ADVANCED GLYcation END PRODUCTS IN DIABETES AND TOBACCO AFFECT COLLAGEN PHENOTYPE IN ROTATOR CUFF INJURY HEALING RESPONSE

By

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A THESIS

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Abstract

Rotator cuff tendon injuries are common, particularly amongst patients who suffer from type 2 diabetes mellitus and patients with a history of smoking. In these patients, rotator cuff injuries also heal more slowly and are more likely to recur. The cellular and molecular processes underlying the tendon healing process, at times inflammatory by nature, are well-known. However, where along that process diabetes and tobacco use exacerbate the problem has yet to be understood.

Advanced glycation end products (AGEs) may be the culprit. AGE accumulation is increased in both diabetic and tobacco-using groups, and AGEs are known to exacerbate the more inflammatory or critical stages of the cellular and molecular healing process. Collagen phenotype, expressed as the ratio of Collagen type 1 to Collagen type 3, is the primary determinant underlying tendon injury and impaired healing. This study was conducted to assess AGEs as a causal event in impaired tendon healing, particularly by way of increasing levels of matrix metalloproteinases, namely MMP2 and MMP9.

Human tendon tissue recovered as surgical waste was utilized to assess the correlation between these and other relevant molecules in the injured tendon. Following, swine tenocytes in cell culture were treated with cigarette smoke condensate, AGE inhibitor (pyridoxamine), and both in order to assess AGEs as a causal factor in terms of expression change in MMP2, MMP9, and the Pro-Collagen 1:3 ratio.

Cigarette smoke condensate containing tobacco-derived AGEs was found to increase MMP2 and decrease the Pro-Collagen 1:3 ratio, suggesting that increased AGE accumulation does underlie impaired healing processes in diabetic and smoking patients with rotator cuff
injuries. Findings suggest that this is accomplished through MMP2-mediated suppression of Pro-Collagen 1:3 ratio.

The results of this work are encouraging and set the stage to uncover the pathways that interfere with tendon healing. Verification of our results by replication in a larger sample of patients’ tissues as well as considering in vivo studies is necessary to draw a strong conclusion. In addition, the results may show that Pyridoxamine, which functions to inhibit the production of AGEs, may not be protective against already-synthesized AGEs due to the history of tobacco smoking and diabetes. Nonetheless, further search should be done into developing a more clinically relevant AGE inhibitor.
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<table>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate Protein Kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>COL1</td>
<td>Collagen 1</td>
</tr>
<tr>
<td>COL3</td>
<td>Collagen 3</td>
</tr>
<tr>
<td>CSC</td>
<td>Cigarette Smoke Condensate</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-Associated Molecular Pattern</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FW</td>
<td>Forward</td>
</tr>
<tr>
<td>GA</td>
<td>Glenohumeral Arthritis</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>HBS</td>
<td>Horse Blocking Serum</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat Induced Epitope Retrieval</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-Mobility Group Box Protein 1</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1, family</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus Kinase/Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>LH</td>
<td>Lysine hydroxylase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescent Intensity</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix Metalloproteinase 2</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix Metalloproteinase 9</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa Beta</td>
</tr>
<tr>
<td>p38</td>
<td>Class of MAPK</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor suppressor pathway; TP53 gene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-Buffered Saline with Triton</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PH</td>
<td>Proline Hydroxylase</td>
</tr>
<tr>
<td>PM</td>
<td>Pyridoxamine</td>
</tr>
<tr>
<td>Pro-Col1</td>
<td>ProCollagen1</td>
</tr>
<tr>
<td>Pro-Col3</td>
<td>ProCollagen3</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End products</td>
</tr>
<tr>
<td>RC</td>
<td>Rotator Cuff</td>
</tr>
<tr>
<td>RCI</td>
<td>Rotator Cuff Injury</td>
</tr>
<tr>
<td>RCT</td>
<td>Rotator Cuff Tear</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RW</td>
<td>Reverse</td>
</tr>
<tr>
<td>SLAP</td>
<td>Superior Labral Anterior to Posterior</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TPM</td>
<td>Total Particulate Matter</td>
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**Introduction**

Type II Diabetes Mellitus (T2DM) accounts for between 90 and 95% of the 422 million diabetics globally and 30.3 million diabetics in the United States [1,2]. The most recent data, from a 2016 report, suggest that diabetes was directly responsible for 1.5 million deaths around the world in 2012 and indirectly responsible for an additional 2.2 million deaths due to diabetes complications [1]. Reports estimate the incidence of shoulder disorders, defined as adhesive capsulitis or rotator cuff (RC) disease, to be 27% among the diabetic population as compared to a 5% incidence rate among the general population [3].

Specifically, the estimated prevalence of RC tendinopathy ranges from 0.3-5.5% of the population [3,4] and its incidence increases with age; only 9.7% of RC tendinopathy patients are aged 20 years or younger, whereas 62% of patients are aged 80 or older [5]. The presenting symptoms of this disorder are pain and weakness, particularly with elevation and external rotation [3,6] along with decrease in the shoulder’s range of motion [3]. These decreased functional properties are accompanied by local tenderness, inflammation, and reduced mechanical strength [7]. Therapies are broad and expensive, including electrotherapy, non-steroidal anti-inflammatory drugs, and physical therapy. More invasive options include corticosteroid injections [6] and nerve blocks, which are still considered conservative treatment options [8]. In more severe cases, surgery may be required [3].

In addition to experiencing shoulder disorders at a higher rate than non-diabetics, diabetic patients are also more likely to undergo spontaneous tendon rupture [9] and experience impaired healing after an injury [10,11]. Certain factors for the increased prevalence of shoulder disorders among diabetic patients have been examined. Insulin as a management strategy, for instance, has been investigated and found to have no significant effect on patients’ increased risk [3]. While
the link between tendinopathy and T2DM is clinically well-established, the cellular and molecular mechanisms linking these two disorders have not been fully elucidated. Some possibilities have been considered, including the prevalence of advanced glycation end-products (AGEs) and impaired microcirculation [12,13].

Tobacco use has also been associated with increased prevalence and impaired healing of rotator cuff injury (RCI) and is another common comorbidity. The most recent data indicated that 15.5% of Americans, or 37.8 million people, regularly smoked cigarettes in 2016. This includes 17.5% of American men and 13.5% of American women [14]. On a global level also, more men (36.1%) than women (6.8%) smoke cigarettes. Smoking claims over seven million lives annually, including nearly one million deaths due to secondhand smoke [15].

Smoking and RC tears have been found to be correlated with a dose-dependent relationship. Smokers also are at increased risk for larger RC tears than their non-smoking counterparts [16]. Measured by MRI, RC healing after surgery was found to be statistically decreased in smokers as well as impaired repair quality and mechanical properties of the tendon [17]. The American Association of Orthopaedic Surgeons asserts that smoking is a risk factor for surgical outcomes [18]. As with diabetes, the link between tobacco usage and sustained RCI is clinically well-established but does not have a clearly defined molecular mechanism. A major factor underlying sustained injury is a high level of maintained inflammation. Tobacco smoke has been implicated strongly in lung and airway inflammation [19], one of the downstream effects of AGE accumulation. Some AGEs are tobacco-derived [20], indicating that AGE accumulation may be a causative factor in increased susceptibility and decreased healing of RCI in smoking patients.
Typical Tendon Healing Process

Tissue and Cellular Level

In an injured but otherwise healthy tendon, the healing process broadly follows three phases: substrate phase, fibroplasia phase, and remodeling phase. The substrate phase is characterized by hematoma formation and an inflammatory reaction, which activates the healing process [21]. During the fibroplasia phase and its proliferative sub-phase, fibrils reorganize to form fibers that in turn form connective tissue. The soluble protein fibrinogen forms fibrin, which then forms a provisional matrix. In the proliferative sub-phase of the fibroplasia phase, Collagen 3 synthesizes to Collagen 1 [21,22]. Collagen 1 is stronger and less flexible than Collagen 3 and the step of increasing the Collagen 1 to Collagen 3 ratio is vital to tendon healing [23]. Finally, during the remodeling phase collagen and fibroblasts reorient, restoring function to the tendon [21].

