. Animal or *ex vivo* data supporting the assertion of enhanced AGE-mediated cross-linking generally rely on non-physiological representations of diabetes and no perfect animal model of T2-DM exists (40, 41). There is a significant need for the exploration of alternative mechanisms to explain diabetic tendon pathology, especially in humans. It is well established is that AGE-mediated activation of RAGE is a source of several diabetes-related complications such as cardiomyopathy, retinopathy, nephropathy, and endothelial dysfunction (42, 43). However, this claim has not been explored in the context of human diabetic tendon. Our previous *in vitro* work describing the AGE-RAGE mediated impairments to tendon fibroblasts lend some support to the idea that RAGE activation in tendon may be responsible for the high incidence of observed diabetic tendon pathology. In order to establish this new line of research, we explored, in a cross-sectional manner, the

possibility that tendon CSA and signal intensity would be correlated to diabetes-related serum markers as well as serum AGEs.

Subjects were grouped by both current HbA1c and by previous diagnosis. Subjects with a previous diagnosis in many cases had implemented lifestyle modifications or began to treat Pre-DM and T2-DM with medication and were, therefore, no longer in the same HbA1c category of their original diagnosis. The rationale for stratifying the data both ways is to allow for a stronger picture of variables that have previously been influenced by presence of diabetes. Also some variables are more understood compared to others, and it is not known how long some variables would take to recover to a "non-diabetic" state. For that reason, all data, with the exception of the correlation matrix, are presented using both subject-grouping formats.

AGEs are known to accumulate in the serum of diabetics due to hyperglycemia, high dietary intake of AGE containing foods, and impaired AGE clearance (44-53). Two noteworthy AGE products commonly implicated in diabetes-related complications are N^{ε}-(carboxymethyl)lysine (CML) and methylglyoxal-derived hydroimidazolone (MG-H1). Specifically, CML is elevated in diabetic serum due to dietary choices that include highly processed foods or foods cooked with high heat (54). MG-H1 on the other hand is elevated in diabetic serum as a result of spontaneous oxidation of the glycolytic intermediate methylglyoxal (MG) (55, 56). Generally, MG is cleared in the form of D-lactate, but a deficiency in triosephosphate isomerase and the glyoxalase detoxification system results in impaired MG accumulation and later spontaneous oxidation to MG-H1 (50, 56). Once in circulation, both CML and MG-H1 are free to interact with RAGE and induce downstream activation (57, 58).

Despite strong evidence indicating that serum AGEs are elevated in diabetic serum, we observed no difference in CML in both cases of data stratification (Figure 47 k and l). Additionally, despite higher HbA1c levels in the Pre-DM and T2-DM groups (Figure 47 a and b), no correlation was observed between CML and HbA1c (Figure 51). Further CML did not correlate with any of the measured patellar tendon dimension variables or pathology estimates (Fig 51). Although evidence indicates CML is elevated with diabetes, others have found that fasted CML levels are not different in those with impaired fasting glucose when compared to euglycemic controls (54, 59).

It was no surprise that HbA1c, fasting glucose, EAG, and HOMA-IR were greater in the T2-DM subjects in both cases of data organization (Figure 47 a-d and g-j). HOMA-IR is a

powerful tool to estimate insulin resistance. Values above 1.9 generally indicate early insulin resistance while values above 2.9 indicate significant insulin resistance. When stratified by diagnosis, we note that HOMA-IR was 2.4 ± 0.5 in Pre-DM subjects and 4.2 ± 1.0 in T2-DM subjects. Independent of current HbA1c and previous diagnosis, both Pre-DM and T2-DM subjects display evidence of significant insulin resistance (28). Not surprisingly, HOMA-IR showed a positive correlation with body weight, BMI, fasting glucose levels, and fasting insulin levels (Figure 51). However, we were surprised to learn that HOMA-IR was negatively correlated with body weight normalized (BWN) tendon CSA (Figure 51). BWN CSA allows for comparisons of tendon dimensions across subjects and accurately accounts for body size (31, 32). Tendon thickness has been reported to be greater in diabetics and accordingly we hypothesized that CSA would be greater in diabetic individuals (6, 22, 60). However, contrary to our hypothesis, we observed a lower mean BWN CSA in the T2-DM group compared to non-diabetics when stratified by diagnosis (Figure 49j), but no regional measures of uncorrected tendon CSA by current HbA1c or previous diagnosis stratification were different between groups (Figure 49 a-i). While a HOMA-IR value greater than 1.9 alone does not constitute diabetes diagnosis, it does provide a more clear picture of metabolic syndrome when combined with HbA1c, fasting glucose, and fasting insulin data. The relationship between HOMA-IR and BWN CSA requires more investigation, but our findings provide more reason to believe that the diabetic tendon pathology literature is an evolving area of research.

In challenge to the hypothesis that diabetes increases tendon CSA, it is possible that activation of RAGE leads to tissue degeneration, which has been observed in non-tendon systems. Specifically, activation of RAGE by AGE leads to increased catabolic activity in cartilage and acceleration of osteoarthritis (61). While this may be a valid theory for diabetic tendon, we did not observe any changes to CML content between groups (Figure 47 k and l). CML represents only one AGE species, and CML in particular is sensitive to feeding. Our plasma samples were collected after a 12-hour fast, and it is possible that we are missing the peak CML levels that would be experienced by sensitive tissues throughout the day. However, diabetic subjects also have reduced AGE clearance capabilities, and we would have expected these to accumulate in the circulation independent of fasted blood collection (25).