Molecular Level

Healing also takes place on the molecular level, involving several microscopic factors (See Figure 1). The first of these is that the extracellular matrix (ECM) is disorganized through the injury [24]. This disorganization decreases the tendon’s ratio of Collagen 1 to Collagen 3 [25]. In a normal, healthy, uninjured tendon, the ratio is approximately 90:10, but in an injured tendon this ratio decreases by two and a half to three-fold between the time of injury and the two weeks after. This ratio maintains until at least four weeks after the time of injury [26], which indicates structural deficiencies. Collagen 3 is weaker than Collagen 1, pathological in excess, and is flexible to the point of detriment [11,23]. With Collagen 3 as the primary collagen type in a tendon, tensile strength and mechanical load-to-failure decrease while inflammation persists.
These impaired mechanical properties serve to increase defects in forming collagen crosslinks, which in turn leads to further impaired mechanical properties [24,27,28]. A lowered ratio of Collagen 1 to Collagen 3 also increases expression of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) [29] and increases oxidative stress [30,31], which in turn increases activation of the receptor for advanced glycation end products (RAGE) by such ligands as high mobility group box protein 1 (HMGB1) [32].

MMP2 is involved in both collagen degradation and remodeling while MMP9 is involved solely in collagen degradation [9,33]. Thus, an increase in MMP2 and MMP9 disrupts the regular process of collagen and ECM turnover, leading to further exacerbations in the changed Collagen 1 to Collagen 3 ratio and further exacerbated ECM disorganization [25,34,35]. Inhibition of MMP2 and MMP9 has been shown to prevent impairment of mechanical properties in tendons [36], suggesting that upregulation such as in injury would cause mechanical impairment.

Defective collagen crosslinks as formed by decreased mechanical properties and low collagen turnover result in collagen fibers that are too stiff for typical function [27]. Lysyl oxidase mediates these crosslinks and is implicated in collagen crosslink dysfunction across tissue types, including in tumors [27,37]. The enzyme family lysyl hydroxylase catalyzes the conversion of the essential amino acid lysine to hydroxylysine, a modified amino acid specific to collagens [38]. Pathologic tendons show elevated levels of both hydroxylated lysine and hydroxylated proline residues [39,40], indicating involvement of the prolyl hydroxylase enzyme family as well. Similar to lysyl hydroxylase and lysine, the prolyl hydroxylase family acts on proline. Prolyl hydroxylase catalyzes proline’s conversion to hydroxyproline, which comprises about 13.5% of collagen amino acids [41]. Approximately 300 hydroxyproline residues are
present in each crosslinked collagen molecule [40] and as such, both hydroxylysine and hydroxyproline may be used as indicators of dysfunctional collagen crosslinking.

Figure 1: Molecular Mechanisms of Tendon Healing. An injured tendon suffers from ECM disorganization, which leads to a decreased Collagen 1 to Collagen 3 ratio with a threefold effect of increased oxidative stress, increased MMP 2 and MMP 9, and decreased mechanical properties. This typical healing process leads to a moderate healing response.

A third result of a lowered Collagen 1 to Collagen 3 ratio is increased levels of stress. While tendon stress takes the form of damage, metabolic, and oxidative stress [42,43], oxidative stress is the primary form and is characterized by elevated levels of reactive oxygen species (ROS) [44]. ROS are species produced by reduced molecular oxygen and include both free radicals such as hydroxyl radical and nonradicals such as hydrogen dioxide [45]. When ROS are present in an injured tendon environment, cellular damage occurs that indicates the release of damage associated molecular patterns (DAMPs), especially HMGB1 [46]. When HMGB1 binds to RAGE, the primary downstream effect is increased inflammation by activation of the
JAK/STAT, NF-kB, or MAPK signaling pathways and the resultant production of tumor necrosis factor alpha (TNF-α) [47–51]. This inflammation decreases as the tendon heals. A provisional matrix occurs from fibrin produced by soluble fibrinogen and increases the ratio of Collagen 1 to Collagen 3 [52–54]. With the cycle broken by restored collagen ratio, inflammation subsides, and the tendon heals. With persistent inflammation, however, the condition persists as well. A primary effect of the AGE-RAGE axis is downstream inflammation [47], suggesting that elevated levels of AGEs may impair tendon healing response.

**Advanced Glycation End products and Receptor for Advanced Glycation End products**

Non-enzymatic reactions occur between proteins, lipids, or nucleic acids with reducing sugars such as glucose. The reactive sugars’ carbonyl groups react with free amino groups or amino acids in the reactive proteins, lipids, or nucleic acids, forming an unstable Schiff base. This unstable base may then further rearrange to create a stable ketoamine, or Amadori product. Both Schiff bases and Amadori products are reversible. However, both may react irreversibly with peptide or protein amino acid residues, forming protein adducts or protein crosslinks. These are advanced glycation end products (AGEs) [55]. AGEs accumulate in normal aging, though levels of accumulation are significantly increased in diabetes [39] and in smokers [20]. As AGE is known to have detrimental effects overall, AGE inhibitors have been developed. These include pyridoxamine, which acts by inhibiting the formation of AGEs from Amadori products [56].

The AGEs with the highest affinity for RAGE are the three structural isomers of methylglyoxal-derived hydroimidazolones [57,58]. Regardless of the specific ligand, AGE-RAGE binding activates the proinflammatory JAK/STAT and NF-kB pathways [47] as well as
the apoptotic JNK pathway [59]. The AGE-RAGE axis also activates the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway, which produces reactive oxygen species (ROS) [59,60]. HMGB1 binds to RAGE [32] and activates the JAK/STAT, NF-kB, p53, and MAPK signaling pathways. Activation of these pathways leads to downstream inflammation by production of tumor necrosis factor alpha (TNF-α), IL-1, IL-6, and IL-8, or to downstream cell death via apoptosis and pathological cell cycle arrest [47–51].

While RAGE has several ligands, the AGE-RAGE, and to a lesser extent the HMGB1-RAGE, axes are of particular interest. Methylglyoxal-derived hydroimidazolones are an example of AGES, and form three structural isomers. Their high affinity and specificity for RAGE suggest that they may be the primary AGE protein adduct [57]. AGE-RAGE binding has varying effects based on the location of the binding site in the body, but in fibroblasts AGE-RAGE binding specifically modulates the expression of proinflammatory molecules. Further, a major result of AGE-RAGE binding is the generation of ROS [60,61]. Interestingly, while methylglyoxal-derived hydroimidazolones bind to RAGE with a particularly high affinity, AGES as a whole bind with a relatively low affinity of approximately 10 µM [58].

HMGB1, when intracellular, is involved in such processes as transcription, autophagy, and cell development, but when released from dying cells it takes on the role of damage-associated molecular pattern, DAMP [59]. In this condition, it has been shown to bind to RAGE with an affinity greater than 10 µM, which is a higher affinity than AGES [32].
Figure 2: Downstream effects of RAGE ligands AGE and HMGB-1. AGE, a primary RAGE ligand, activates the NADPH oxidase, JAK/STAT, NF-kB, and JNK pathways leading to ROS production, activation of TNF-α, IL-1, IL-6, IL-8, and downstream molecular damage, pathological Collagen 1 synthesis, and inflammation. The HMGB1-RAGE axis activates the JAK/STAT, NF-kB, p53, and MAPK pathways, inducing TNF-α, IL-1, IL-6, IL-8, apoptosis, inappropriate cell proliferation, and cell cycle arrest, leading to downstream pathological Collagen 1 synthesis, inflammation, and cell death.

RAGE activation, therefore, whether by HMGB1 or AGE, is detrimental as a whole. AGE-RAGE binding leads specifically to ROS production and therefore molecular damage, HMGB1-RAGE binding leads to cell death, and both axes result in downstream inflammation [26,48,51,59,62–65]. As inflammation is a strong suspect as an underlying cause of prolonged injury and impaired healing [7,66], RAGE activation is highly likely to be responsible for much of the impaired healing process demonstrated in both T2DM and in tobacco users. Though both HMGB1 and AGEs are strong ligands for RAGE and its downstream proinflammatory effects,
and both bind with RAGE in an injured tendon, AGE levels are demonstrably elevated in T2DM patients and in tobacco users [67,68]. As such, AGE-RAGE binding and therefore AGEs are likely to play the causative role in the impaired healing process of rotator cuff tendon in T2DM patients and in smoking patients.