Presence of pathology was estimated by signal intensity, or mean gray value (MGV) generated by MRI, which provides an indication of tendon pathology (62). MGV can be used as

a reliable indication of tendon pathology, with a higher signal indicating greater pathology (63, 64). Despite stratifying data by current HbA1c and previous diagnosis, no difference in MGV was observed regionally or as an average of the whole patellar tendon (Figure 50 a-h). These findings were unforeseen as we were expecting to see some indication of pathology in the diabetes groups. Specifically, it has been reported that up to ~90% of diabetic patients have evidence of tendinopathy (1, 2). MGV represents only one indicator of pathology. In fact, it is designed to be used as an adjunct tool rather than a primary clinical indicator of tendon pathology (63). Despite no observed differences across groups of diabetes status, BMI demonstrated a significant positive correlation to proximal MGV, distal MGV, and whole patellar tendon MGV, confirming literature which demonstrates that obesity plays a role in tendon pathology (65).

For the first time we have explored the role of serum AGEs on diabetic tendon properties. Gaps in our understanding of diabetic tendon pathology are slowing the development and timely implementation of therapeutic measures in the growing diabetic population. A new approach on the impact of serum AGEs on tendon properties will provide new perspective of this growing problem. Our findings demonstrating that HOMA-IR is negatively correlated to patellar tendon BWN CSA are novel and establish that metabolic syndrome does not necessarily constitute an increase in tendon CSA nor an increase in circulating CML. Additionally, these data suggest that factors aside from AGE cross-linking may impact tendon dimensional properties and signal intensity. With the exploration of additional circulating AGEs we hope develop a more insightful correlation matrix demonstrating a potential role of RAGE mediated tendon pathology with diabetes.

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Table 9: Subject Stratification

	By Current HbA1c			By Previous Diagnosis		
	Control	**Pre-DM**	**T2-DM**	**Control**	**Pre-DM**	**T2-DM**
n	13	14	5	9	11	12
HbA1c	5.3 ± 0.1	5.9 ± 0.1	7.6 ± 0.5	5.3 ± 0.1	5.7 ± 0.1	6.6 ± 0.3
Age, yr	37.2 ± 4.7	55.9 ± 2.9	54.2 ± 7.2	35.9 ± 5.4	52.0 ± 5.6	53.4 ± 3.1
Height, cm	167.8 ± 2.0	167.6 ± 3.1	169.4 ± 6.3	168.6 ± 2.7	167.8 ± 3.3	167.8 ± 3.3
Weight, kg	73.9 ± 4.7	86.1 ± 6.7	93.6 ± 11.2	65.1 ± 3.5	83.0 ± 6.3	94.6 ± 6.9
BMI	26.2 ± 1.6	30.6 ± 2.4	32.4 ± 2.4	22.8 ± 0.8	29.5 ± 2.1	33.5 ± 2.1

Data presented at mean ± SE.