AGEs have direct impacts on the normal tendon healing process in three primary ways: 1) increasing oxidative stress, 2) increasing RAGE activation, and 3) increasing the defective, nonspecific collagen crosslinks [10,57,60,61] (See Figure 3). Firstly, AGE-induced oxidative stress, ROS, is accomplished through the activation of the NADPH oxidase pathway. When AGE binds with RAGE and activates this pathway, NADPH oxidases initiate the transfer of electrons from NADPH to molecular oxygen, generating ROS [59,60]. Specifically, activation of the NOX family of NADPH oxidases has been specifically demonstrated to be an effect of AGE-RAGE binding [59,69]. ROS, which include such species as nitric oxide and hydrogen dioxide [44], damage lipids, modify proteins, and cleave DNA strands [70,71]. ROS have specifically been implicated in tendinopathy, though due to their complex nature have not been fully deemed a causal factor. However, their harmful effects are seen in tendon tissue as well as in various tissue types across the body system, suggesting direct involvement in cellular and molecular damage [71–74]. Increased ROS levels lend themselves to further release of DAMPs such as HMGB1, activating RAGE and leading to exacerbated inflammation [46]. Secondly, cells treated with increased concentrations of AGEs express increased RAGE levels [59] which, as discussed, directly leads to increased inflammation that is also present in sustained tendon injury [47,59]. Finally, AGEs convert specific, enzymatic collagen crosslinks to non-specific, nonenzymatic crosslinks by altering the surface of the collagen cells [10]. These dysfunctional crosslinks
impair the tendon’s mechanical properties by decreasing load-to-failure, increasing stiffness, and altering protein binding sites [24,28].

Figure 3: Impact of AGE accumulation on tendon healing. AGE accumulation impacts an injured tendon by elevating already increased oxidative stress, RAGE, and defective collagen crosslinking. This impaired healing process leads to a poor healing response.

AGEs naturally accumulate as a person gets older, but persistent hyperglycemia results in an increased glycation rate and therefore an increased accumulation of AGEs [75]. Natural AGE accumulation may offer an explanation as to why tendinopathy rates are so much more prevalent in the older population.
**Diabetes and Tendon Healing**

Diabetic tendons have been demonstrated to have poorer surgical outcomes as compared to their otherwise healthy counterparts [10,11]. Changes in healing response of the diabetic tendon as contrasted with the nondiabetic tendon occur on a macro level as well as on the micro level. As continually high glucose levels result in an increase in AGE accumulation [75], AGEs may be the reason for the impaired healing response.

*Tissue and Cellular Level*

In the diabetic tendon, the inflammatory processes of the substrate phase are elevated. This prolonged inflammation delays healing, and the extent of inflammation seen in healing tendons is positively correlated with levels of insulin resistance, a hallmark of T2DM [66]. Hyperglycemia, an additional T2DM hallmark, has been shown to encourage several inflammatory mediators, including fibrinogen, tumor necrosis factor α (TNFα), IL-1β, and IL-6 [12,76,77].

In the fibroplasia phase, the necessary levels of fibrinogen are exceeded in T2DM; this excess leads to further increased inflammation and dysregulation of tendon healing [52,53]. While the proliferative phase of healing increases the Collagen 1 to Collagen 3 ratio, in T2DM the remodeling process is significantly less efficient and as such, Collagen 3 remains the primary collagen type rather than Collagen1 [11,22]. The elevated levels of the weaker, more flexible Collagen 3 lead to decreased tensile strength, delayed healing, persistent inflammation, and decreased mechanical load-to-failure [10,24].

During the remodeling phase, typical tendons experience reorganization of collagen and fibrils [21]. In T2DM, however, collagen fiber organization is significantly decreased [22].
Fibrils in T2DM demonstrate increased and irregular diameters as well as closer packing [78] along with significantly reduced fibrocartilage, demonstrating an impairment in ability to reorganize fibrils [10,22]. As a result, tendon function is not fully restored in diabetic patients as compared with otherwise healthy patients.

*Molecular Level*

Injured diabetic tendons experience a higher level of ECM disorganization [79], thus beginning the healing pathway with a disadvantage, setting off exacerbated changes throughout the rest of the process. With exacerbated ECM disorganization, it follows that the rest of the cascade, meaning Collagen 1 to Collagen 3 ratio decreases, MMP2 and MMP9 increases, mechanical property decreases, defective crosslink increases, oxidative stress increases, RAGE binding, and inflammation increases, is exacerbated as well. However, this alone is not enough to account for the poor healing outcome seen in diabetic patients as increased ECM disorganization may be likened to worse injury. Worse injury on its own has not been demonstrated to have a significant impact on surgical outcome [80].

One possible explanation, then, is the increased presence of AGEs in diabetic patients [12,39,75]. The combined effect of increased ECM disorganization, AGEs, and increased AGEs caused by the diabetic state may be sufficient to account for the poor healing response. While the entire cascade is elevated due to increased ECM disorganization, ROS, RAGE, defective collagen crosslinking, and events downstream of these are exacerbated even further by the presence not only of AGEs, but of large amounts of AGEs. Therefore, the healing response is poorer than that of a tendon experiencing impacts of AGEs alone (see Figure 4). While the steps are not changed and new steps not added to this cascade, it follows that the combination of effects amplifies each step to the extent that a very poor healing response follows.
Figure 4: AGEs and T2DM in healing tendon. Increased AGE accumulation, combined with increased ECM disorganization, serves to exacerbate the inflammatory mechanisms of a healing rotator cuff tendon in diabetic patients. Each effect is amplified, resulting in a very poor healing response.

**Tobacco and Tendon Healing**

Two of the difficulties in studying the impact of tobacco on various body systems are the large number of chemicals found in cigarette smoke and the various methods of tobacco consumption. Tar, the particulate matter of cigarette smoke, is the part of the smoke that contains most of the carcinogenic and mutagenic compounds [81]. While the chief addictive ingredient in cigarettes is nicotine, over 4000 chemicals have been identified in tobacco smoke and over 70 have been specifically classified as carcinogenic, including arsenic, benzene, cadmium,
formaldehyde, lead, and nickel [82]. Which of these ingredients is specifically responsible for harmful effects is difficult to elucidate and the broad scope of mechanisms for tobacco consumption, including smoking cigarettes, chewing tobacco, and the recently popular e-cigarettes makes it difficult to determine whether specific forms of tobacco consumption or tobacco broadly is responsible.

In addition to T2DM, smoking tobacco has also been correlated with elevated rates of both rotator cuff tears and rotator cuff dysfunction, as well as poorer surgical outcomes such as decreased rates of healing [18]. Inflammation has been largely connected to the persistence of rotator cuff tendon injury and impaired healing processes. Tobacco has well-established inflammatory effects, chiefly investigated in terms of lung and airway inflammation; inflamed airways have been reported to be both caused and maintained by cigarette smoking [19]. As inflammation is caused and maintained by many of the same pathways throughout the body regardless of anatomical location, it would follow that rotator cuff tendon inflammation would be exacerbated, caused, and/or maintained in tobacco smokers thus impairing the healing process of these injuries. Tobacco smoke has been specifically implicated in the maintenance of inflammatory processes assessed in typical rotator cuff tendon injury, including MMPs, HMGB1, ROS, and AGEs (see Figure 5).

**MMP2 and MMP9**

Increases in MMP2 and MMP9 have been reported to be directly connected to the maintenance of RCI [25,29,34,35] and these MMPs are also directly connected to cigarette smoke exposure. Patients who smoke cigarettes are therefore more likely to have affected MMP2 and MMP9 levels even before enduring a rotator cuff tendon injury, leaving them more susceptible to exacerbated injury and to impaired healing. MMP2, responsible for collagen
degradation and remodeling, has been demonstrated to be overexpressed in both RCI and in cigarette smokers. It is possible that MMP2 response to tobacco is tissue-dependent; it is reported that aqueous extract of cigarette smoke leads to an increase in pro-MMP2 levels in aortic vascular smooth cells [83], while prenatal nicotine exposure is reported to increase MMP2 expression in newborns with ROS levels acting as a mediating factor. Further, nicotine specifically has been reported to increase MMP2 levels in adult cardiac tissue [84]. However, lung fibroblasts have been reported to experience a decrease in MMP2 activity after cigarette smoke exposure, though whether the expression of MMP2 itself is inhibited or the activity is specifically inhibited [19].