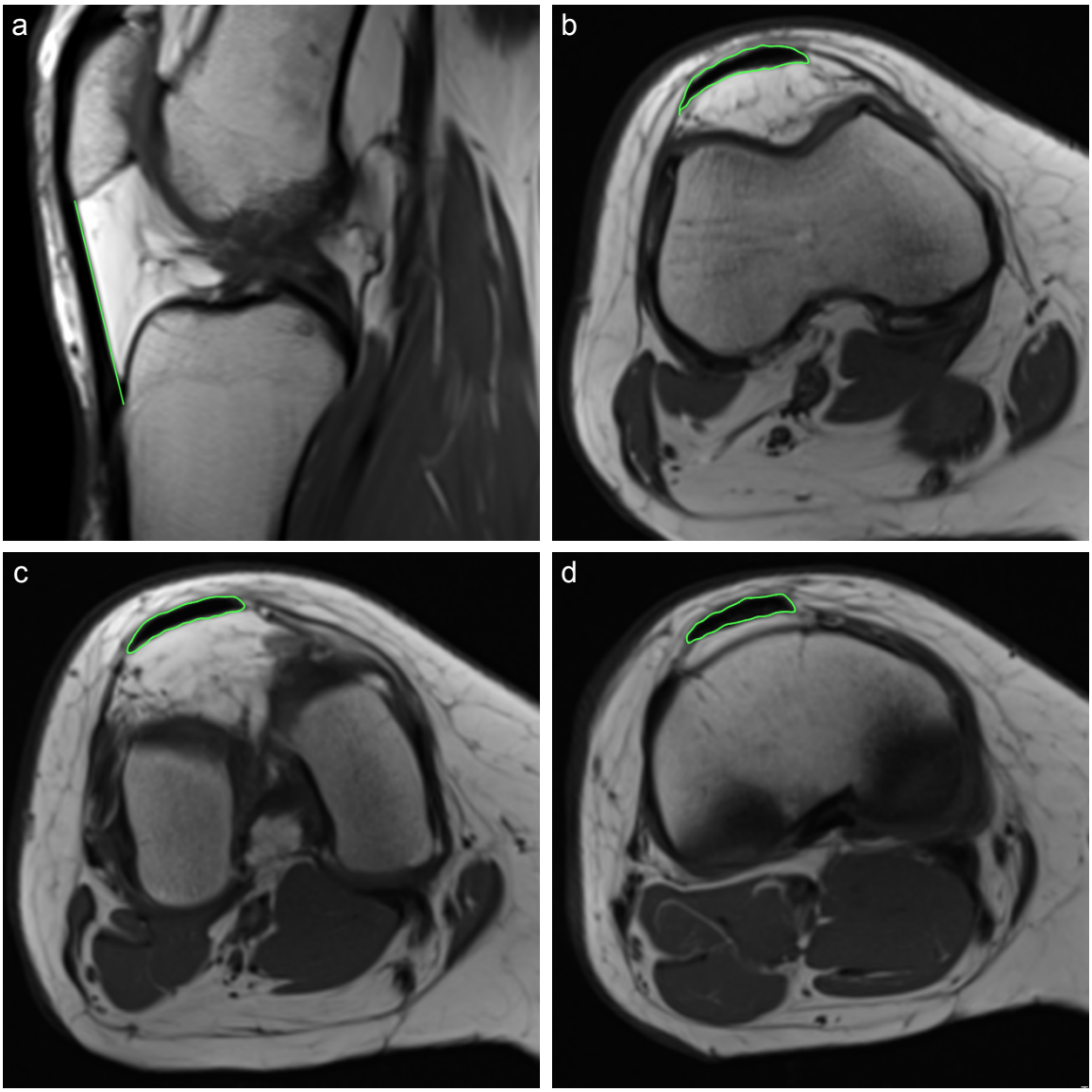

Figure 45: Representative MRI Planimetry

a. Sagittal image of patellar tendon. **b.** Axial image of patellar tendon at proximal region. **c.** Axial image of patellar tendon at mid region. **d.** Axial image of patellar tendon at distal region. Green tracing represents manual planimetry conducted in OsiriX (Version 11.0.2).

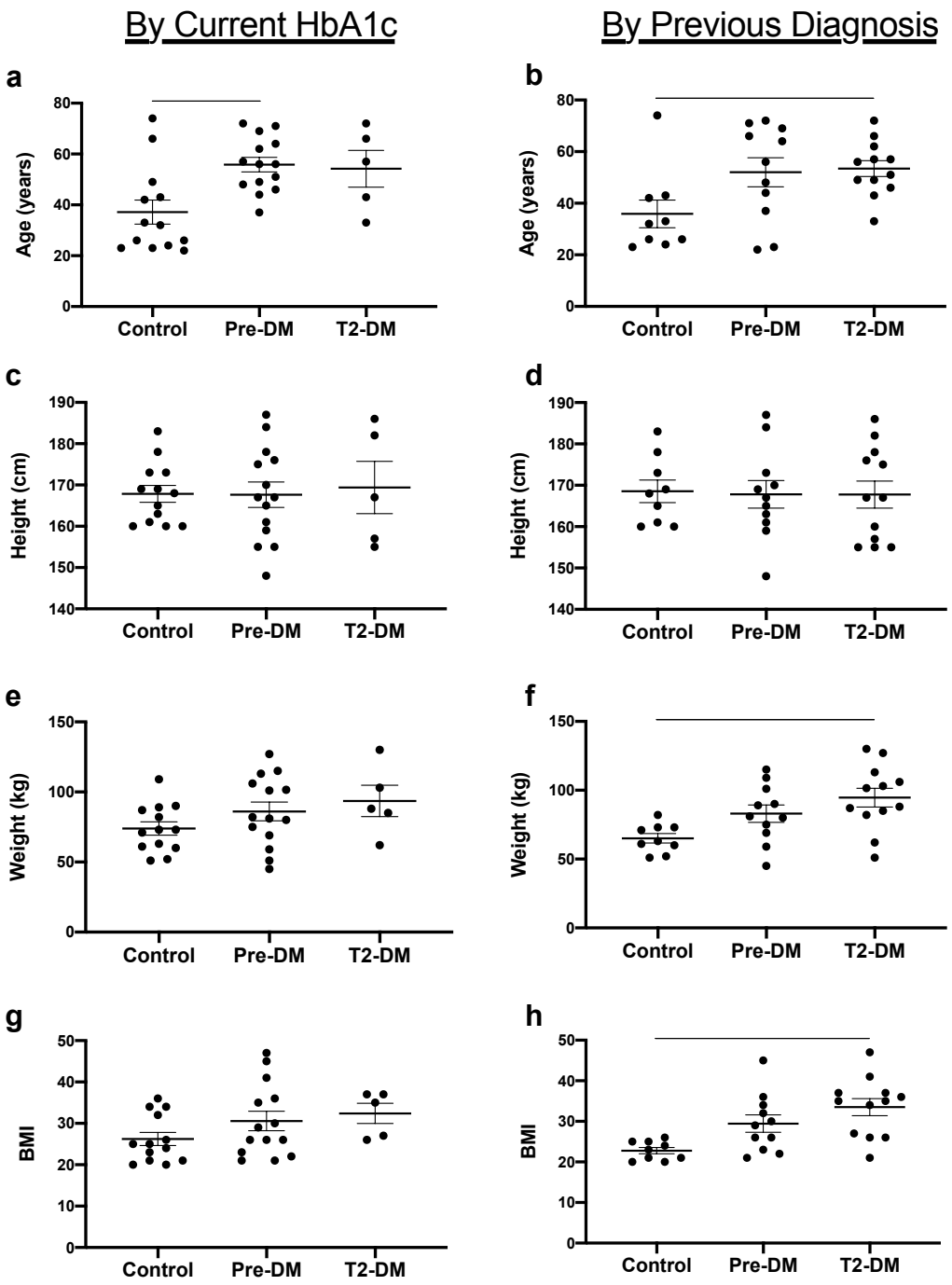

Figure 46: Subject Characteristics

a. Age by current HbA1c. **b.** Age by previous diagnosis. **c.** Height by current HbA1c. **d.** Height by previous diagnosis. **e.** Weight by current HbA1c. **f.** Weight by previous diagnosis. **g.** BMI by current HbA1c. **h.** BMI by previous diagnosis. Data presented as mean ± SE. Significance between groups is indicated with solid black lines ($p<0.05$).