MMP9, responsible for collagen degradation, is typically overexpressed in RCI and results in an excess of collagen degradation without sufficient replenishment. Underexpression of MMP9, on the other hand, indicates an inability to produce new healthy collagen [85]. The effect of smoking on MMP9 levels is unclear; MMP9 has been reported to be both increased [83,86–89] and decreased [85,90] in people who smoke. The many ingredients found in tobacco smoke complicate investigations on the subject as researchers isolate different portions of the smoke to test for effects; recorded MMP9 expression may be dependent on the specific smoke ingredient tested. For instance, whole tobacco smoke, aqueous extract of cigarette smoke, and cadmium each demonstrated a positive effect on MMP9 expression [83,86–89], while whole tobacco smoke and nicotine demonstrated a negative effect [85,90]. Whole tobacco smoke has been implicated in both increased and decreased levels of MMP9 expression, suggesting that a variance in levels of other ingredients present in whole smoke such as nicotine and cadmium may be responsible for these differences. When nicotine specifically was associated with decreased MMP9 levels, the association was dose-dependent [90]. Expression of MMP9 related
to cadmium was found to be a 44 to 48% increase after exposure to 50 µM of cadmium [89] while the average amount of cadmium inhaled in a full 20-cigarette pack is between 24.65 and 87.36 µM [91]. One possible explanation for the variance in whole-smoke study results is differing levels of nicotine and cadmium present in the whole smoke used in each study. While the overall consensus appears to be that cigarette smoke exposure elevates MMP9 levels, there is enough conflicting evidence that does not allow a definite conclusion.

**Oxidative Stress**

Oxidative stress is a key component of rotator cuff injury as it affects the release of damage associated molecular patterns (DAMPs), particularly HMGB1. This results in RAGE activation and downstream inflammation. Oxidative stress specifically refers to ROS and is a particularly common effect of cigarette smoking. For example, while nicotine in cigarettes is primarily known for its addictive properties, it also increases ROS production leading to cellular senescence and apoptosis [44,84,92]. Tar, the particulate matter of smoke, is hypothesized to contain ROS that has already been synthesized [81,93], which would suggest that ROS produced as the result of other ingredients such as nicotine is secondary to tar ROS. ROS have also been demonstrated to generate in response to cigarette smoke exposure [94], indicating a third way by which smokers may experience increased ROS levels.

ROS is not limited to firsthand smoke. Secondhand smoke also contains ROS, though in decreased amounts. Light cigarettes also contain decreased amounts of ROS as compared to regular cigarettes; firsthand smoke has been reported to contain 120-150 nmol of ROS in regular cigarettes and 90-110 nmol of ROS in light cigarettes, while secondhand smoke was reported to contain 60-90 nmol of ROS in regular cigarettes and 30-70 nmol of ROS in light cigarettes [95]. Since nearly twice the amount of already-synthesized ROS is present in firsthand smoke as
opposed to secondhand smoke, it would follow that particles necessary to induce further ROS production, such as nicotine, would also be elevated in firsthand smoke as compared to secondhand smoke. This effect has been seen in rats; rat models have shown that nicotine-dependent ROS production increases with increase in dose [44], though whether this generalizes to humans is not yet known.

**High Mobility Group Box Protein 1 (HMGB1)**

High Mobility Group Box Protein 1, or HMGB1, has both intracellular and extracellular effects. Intracellularly, it is involved in processes such as transcription, autophagy, and cell development. When released from dying cells, however, it takes on the role of damage-associated molecular pattern, DAMP [59]. It is as a DAMP that HMGB1 is damaging. This extracellular release of HMGB1 is a demonstrated effect of oxidative stress as well as a direct effect of cigarette smoke exposure [96–98]. In mice, exposure to cigarette smoke amounts equivalent to one pack per day for three days resulted in HMGB1 translocation from the nucleus to the cytoplasmic space in lung epithelial cells. This also resulted in inflammatory JNK and p38 pathway activation [96].

The effect of cigarette smoke on translocating HMGB1 as a DAMP has also been demonstrated in skin tissue in a rat model as well as in skeletal muscle [97,99], indicating the potential for further generalization of these findings. HMGB1 elevation even occurs with secondhand cigarette smoke exposure [97], demonstrating its sensitivity to the smoke. In a rat model, secondhand smoke exposure increased HMGB1 and decreased collagen such that skin aged prematurely [99]. Interestingly, collagen is a major factor in tendon restoration; the role of HMGB1 in rotator cuff tendon collagen phenotype warrants further investigation.
Figure 5: Impact of tobacco on tendon healing. Tobacco usage impacts an injured tendon by elevating already increased MMP2, MMP9, oxidative stress, and DAMPs. This impaired healing process leads to a poor healing response.

**Advanced Glycation End products (AGEs)**

AGEs exacerbate inflammatory pathways, primarily through ROS, RAGE, and dysfunctional collagen crosslinking [10,57,60,61]. Tobacco smoke contains reactive glycation products that operate much as glucose does in terms of forming AGEs; these reactive glycation products react with proteins to form tobacco-derived AGEs [20]. That some forms of AGEs are tobacco-derived indicates a base-level increase in tobacco users’ AGE accumulation. This further suggests exacerbation of AGE-mediated inflammatory processes. Tobacco-derived AGEs have been specifically demonstrated to accumulate on the lens of the eye and on low density lipoproteins that form the vascular wall structure [68]. These AGEs have been implicated in atherosclerosis, coronary artery disease, and peripheral vascular disease [100], which indicates
that tobacco-derived AGEs do play a critical role in disease processes. Some studies suggest that this type of AGEs are culpable in enduring tissue injury even in former smokers [101]. Tobacco-derived AGE accumulation indicates that rotator cuff tendons of smokers are subjected to increased inflammation both due to the direct effects of tobacco and due to the increased levels of AGEs (see Figure 6), thus leading to further impaired healing outcomes in injured tendon.

**Figure 6: AGEs and tobacco in healing tendon.** An injured tendon experiences increased oxidative stress, MMP2, and MMP9 due to tobacco’s direct effects. Tobacco-derived AGE accumulation further exacerbates oxidative stress, RAGE activation, and defective collagen crosslinking. This impaired healing process leads to a very poor healing response.

Current literature does not directly connect tobacco usage to nonspecific collagen crosslinking though it has connected smoke to RAGE expression and oxidative stress, the other two primary effects of AGEs in the tendon healing process. RAGE expression is increased simply following cigarette smoke exposure [102,103]. Tobacco smoke particulates specifically
activate RAGE, though the exact molecules are yet unknown [102]. As such, it is possible that tar may include inflammatory RAGE ligands such as HMGB1.

**Outstanding Questions**

While a connection between T2DM and impaired RCI healing response and a connection between tobacco usage and impaired RCI healing response are clinically well-established, the cellular and molecular mechanism by which these occurrences are linked is yet unknown. Much is understood about the connection between AGEs and specific inflammatory pathways in the tendon healing process, direct connections between AGEs and tendons have yet to be elucidated. AGEs may form the missing link to explain this impaired healing among both T2DM and smoking populations.

Additionally, the primary event in the molecular healing cascade is a decreased Collagen 1 to Collagen 3 ratio. This ratio mediates the rest of the healing pathway. Thus far, literature has considered the impact of AGEs on processes further downstream in the pathway. Whether AGEs affect the Collagen 1 to Collagen 3 ratio, and if so then by what mechanism, could be the missing link in the understanding of the impaired healing outcomes in patients with higher AGE accumulation.
Hypothesis and Aims

Central hypothesis

Increased levels of AGEs positively correlate with MMP2 and MMP9 expression and with decreased Collagen 1:3 ratio in rotator cuff tendon.

Specific Aims

1. Determine the relative expression of AGEs, RAGE, MMP2, MMP9, Collagen 1, Collagen 3, lysine hydrolase (LH), proline hydroxylase (PH), and AMPK in tissue samples from RCI repairs of patients with T2DM and a history of smoking.

2. Determine the relative expression of AGEs, RAGE, MMP2, MMP9, HMGB1, IL-1B, Collagen 1, Collagen 3, Pro-Collagen 1, Pro-Collagen 3, LH, and PH in swine and human tenocytes treated with cigarette smoke condensate.

3. Inhibit AGEs in vitro using pyridoxamine to determine the effect on RAGE, MMP2, MMP9, HMGB1, IL-1B, LH, PH, and Collagen 1 to Collagen 3 ratio by measuring these factors before and after AGE inhibition.
Materials and Methods

Tissue Collection and Processing

Tissue from twenty-seven patients with RCI were collected for the study in an anonymous manner. The only information about the patients that was provided was: smoker or non-smoker, healthy or history of T2DM, presence or absence of glenohumeral arthritis, and the surgery to repair tendon. Nine of the subjects had a history of smoking, five patients had T2DM, and one patient had both T2DM and a history of smoking. The remaining twelve patients had RCI but neither T2DM nor a history of smoking. Each patient was undergoing shoulder surgery and biceps tendon or rotator cuff tendon was harvested. The tendon tissue samples from the patients were labeled as PT3 to PT119. The patients were classified into four groups. Group 1 included 12 patients (PT3, PT5, PT8, PT9, PT10, PT15, PT16, PT17, PT18, PT25, PT30, PT33) with rotator cuff tear (superior labral anterior to posterior; SLAP) without a history of smoking or T2DM. Group 2 included 9 patients (PT4, PT6, PT12, PT13, PT21, PT31, PT101, PT112, PT114) with SLAP and with a history of smoking. Group 3 included 5 patients (PT115, PT116, PT117, PT118, PT119) with SLAP and T2DM. Group 4 included 1 patient (PT113) who had SLAP and both T2DM and a history of smoking.

Rotator cuff tears were intraoperatively confirmed during routine shoulder arthroscopy. Subpectoral biceps tenodeses were performed in all patients for clinically symptomatic bicipital symptoms. The intra-articular portion of the biceps was resected and obtained for tissue processing. Specimens were collected in the UW (University of Wisconsin) solution for temporary storage and transportation, and the proximal portions were taken for analysis.
**Histology**

Tissue was fixed in 10% formalin at room temperature for 24 hours. Following, tissue was embedded in paraffin wax and sectioned into 5 µm thick sections using microtome (Leica, Germany) onto microscopic slides for analysis. Sections were deparaffinized with xylene and dehydrated with ethanol concentrations decreasing from 100% to 70%. These sections were then used for Hematoxylin and Eosin (H&E) and Trichrome staining.

For H&E stains, deparaffinized sections were placed in Harris Hematoxylin Alum (Sigma-Aldrich) for 2 minutes then rinsed in distilled water to remove remnants of the hematoxylin. Sections were then differentiated in Bluing Solution (ThermoScientific) for 10 seconds and again rinsed in distilled water. Sections were rinsed briefly in 90% ethanol before being counterstained in Eosin Y solution (Sigma-Aldrich) for 2 minutes then rehydrated in 90% ethanol. Sections were dipped in two changes of 100% ethanol then into a 1:1 xylene and ethanol solution before mounting.

For trichrome stains, deparaffinized sections were rinsed in distilled water then submerged in Bouin’s fluid (LabChem) for 1 hour. Sections were then rinsed in distilled water before being placed in Weigert’s Fe Hematoxylin stain for 10 minutes. Sections were rinsed in distilled water again then placed in Biebrich scarlet-acid fuchsin solution for 5 minutes. Sections were rinsed in distilled water an additional time then placed in Phosphomolybdic Acid–Phosphotungstic Acid for 5 minutes and Analin blue for 5 minutes. Following, sections were placed in 1% acetic acid solution for one minute then rehydrated in two changes of 100% ethanol for one minute each. Sections were placed in a 1:1 xylene and ethanol solution for one minute, xylene for one minute, and then mounted.
Stained sections were mounted using a xylene-based mounting media (CytoSeal, ThermoScientific) and imaged using an inverted microscope with attached CCD camera (Olympus BX51; Olympus America, Center Valley, PA) in brightfield mode.

**Immunofluorescence**

Protein expression in the tendon tissue sections was analyzed by immunofluorescence (IF) staining. Sectioned slides were deparaffinized following a dehydrating and rehydrating procedure, submerging the slides in xylene followed by 100%, 90%, 80%, and 70% ethanol dilutions. Sections then underwent antigen retrieval by heating at 95°C for 20 minutes in HIER buffer (Heat Induced Epitope Retrieval) (TA-135-HBM). Following, sections were incubated in blocking solution (0.25% Triton X-100 and 5% horse serum [Vector Laboratories]) in PBS [Phosphate Buffered Saline]) for two hours at room temperature. Primary antibodies against COL1, COL3, MMP2, MMP9, LH, PH, AGEs/AGE-BSA-protein, AMPK, RAGE, and Scleraxis were applied in a 1:300 dilution. Corresponding fluorochrome-conjugated secondary antibodies were used to bind the primary antibodies in a 1:300 dilution. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (H-1200). A negative control slide was maintained in the same way with secondary antibodies alone to distinguish background intensity and fix microscope exposure time. Slides were imaged using a fluorescent microscope (Olympus BX51; Olympus America, Center Valley, PA). Fluorescence intensity was quantified using ImageJ software and cross-referenced with nucleus count to express reported results as mean fluorescence intensity (MFI).
Cell Culture

Skin in the shoulder region of Yucatan microswine (Sinclair Research) (female, 6-8 months old) was cleaned twice with iodine solution and with 70% isopropanol. With a sterile scalpel blade (#20), an incision was made at the shoulder and infraspinatus tendon tissue was harvested. The harvested tissue was washed in serum free DMEM (Dulbecco’s Modified Eagle Medium) (D-6429, Sigma Aldrich) containing 3% antibiotics (Anti-Anti, Gibco). Tissue was then minced and digested with collagenase-1 for two hours at 37° C with intermittent shaking. The resultant mixture was then centrifuged for five minutes at 200 G to settle the undigested tissue. The tendon tissue was then placed in a culture flask for explant culture. Tendon cells migrate and began to colonize five to six days after seeding and became confluent after two weeks. Isolation and maintenance of the cells was accomplished using DMEM containing 20% FBS (Fetal Bovine Serum, ThermoFisher) and 1% antibiotics (Anti-Anti, Gibco). The culture was maintained at 37° C and 5% CO₂ in a humidified incubator for six days. Unattached cells were then removed, and media was replaced with fresh DMEM. Cells were isolated from four microswine, pooled (passage 2-4), and stored under liquid nitrogen. Cells were revived for each experiment. Revived cells were cultured using collagen-1-coated (Zen-Bio) culture flasks.

Cigarette Smoke Condensate and Pyridoxamine Dihydrochloride

Cigarette smoke condensate (CSC) was obtained from the University of Kentucky. TPM (total particulate matter) was collected from a 3R4F reference cigarette on a Cambridge filter pad. The reference cigarette was smoked on an ISO smoke regime of a 35mL puff volume, 60 second puff frequency, and 2 second puff duration. TPM collected was 211.90 mg. CSC extract
was prepared in DMSO and stock solutions were prepared in serum free media with 1% antibiotic.

Pyridoxamine dihydrochloride (PM) was obtained from Fischer Scientific, Alfa Aesar. PM was prepared in DMSO and stock solution was prepared in serum free media with 1% antibiotic.

**MTT Assay**

A yellow tetrazolium MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used in the MTT assay to determine effective concentrations for both cigarette smoke condensate and pyridoxamine dihydrochloride. In this reaction, the yellow tetrazolium MTT is reduced by metabolically active cells to generate NADH and NADPH due to the action of dehydrogenase enzymes. The resulting quantification of opacity is assessed for cell viability.

In previous studies, cigarette smoke condensate has been utilized in cell culture in concentrations of 10mg TPM/mL (total particulate matter per mL) [104]. Pyridoxamine dihydrochloride has been utilized in cell culture in concentrations ranging from 10 to 100 µM [105,106]. Cells were seeded into 48-well plates and the MTT assay was conducted two times. Stock solutions of cigarette smoke condensate (Reference Cigarette 3R4F, University of Kentucky) and pyridoxamine dihydrochloride (Alfa Aesar) were prepared in serum free media with 1% antibiotic. Concentrations of 6.25, 12.5, 25, 50, 100, and 200 µM of PM and mg TPM/mL of CSC were added to each well and the cells incubated at 37° C and 5% CO₂ overnight.
All media was then removed from wells. 50μL of MTT (5mg/mL in PBS) was added to each well and cells incubated for 3 hours at 37° C and 5% CO₂. Following, 100μL of DMSO (dimethyl sulfoxide, ThermoScientific) were added to each well and the plate sat for 10 minutes at room temperature. Contents of each well were briefly washed inside a pipette then transferred to a 1mL centrifuge tube. Tubes were centrifuged for 2 minutes at 11 rpm. 75μL of each tube was then added to each well of a 96-well plate, allowing for each original well to be analyzed in triplicate. The plate was then loaded into a plate reader (PerkinElmer, EnSpire 2300 Multilabel Reader) and analyzed using EnSpire software.

**Cytoimmunofluorescence**

Stock solutions of cigarette smoke condensate (Reference Cigarette 3R4F, University of Kentucky) and pyridoxamine dihydrochloride (Alfa Aesar) were prepared in serum free media with 1% antibiotic.