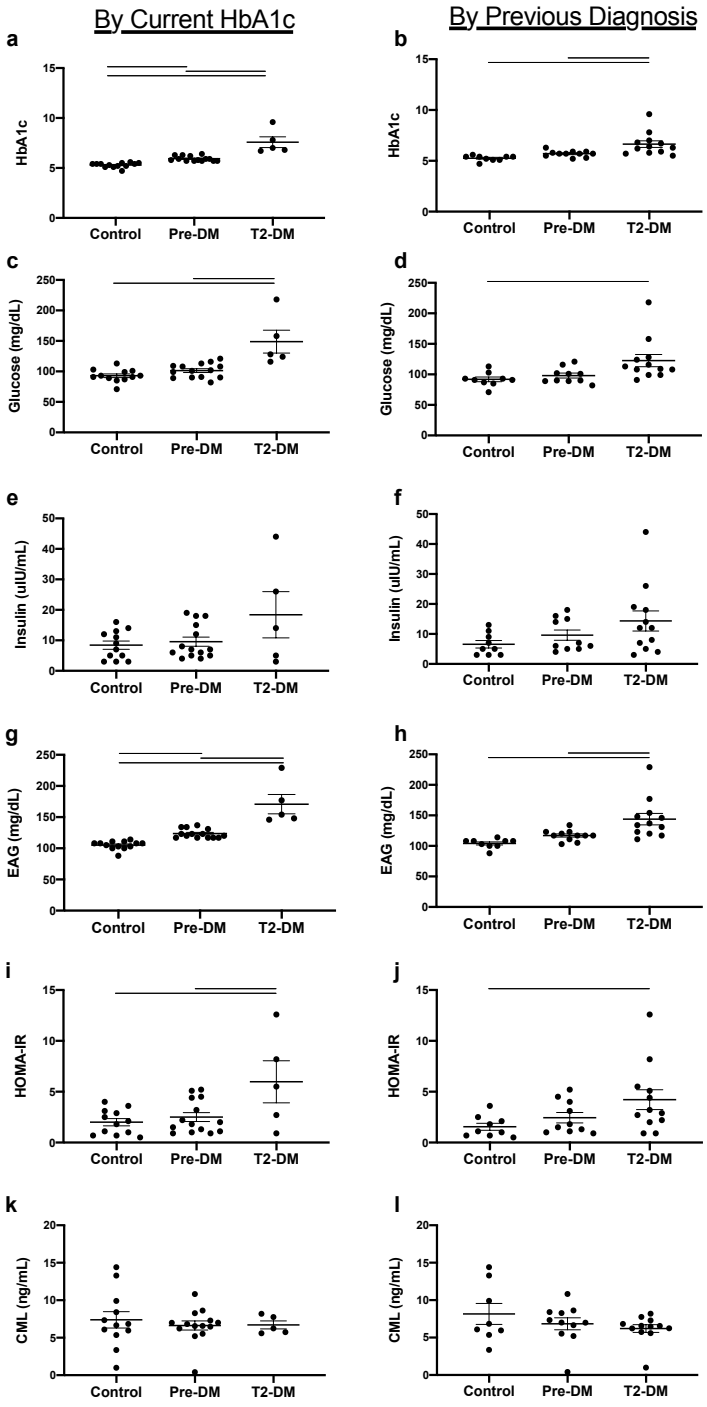

Figure 47: Diabetes-Related Serum Markers

a. HbA1c by current HbA1c. **b.** HbA1c by previous diagnosis. **c.** Glucose by current HbA1c. **d.** Glucose by previous diagnosis. **e.** Insulin by current HbA1c. **f.** Insulin by previous diagnosis. **g.** EAG by current HbA1c. **h.** EAG by previous diagnosis. **i.** HOMA-IR by current HbA1c. **j.** HOMA-IR by previous diagnosis. **k.** CML by current HbA1c. **l.** CML by previous diagnosis. Data presented as mean ± SE. Significance between groups is indicated with solid black lines ($p<0.05$).