Cultured tenocytes were split into four chamber slides (Lab-Tek, Rochester, NY). One slide was held as control in high glucose Dulbecco’s Modified Eagle Media (3.7g/L), 20% fetal bovine serum. In one slide, media was replaced with serum free media with 1% antibiotic and each well was treated with 1μM solution of cigarette smoke condensate (University of Kentucky). In another slide, media was replaced with serum free media with 1% antibiotic and each well was treated with 1mg/mL solution of pyridoxamine dihydrochloride. In the final slide, media was replaced with serum free media with 1% antibiotic and each well was treated with both 1μM solution of cigarette smoke condensate and 1mg/mL solution of pyridoxamine dihydrochloride. Slides were stored at 37° C and 5% CO₂ in a humidified incubator for 16 hours.
Protein expression in the cultured tenocytes was analyzed by immunofluorescent staining. Media was removed from each well then each well was washed with filtered PBS. Following, cells were fixed with formalin; formalin was added to each well and slides were incubated at room temperature for 30 minutes. Each well was then washed with filtered PBST (1 liter of PBS with 1 drop of Triton X) and kept at room temperature for 3 minutes. Following, HBS for cytoimmunofluorescence (PBST with 1% horse serum) for 30 minutes. Primary antibodies against AGE, PH, LH, HMGB1, ProCol1, ProCol3, IL-1B, RAGE, COL1, COL3, MMP2, and MMP9 were applied in a 1:400 dilution, 100µL per well, and slides incubated in the dark for 1 hour. Following, slides were washed twice with filtered PBST and corresponding secondary antibodies were applied in a 1:400 dilution, 100µL per well. Slides were again incubated in the dark for 1 hour. Subsequently, slides were washed twice with filtered PBST and the chambers were removed. Slides were allowed to sufficiently dry before nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (H-1200). Negative control wells were maintained in the same way with secondary antibodies alone to distinguish background intensity and fix microscope exposure time. Slides were imaged using a fluorescent microscope (Olympus BX51; Olympus America, Center Valley, PA). Fluorescence intensity was quantified using ImageJ software and cross-referenced with nucleus count to express reported results as mean fluorescence intensity (MFI).

Polymerase Chain Reaction (PCR)

First, RNA was isolated from swine tenocytes. All media was removed from the cell culture flasks, followed by the addition of 1mL tri reagent (Trizol, Ambion Life Technologies). The resultant solution was transferred to a small centrifuge tube. After sitting for 5 minutes, 200 µL of chloroform (Sigma-Aldrich, St. Louis, MO) was added and the
sample was vortexed. Following, the sample was centrifuged at 12000 RCF for 15 minutes. The aqueous phase was then transferred to a new small centrifuge tube and 500µL of 2-propanol (Sigma-Aldrich, St. Louis, MO) was added to each tube. Samples were vortexed and then centrifuged at 12000 RCF for 15 minutes. 2-propanol was removed and 1mL of 70% ethanol was added to each tube followed by an additional centrifuge at 12000 RCF for 10 minutes. Ethanol was then removed, and resultant pellets were allowed to air dry before adding 200µL of RNAse-free water and mixing with the pellet. RNA was then stored at -80°.

Secondly, complementary DNA (cDNA) was made from RNA. The spectrophotometer (NanoDrop 2000c, ThermoScientific) was cleaned using 1µL RNAse free water. Following, each sample was centrifuged and 1µL of sample was added to spectrophotometer to measure yield. Based on the RNA concentration, sufficient amount of additional RNA, RNAse free water, and oligo DT were added for a mix with total volume of 10µL. Each mix was centrifuged briefly then loaded into the thermal cycler (T100, Bio-Rad) for pre-reverse transcriptase.

Thirdly, primers were diluted at a ratio of 10 parts primer to 90 parts RNAse free water. Then a mix was made for genes HMGB1, COL1A1, COL3A1, MMP2, RAGE, and housekeeping gene GAPDH. Each was prepared with a ratio of 2µL FW, 2µL RW, and 10µL supermix (SYBRgreen, Bio-Rad), and 14µL of the appropriate mix was added to the wells of a PCR plate along with 8µL of cDNA. Once the plate was loaded, it was sealed, shaken briefly, and run in an additional thermal cycler (C100 thermal cycler, Bio-Rad). Experiments were run in quadruplicate, such that each gene was assessed in a control, CSC, PM, and CSC+PM condition four times.
Statistical Analyses

Statistical analyses were conducted using GraphPad Prism software (GraphPad, San Diego, CA). In both tissue IF and cell IF, analysis of variance (ANOVA) and Kruskal-Wallis tests were run for each gene marker. If results were determined to be statistically significant, Dunn’s multiple comparisons tests were conducted to determine between which groups there was a statistically significant change. Tissue samples included several patients with severe glenohumeral arthritis (GA). As such, analyses were run with and without these patients to address the potential confound of an additional severe inflammatory condition.
Results

Tissue

One-way analyses of variance (ANOVAs) were conducted for each gene marker in human tissue samples, both including and excluding patients with severe glenohumeral arthritis. In both sets of analysis, LH was not significant ($p > 0.05$) (see Figure 7, Figure 8), PH was not significant ($p > 0.05$) (see Figure 9, Figure 10), MMP2 was not significant ($p > 0.05$) (see Figure 11, Figure 12), MMP9 was not significant ($p > 0.05$) (see Figure 13, Figure 14), AGE was not significant ($p > 0.05$) (see Figure 15, Figure 16), Collagen 1 to Collagen 3 ratio change was not significant ($p > 0.05$) (see Figure 17, Figure 18), and RAGE expression was not significant ($p > 0.05$) (see Figure 19, Figure 20).

When excluding patients with GA, AMPK, the measure utilized for glucose metabolism, was significantly decreased in the T2DM group as compared to the smoking group ($p < 0.05$) (see Figure 21, Figure 22).
Figure 7: LH Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 8: Immunofluorescence for LH. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 9: PH Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 10: Immunofluorescence for PH. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 11: MMP2 Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 12: Immunofluorescence for MMP2. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 13: MMP9 Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 14: Immunofluorescence for MMP9. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 15: AGE Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 16: Immunofluorescence for AGE. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 17: Collagen 1:3 ratio in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 18: Immunofluorescence for Collagen 1:3 Ratio. The varying number of nuclei accounts for comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 19: RAGE Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. There was no significant difference across groups, regardless of the inclusion or exclusion of patients with comorbid GA.

Figure 20: Immunofluorescence for RAGE. The varying number of nuclei accounts for the comparable MFI per nucleus despite the qualitatively higher expression in some conditions.
Figure 21: AMPK Expression in Patient Tissue. Group 1 represents patients with RCI alone. Group 2 represents patients with a history of smoking. Group 3 represents patients with T2DM. Group 4 represents two samples from two separate tendons from one patient with both T2DM and a history of smoking. Excluding patients known to have comorbid GA showed significance between the diabetic group and the smoking group.
Figure 2: Immunofluorescence for AMPK. There was no significant change across groups unless GA was excluded from analysis.

Swine Tenocytes: MTT

The MTT assay revealed an EC\textsubscript{77} (effective concentration with 77\% viability) of 100mg TPM/mL of CSC (see Figure 23) and an EC\textsubscript{75} of 100\mu M PM (see Figure 24). Typical use is EC\textsubscript{50}, though due to the clustering of MTT results and the ability to see a greater effect with greater survival of cells, the EC\textsubscript{75-77} was utilized in experiments.
Figure 23: MTT Results for CSC. Group 1 represents a concentration of 12.5 mg TPM/mL, Group 2 represents a concentration of 25 mg TPM/mL, Group 3 represents a concentration of 50 mg TPM/mL, Group 4 represents a concentration of 100 mg TPM/mL, and Group 5 represents a concentration of 200 mg TPM/mL.

Figure 24: MTT Results for PM. Group 1 represents a concentration of 6.25 µM, Group 2 represents a concentration of 12.5 µM, Group 3 represents a concentration of 25 µM, Group 4 represents a concentration of 50 µM, Group 5 represents a concentration of 100 µM, and Group 6 represents a concentration of 200 µM.
Cytoimmunofluorescence

Three rounds of cytoimmunofluorescence were conducted. Due to the variation in each experimental repeat (see Table 1, Figures 25-33), analysis was conducted on mean percent change across groups. MMP2 expression was statistically significant ($p = 0.0013$), with MMP2 expression significantly increased in the CSC condition as compared to the control (M = 815.38%) (see Figure 34, Figure 35). MMP9 was not significant ($p > 0.05$) (see Figure 36, Figure 37). Pro-Collagen 1 to Pro-Collagen 3 ratio was statistically significant ($p = 0.0447$) with significant decreases in the CSC condition as compared to the control condition (M = -29.8%) (see Figure 38, Figure 39).

RAGE expression was statistically significant ($p = 0.002$), with the CSC+PM group having significantly decreased RAGE activation as compared to the control group (M = 57.74%) (see Figure 40, Figure 41). HMGB1, IL-1B, LH, and PH were not statistically significant ($p > 0.05$) (see Figures 43-51). In one experimental repeat, MFI of AGE per nucleus was significantly decreased in the PM group as compared to the CSC group ($p = 0.0036$) (see Table 1).

<table>
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<th>Repeat</th>
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<th>RAGE</th>
<th>LH</th>
<th>PH</th>
<th>AGE</th>
<th>PROCOL</th>
<th>COL</th>
<th>HMGB1</th>
<th>MMP9</th>
<th>MMP2</th>
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Table 1: Cytoimmunofluorescence Significance by Repeat. Statistical significance between groups varied by experimental repeat. Consistent findings are noted in red.
Figure 25: Average MMP2 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 26: Average MMP9 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 27: Average HMGB1 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 28: Average PH Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 29: Average Pro-Collagen 1:3 Ratio in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 30: Average LH Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 31: Average Collagen 1:3 Ratio in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 32: Average RAGE Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 3.3: Average IL-1B Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes with no treatment. Group 2 represents tenocytes treated with CSC. Group 3 represents tenocytes treated with PM. Group 4 represents tenocytes treated with CSC and PM. There was no significant change across groups.
Figure 34: Average Percent Change in MMP2 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. MMP2 expression was significantly increased in the CSC group.

Figure 35: Comparison of MMP2 Cytoimmunofluorescent Expression Across Groups. Average expression was highest in the CSC group.
Figure 36: Average Percent Change in MMP9 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 37: Comparison of MMP9 Cytoimmunofluorescent Expression Across Groups. Average expression was consistent across groups.
Figure 38: Average Percent Change in Pro-Collagen 1:3 Ratio in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. Pro-Collagen 1:3 ratio was significantly decreased in the CSC group.

Figure 39: Comparison of Pro-Collagen 1:3 Cytoimmunofluorescent Expression Across Groups. Pro-Collagen 1:3 ratio was significantly decreased in the CSC group.
Figure 40: Average Percent Change in RAGE Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. RAGE was significantly decreased in the CSC+PM group.

Figure 41: Comparison of RAGE Cytoimmunofluorescent Expression Across Groups. RAGE expression was significantly decreased in the CSC+PM group.
Figure 42: Average Percent Change in HMGB1 Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 43: Comparison of HMGB1 Cytoimmunofluorescent Expression Across Groups. Average expression was not significantly changed across groups.
Figure 44: Average Percent Change in IL-1B Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 45: Comparison of IL-1B Cytoimmunofluorescent Expression Across Groups. Average expression was not significantly changed across groups.
Figure 46: Average Percent Change in LH Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 47: Comparison of LH Cytoimmunofluorescent Expression Across Groups. Average expression was not significantly changed across groups.
Figure 48: Average Percent Change in PH Expression in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 49: Comparison of PH Cytoimmunofluorescent Expression Across Groups. Average expression was not significantly changed across groups.
Figure 50: Average Percent Change in Collagen 1:3 Ratio in Swine Tenocytes Across Three Experimental Repeats of Cytoimmunofluorescence. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change across groups.

Figure 51: Comparison of Collagen 1:3 Cytoimmunofluorescent Expression Across Groups. Average Collagen 1:3 ratio was not significantly changed across groups.
PCR

ANOVA were also conducted on fold-change in PCR. HMGB1 fold change was significant ($p = 0.0006$), seen to be increased in the CSC + PM group as compared to the control (see Figure 52). COL1A1 fold change was also significantly increased in CSC + PM as compared to the control ($p = 0.0042$) (see Figure 53). COL3A1 fold change was not significant across groups ($p > 0.05$) (see Figure 54). RAGE fold-change was significantly increased in CSC+PM as compared to the control group ($p = 0.0016$) (see Figure 55). MMP2 fold-change was significantly increased ($p = 0.0003$), with significant increases seen in the CSC group and in the CSC+PM group (see Figure 56).

![Figure 52: Average HMGB1 Fold Change Across Three Experimental Repeats of PCR. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. HMGB1 was significantly elevated in the CSC+PM group.](image-url)
Figure 53: Average COL1A1 Fold Change Across Three Experimental Repeats of PCR. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. Collagen 1 was significantly increased in the CSC+PM group.

Figure 54: Average COL3A1 Fold Change Across Three Experimental Repeats of PCR. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. There was no significant change in Collagen 3 expression across groups.
Figure 55: Average RAGE Fold Change Across Three Experimental Repeats of PCR. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. RAGE was significantly elevated in the CSC+PM group.

Figure 56: Average MMP2 Fold Change Across Three Experimental Repeats of PCR. Group 1 represents tenocytes treated with CSC. Group 2 represents tenocytes treated with PM. Group 3 represents tenocytes treated with CSC and PM. MMP2 was significantly elevated in the CSC and CSC+PM groups.
Discussion

Tissue

That AMPK was decreased in T2DM as compared to the smoking group is surprising; as a regulator of glucose metabolism, AMPK is roughly used as a marker for hyperglycemia, present in T2DM. Measuring for whole AMPK as opposed to phosphorylated AMPK is the most likely explanation for this result.

No other gene was significantly changed across groups. The nature of the samples may explain this consistency. Individual differences complicate any human study. Further, history of these patients is not known. In patients with a history of smoking, how many cigarettes smoked per day and for how long is not known. In patients with T2DM, severity of the disease and whether the disease is controlled are also unknown. Additionally, general history such as AGE- or inflammation-related comorbidities, age, and lifestyle are not known.

Cytoimmunofluorescence

Due to the variation in gene expression by experimental repeat (see Table 1), analyses were conducted on percent change. That MMP2 expression was significantly changed from control in the CSC group indicates that CSC, in the form of total particulate matter, has a positive effect on MMP2 expression. Since neither the PM nor CSC+PM group was significantly changed from the control group, PM must mitigate the effects of CSC on MMP2.

Pro-Collagen 1 to Pro-Collagen 3 ratio was significantly decreased in the CSC group as compared to the control group. This whole collagen present in the cell is a more appropriate measure of collagen ratio in tenocytes than collagen itself, as the tissue would need to be present for an accurate collagen measure. This decreased ratio of Pro-Collagen 1 to
Pro-Collagen 3 indicates that CSC is then responsible for the changes in collagen phenotype in tendon.

RAGE expression, interestingly, was shown to be significantly changed from control in only the CSC+ PM group. One possible explanation is that RAGE, as a multi-ligand receptor, is to some extent activated by PM. If this were so, then PM may serve as a RAGE ligand more quickly or efficiently than it is able to block AGE. In that case, PM may not be the most effective AGE inhibitor in terms of blocking the effects of AGE in addition to the formation of AGEs themselves. Alternatively, that the addition of PM to CSC resulted in a significant decrease in RAGE expression indicates that an interaction occurred between PM and CSC. Knowing that AGEs are directly derived from tobacco, PM seems to inhibit the derivation of these AGEs. However, this does not explain the overall decrease; PM must then have interacted in some way with CSC to decrease the already-synthesized AGEs as well. Further, that RAGE was not significantly overexpressed in the CSC group is surprising considering the existing literature on the subject. Further expression may have been found with an increased incubation, or CSC may have small effects on RAGE that in greater concentration may prove to be significant.

MMP9, on the other hand, was not significantly altered in any group. This may suggest that the impact of CSC on MMP9 expression is delayed, perhaps due to being secondary to another molecule such as MMP2. Similarly, HMGB1 expression was not significantly altered in any group. The process for HMGB1 to be released as a DAMP may also be delayed event. Alternatively, these findings are to some extent consistent with current literature. The effect of whole tobacco smoke on MMP9 in inconclusive, as MMP9 expression has been demonstrated to be both increased and decreased in the presence of whole tobacco smoke.
HMGB1, on the other hand, is postulated to be increased in rotator cuff tendon injury due to its demonstrated tobacco-dependent increase in skin collagen levels [99] but has not been directly studied in this context. Qualitatively, cytoplasmic expression of HMGB1 is increased in each condition as compared to the control condition (see Figure 43). An alternative explanation for the consistency of MMP9 expression across groups is that while MMP2 is involved in collagen degradation and remodeling, MMP9 is involved solely in collagen degradation [9,33]. Collagen remodeling, then, may be the activity specifically impacted by CSC.