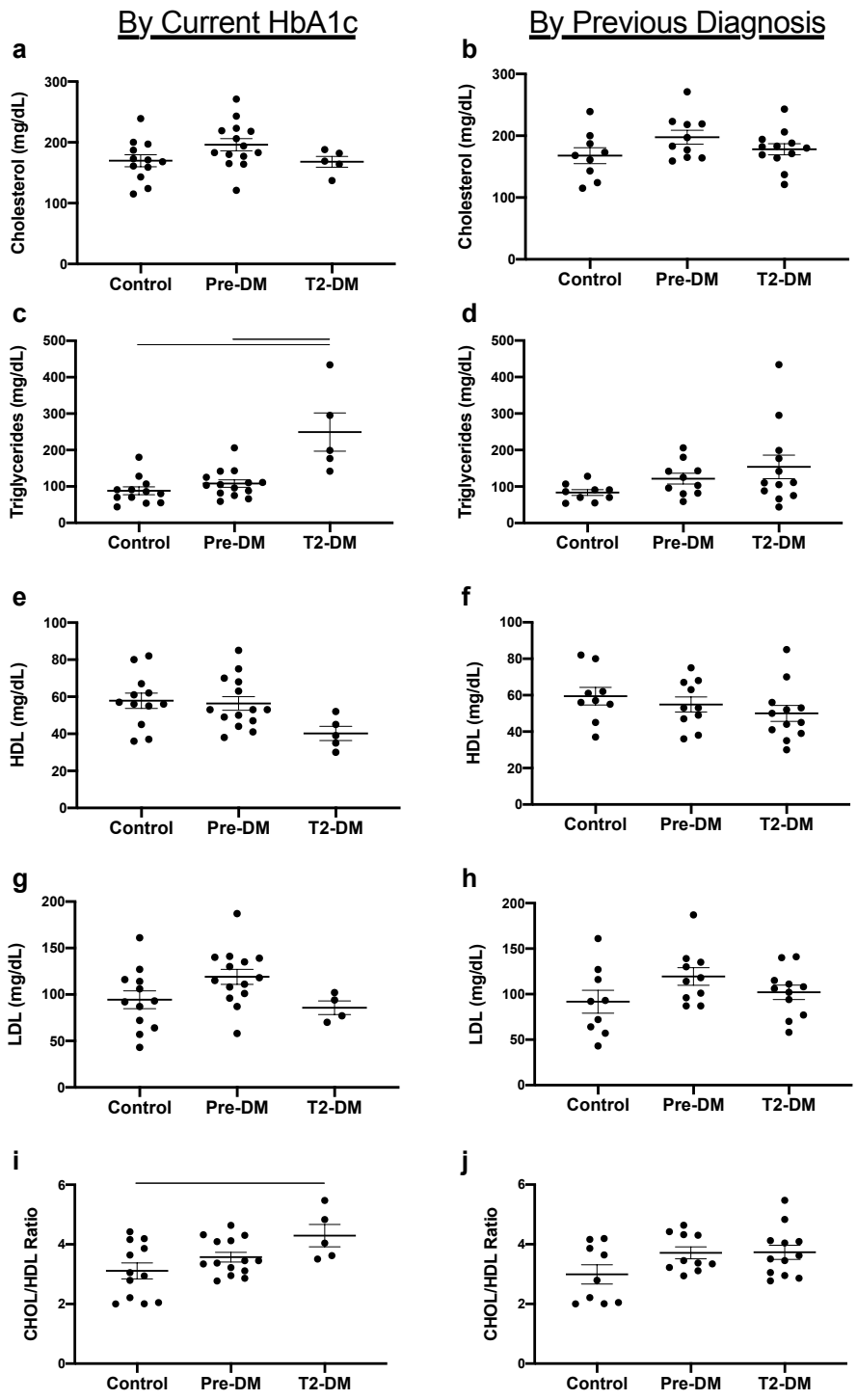

Figure 48: Lipid Panel

a. Cholesterol by current HbA1c. **b.** Cholesterol by previous diagnosis. **c.** Triglycerides by current HbA1c. **d.** Triglycerides by previous diagnosis. **e.** HDL by current HbA1c. **f.** HDL by previous diagnosis. **g.** LDL by current HbA1c. **h.** LDL by previous diagnosis. **i.** CHOL/HDL by current HbA1c. **j.** CHOL/HDL by previous diagnosis. Data presented as mean ± SE. Significance between groups is indicated with solid black lines ($p<0.05$).

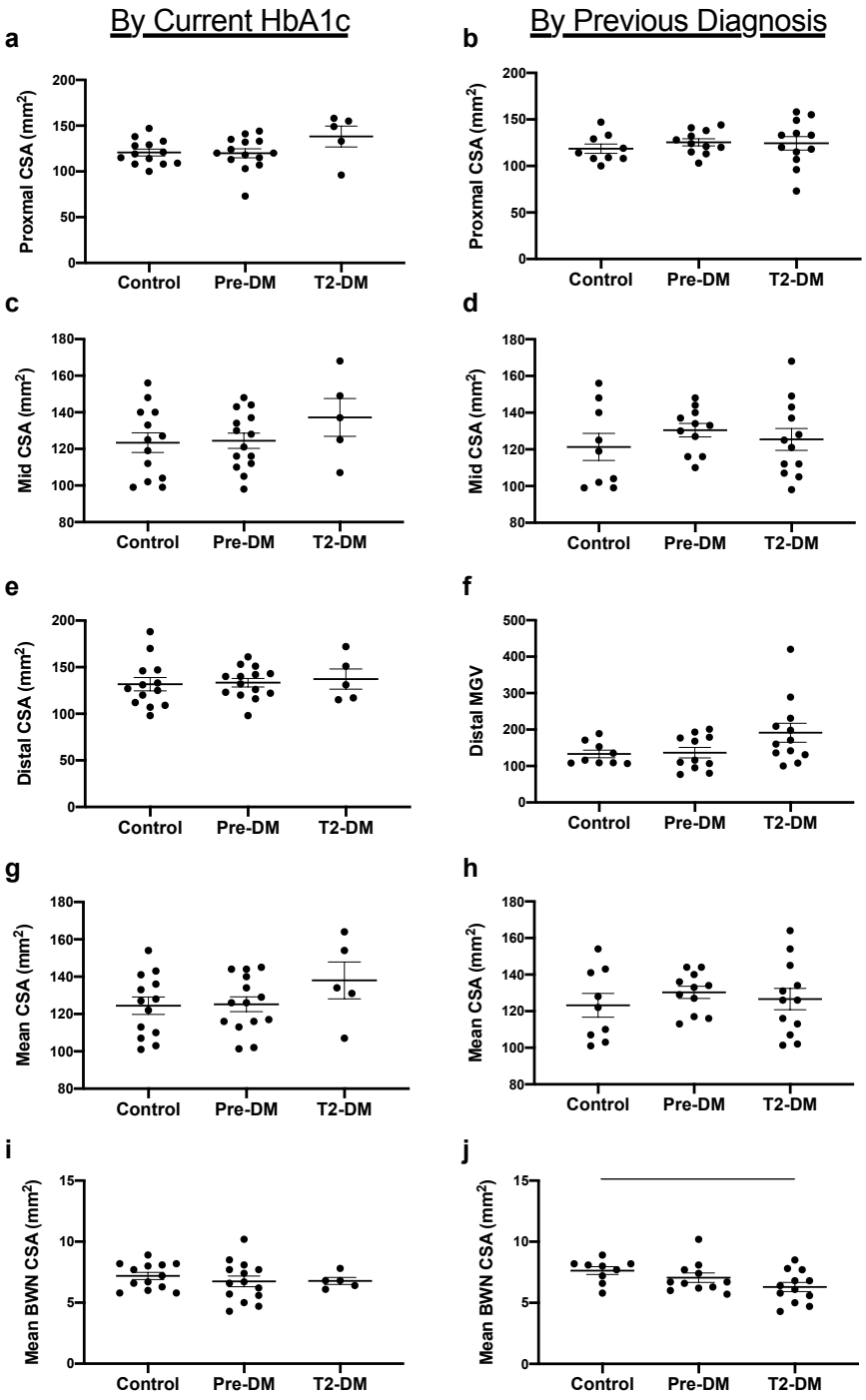

Figure 49: Patellar Tendon Dimensions

a. Proximal CSA by current HbA1c. **b.** Proximal CSA by previous diagnosis. **c.** Mid CSA by current HbA1c. **d.** Mid CSA by previous diagnosis. **e.** Distal CSA by current HbA1c. **f.** Distal CSA by previous diagnosis. **g.** Mean CSA by current HbA1c. **h.** Mean CSA by previous diagnosis. **i.** Mean BWN CSA by current HbA1c. **j.** Mean BWN CSA by previous diagnosis. Data presented as mean ± SE. Significance between groups is indicated with solid black lines ($p<0.05$).

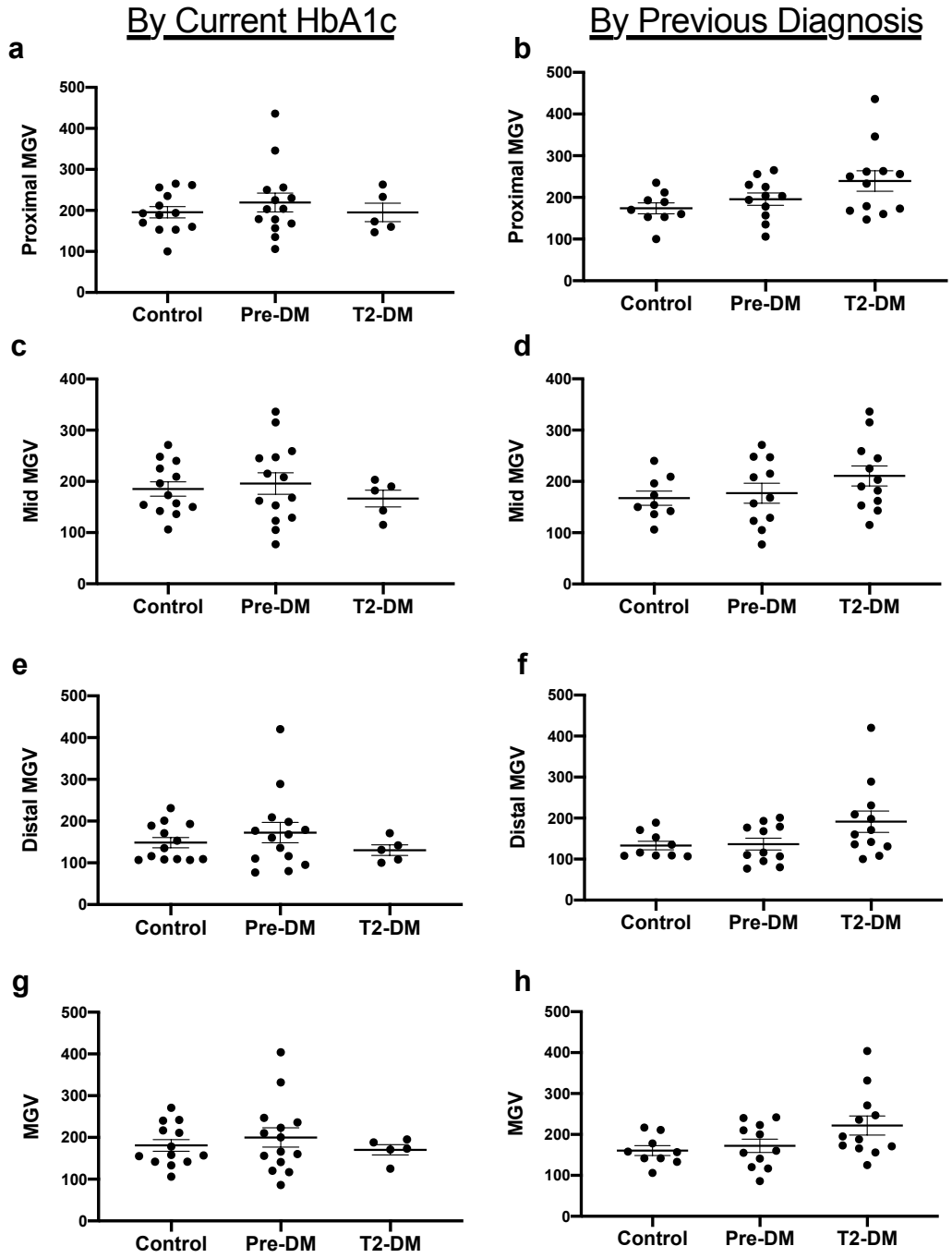

Figure 50: Patellar Tendon Mean Gray Value

a. Proximal MGV by current HbA1c. **b.** Proximal MGV by previous diagnosis. **c.** Mid MGV by current HbA1c. **d.** Mid MGV by previous diagnosis. **e.** Distal MGV by current HbA1c. **f.** Distal MGV by previous diagnosis. **g.** MGV by current HbA1c. **h.** MGV by previous diagnosis. Data presented as mean ± SE. Significance between groups is indicated with solid black lines ($p<0.05$).

	Age (yr)	Height (cm)	Weight (kg)	BMI	Glucose mg/dL	Insulin uIU/ml	HbA1c	EAG (mg/dL)	HOMA-IR	CML (ng/mL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	CHOL/HDL Ratio	Proximal CSA (mm^2)	Mid CSA (mm^2)	Distal (mm^2)	Mean CSA (mm^2)	BWN CSA (mm^2)	Proximal MGV	Mid MGV	Distal MGV	MGV
Age (yr)							0.4	0.4																
Height (cm)			0.4													0.4	0.6	0.6	0.6					
Weight (kg)		0.4		0.9	0.4	0.5			0.5				-0.4							-0.7				
BMI			0.9			0.6			0.6											-0.8	0.5		0.5	0.4
Glucose mg/dL			0.4				0.9	0.9	0.4			0.4	-0.4		0.4	0.4	0.4		0.5					
Insulin uIU/ml			0.5	0.6					1.0											-0.4				
HbA1c	0.4				0.9			1.0				0.6	-0.4		0.4	0.4								
EAG (mg/dL)	0.4				0.9		1.0					0.6	-0.4		0.4	0.4								
HOMA-IR			0.5	0.6	0.4	1.0						0.4								-0.4				
CML (ng/mL)													0.5		-0.5									
Cholesterol (mg/dL)													0.4	0.9										
Triglycerides (mg/dL)					0.4		0.6	0.6	0.4				-0.5		0.7									
HDL (mg/dL)			-0.4		-0.4		-0.4	-0.4		0.5	0.4	-0.5			-0.7									
LDL (mg/dL)											0.9				0.5									
CHOL/HDL Ratio					0.4		0.4	0.4		-0.5		0.7	-0.7	0.5										
Proximal CSA (mm^2)		0.4			0.4		0.4	0.4									0.8	0.5	0.8					
Mid CSA (mm^2)		0.6			0.4											0.8		0.8	1.0			-0.4	-0.4	-0.4
Distal (mm^2)		0.6														0.5	0.8		0.8		-0.4	-0.4	-0.4	-0.4
Mean CSA (mm^2)		0.6			0.5											0.8	1.0	0.8		0.4		-0.4	-0.5	-0.4
BWN CSA (mm^2)			-0.7	-0.8		-0.4			-0.4										0.4		-0.5	-0.4	-0.5	-0.5
Proximal MGV				0.5														-0.4		-0.5		0.9	0.9	1.0
Mid MGV																	-0.4	-0.4	-0.4	-0.4	0.9		0.9	0.9
Distal MGV				0.5													-0.4	-0.4	-0.5	-0.5	0.9	0.9		1.0
MGV				0.4													-0.4	-0.4	-0.4	-0.5	1.0	0.9	1.0	

Figure 51: Correlation Matrix

Red and blue indicate positive and negative significant correlations, respectively, $p<0.05$. Pearson's r-value represented within significant correlations.

CHAPTER 7. CONCLUSION

7.1 Research Summary

Evaluation of patellar tendon gene expression in a rat model of chronic (10-weeks) and acute (1-week) uncontrolled hyperglycemia revealed dramatic loss of gene transcripts associated with tendon extracellular matrix remodeling (ECM). Further, gene expression of tendon regulators, such as scleraxis and tenomodulin, were similarly impacted in response to chronic and acute hyperglycemia. While these effects were not observed in the insulin corrected group, these findings demonstrate that dramatic changes to gene expression occur after only one week of uncontrolled hyperglycemia, indicating the importance of blood glucose control on connective tissue properties. Further uncontrolled hyperglycemia contributes to advanced glycation end product (AGE) formation and non-enzymatic cross-linking in long-lived tissue, such as tendon collagen. Although neither serum AGEs or cross-link formation was measured in these animals, our lab previously demonstrated an increase in total collagen content in tendon of rats with 10-weeks of uncontrolled hyperglycemia, providing further evidence of the consequences to connective tissue during cases of hyperglycemia.

To broaden our understanding of the role of high glucose (HG) and AGEs on tendon, a study was designed to separately test each variable on tendon fibroblast homeostasis. Achilles tendon derived fibroblasts exposed to physiological relevant does of AGEs display impaired cell proliferation capacity, ATP production, and electron transport chain efficiency. Not only did this occur independent of HG, but occurred in a dose-dependent manner. This work highlighted that impairments to mitochondrial function were targeted to complex III of the electron transport chain. Increased levels of mitochondrial DNA, a common indication of histopathology, were also observed independent of hyperglycemia. The HG condition did impact proton leaked coupled respiration (ΔpH, proton gradient) and mitochondrial membrane potential ($\Delta\Psi m$) independent of AGEs, demonstrating that HG and AGEs may contribute to the development and progression of diabetic tendon pathology via separate mechanisms. While HG did impact tendon fibroblasts homeostasis, a greater number of variables were impacted by AGEs alone.

To gain a comprehensive understanding of the mechanisms by which AGEs impact tendon fibroblasts, transcriptome wide analysis was completed. Genes associated with cell cycle

progression and ECM maintenance were the most impacted in tendon fibroblasts exposed to AGEs, confirming our previous work (Chapter 3). To guide future research of AGEs and tendon biology, a descriptive approach was implemented to rank the most impacted pathways. Ranking of pathways revealed several pathways to be implicated, but to our surprise, mitochondrial and energy metabolism pathways were only minimally implicated in the dataset, suggesting that previously observed limitations to mitochondrial function (Chapter 3) may occur through a non-transcriptional mechanism.

Genes associated with wound healing were strongly implicated in our descriptive ranking of the transcriptome, and therefore a mouse model of elevated serum AGEs was established to study the role of circulating AGEs on tendon healing. Following patellar tendon injury, genes related to cell proliferation (Mybl2), mitochondrial function (Bcs1l), and growth factors (Fgf2) were shown to be elevated in mice that received daily AGE injections. Further, some mice were assigned to a moderate treadmill training (MTR) group to determine whether mechanical stimulation of tendon could improve healing parameters. Contrary to our hypothesis, MTR did not alter gene markers such as Ctgf and Fgf2, which are generally indicative of improved wound healing. Our findings, however, indicated that circulating AGEs could play a contributing role in delaying tendon healing which is commonly noted in injured diabetic patients.

To expand our understanding of how circulating AGEs impact tendon properties in diabetic humans, a cross-sectional study was designed to correlate serum AGEs and diabetes-related serum factors to patellar tendon dimensional properties. Contradictory to the current literature, diabetic subjects have smaller patellar tendon dimensions after normalization to body mass. While no association of circulating N^{ε}-(carboxymethyl)lysine (CML) to patellar tendon dimensions was found, homeostatic model assessment of insulin resistance (HOMA-IR) was negatively correlated to patellar tendon dimensions, highlighting that metabolic syndrome does not necessarily constitute an increase in tendon cross-sectional area (CSA) nor an increase in circulating CML levels.

7.2 Future Directions

The present work revealed several novel effects of AGEs and hyperglycemia on tendon and tenocyte biology. Our animal and cell culture data demonstrate that AGEs alter homeostatic properties of tendon fibroblasts in a manner that suggests development and progression of

pathology and delayed healing. Further, our human work challenges the notion that diabetes is associated with greater tendon CSA. However, our data describing the role of circulating AGEs on diabetes-related tendon pathology is primarily descriptive, with only introductory mechanistic insight, and therefore, further work in this area is needed.

Our initial work describing the role of AGEs on tendon healing following injury likely requires a bolstered sample size. Further, comprehensive histology and immunohistochemistry of the injured patellar tendon will assist in stronger interpretation of tendon healing in an environment of elevated serum AGEs. Specifically, hematoxylin and eosin staining will allow for determination of cell density at the injury site in the presence and absence of serum AGEs. Collagen peptide staining will provide qualitative data to visualize the ECM remodeling response and further assess degree of healing.

Future human work implicating the role of alternative circulating AGE species, such as methylglyoxal-derived hydroimidazolone (MG-H1) on tendon dimensions and *in vivo* mechanical properties will provide for a more inclusive assessment of AGEs and diabetes-related tendon pathology. Specifically, collection of *in vivo* tendon mechanical properties data may reveal that tissue properties are correlated to circulating AGEs, rather than to tendon dimension data alone. If mechanical properties data are lower in diabetic subjects and correlated negatively to serum AGEs, the AGE-RAGE tissue degeneration hypothesis discussed in Chapter 1 and Chapter 6 may gain momentum.

Linking the receptor for advanced glycation end products (RAGE) to tendon pathology will be essential to strengthening our claim that circulating AGEs are responsible for initiating pathology. Specifically, future *in vivo* mechanistic studies evaluating RAGE activation, downstream signaling events, and functional tendon outcomes will support our claim. It is our hope that this work encourages tendon researchers to consider exploration of alternative mechanisms contributing to the growing number of diabetic tendinopathy cases.