The Collagen 1 to Collagen 3 ratio was also not significantly altered across groups. As discussed previously, tissue needs to be present in order to obtain an accurate measure of collagen expression; the expression of collagen in tenocytes alone may not be accurate or representative. Additionally, it is possible that the pro-collagen had simply not yet been released from the cells at the time that analysis was conducted.

Neither LH nor PH saw a significant percent change from control in any group. As these residues are markers of changes in collagen crosslinks, this may be explained by the lack of collagen itself present in cell culture, as opposed to tissue studies. Alternatively, CSC may not have a direct effect on collagen crosslinks. Literature has shown that AGEs, including those that are tobacco-derived, do have a direct effect, but these findings may indicate that there is a mediator between AGE and LH/PH.

IL-1B was also not significantly changed across groups as compared to the control condition. While IL-1B expression is expected to increase as part of the IL-1 family in response to RAGE activation, it is entirely possible that there was insufficient time in incubation for RAGE to activate IL-1B before immunofluorescence staining was conducted.
Prior to analyzing average percent change, analyses were conducted on each cytoimmunofluorescence repeat in terms of MFI (mean fluorescent intensity) per nucleus. Some rounds had markedly higher or lower expression across the board, indicating a difference in experiment conduction. This led to the percent-change analyses. While there was indeed variation in the MFI per nucleus results, some findings were more consistent.

In two of three repeats, for instance, RAGE was seen to be significantly increased in the PM condition as compared to the control condition. This finding is similar to the findings of percent change. The same explanations then apply: PM, while inhibiting AGE expression, may function as a RAGE ligand, and different findings may have been observed with increased incubation time.

In two of three repeats, LH was found to be significantly varied in the ANOVA, though multiple comparisons post-tests yielded no significant changes between groups. Similarly, in two of three repeats neither AGE, Pro-Collagen 1 to Pro-Collagen 3 ratio, or MMP2 was significantly changed. This is likely due to the overall changes in expression seen across repeats.

Not statistically significant in either MFI per nucleus or percent change were Collagen 1 to Collagen 3, MMP9, HMGB1, IL-1B, PH, and, most surprisingly, AGE. The lack of change in Collagen 1 to Collagen 3 ratio is to some extent expected in tenocytes and is understandable considering the significantly altered ratio of Pro-Collagen 1 to Pro-Collagen 3.

Since tobacco smoke is known to contain synthesized tobacco-derived AGEs, AGE expression was expected to be heavily increased in tenocytes treated with CSC. In only one experimental repeat, however, was any statistically significant change seen; AGE was
inhibited in the PM group as compared to the CSC group. This single finding establishes PM as a successful AGE inhibitor and indicates that AGEs were inhibited from a higher level of expression. While AGEs were not significantly increased in the CSC group as compared to the control group, that they were significantly decreased between CSC and PM suggests that AGEs were elevated in CSC. However, this finding only appeared one time out of three. As AGE is present in CSC and therefore would be expected to be present in CSC-treated cells, one explanation is that the concentration of CSC applied to these tenocytes was insufficient to produce the results seen in previous literature.

**PCR**

That HMGB1 was increased only in the PM group suggests that HMGB1 is a sensitive molecule; as it exists intracellularly in a normal environment, changes to this environment may cause HMGB1 to be released extracellularly. An alternative explanation is that PM requires alterations before clinical use as an AGE inhibitor.

While Collagen 3 fold-change was not statistically significant across groups, Collagen 1 increased significantly in the CSC + PM group. This indicates that the ratio of Collagen 1 to Collagen 3 was elevated in the CSC + PM group. Since the ratio was unchanged in the CSC group, exposure to cigarette smoke may not have a direct impact on collagen phenotype. Similarly, since the ratio did not change in the group treated with PM alone, PM may not alter collagen phenotype without the presence of AGE. Since this change occurred with PM and CSC combined, it is likely that PM was able to act of the AGEs induced by CSC and produce the collagen phenotype differences.
That RAGE was not elevated in CSC comes as a surprise, as RAGE was here used as a measure of AGE activation. Since this change was seen in the CSC+PM group indicates that one of these had a direct effect on RAGE activation. This effect was also demonstrated in immunofluorescent staining. Further, RAGE was increased in the CSC group though not statistically significantly; since PM alone acted on RAGE insignificantly as well, the combined impact of CSC and PM on RAGE may have been enough to increase RAGE expression.

MMP2 was significantly increased in both the CSC and CSC+PM groups, indicating an effect of CSC and no effect of PM. CSC then is demonstrated to directly increase MMP2 expression regardless of the presence of AGE-inhibitor. That the presence of PM does not decrease MMP2 expression in CSC-treated tenocytes may indicate that tobacco-derived AGEs are not the cause of the increased MMP2 or alternatively may suggest that PM specifically, as an inhibitor of AGE formation, may not inhibit already-synthesized AGEs sufficiently to decrease MMP2 expression.

While findings varied across methods of experimentation, the most consistent finding was that MMP2 expression is increased in tenocytes in the presence of CSC.
Limitations

Tissue samples collected from surgical waste are difficult to control. Potential confounding variables include other comorbidities, particularly inflammatory conditions. Further, the consenting process for this collection means that limited information is known about each patient. For instance, in smoking patients the amount and duration of smoking status is unknown and in diabetic patients the severity and whether the condition is controlled is unknown. As with all human samples, individual differences also complicate findings. It is always possible that patients respond differently, metabolize certain proteins differently, or have different base levels when entering a study.

AMPK was used as a marker for glucose metabolism, to establish a baseline comparison for hyperglycemia. However, the minimal difference seen across conditions suggests that the use of phosphorylated AMPK rather than whole AMPK may have been a more useful marker. Similarly, MMP2 and MMP9 were measured in terms of expression rather than in activity. As the literature is unclear as to whether MMP9 is itself inhibited or its activity is inhibited in smoking patients, use of zymography to measure the activity of MMP2 and MMP9 would be useful and perhaps more representative of the underlying processes.

In vitro studies with swine tenocytes may not wholly generalize for a few reasons. Firstly, while the swine model is one of the best-established animal models for human conditions [107,108], there are still differences in anatomy that may impact generalization [109]. Secondly, the experiments were conducted on otherwise healthy RCT tenocytes rather than in injured RCT such as in human patients. Thus, these results may be used to assess the role of AGEs on various processes that are known to be involved in the rotator cuff healing process but are inconclusive in terms of their clinical effect of RCI. This study is a pilot study which points towards fertile
areas where further exploration is warranted. Further repeats of experiments are necessary for conclusive results. Insufficient repeats of experiments are likely behind the large variances seen across findings. Finally, more experimental repeats are necessary; many of the results escaped statistical significance due to large variances in results. These would likely decrease with further experimental repeats.

AGE in particular suffered the fewest experimental repeats due to supply or experimental error concluding run experiments. Downstream effects of AGEs were demonstrated to be significantly changed, but AGE must be directly and efficiently studied in future research.

*In vitro* studies were only conducted with a tobacco model, and not with a diabetic model. A diabetic model ought to be considered for use in the same ways described above for future studies. Then, conclusions may be drawn across conditions.
Conclusions and Future Directions

The most consistent finding was that CSC significantly increases MMP2 in tenocytes. Based on the function of MMP2, CSC then disrupts the regular process of collagen and ECM turnover in tendon cells. The central hypothesis was that increased levels of AGEs elevate MMP2 and MMP9 activation, thereby decreasing the Collagen 1:3 ratio and leading to delayed healing of rotator cuff tendon. Since synthesized AGE in tobacco smoke is an a priori finding based on literature and smoke components, it can be presupposed that AGE was indeed carried over to cultured tenocytes treated with CSC. As such, the central hypothesis was partially supported: Increased levels of AGEs elevated MMP2 activation. While not demonstrated in PCR, cytoimmunofluorescence saw a decrease in Pro-Collagen 1 to Pro-Collagen 3 ratio. Thus, AGEs were shown to elevate MMP2 activation and decrease the Collagen 1:3 ratio. Functioning as a positive pilot study, this work provides a springboard for future research.

Future research is necessary to fully explore this hypothesis. Importantly, an in vivo model is necessary to evaluate a) whether this decreased Pro-Collagen 1 to Pro-Collagen 3 ratio carries over to a decreased Collagen 1 to Collagen 3 ratio and b) whether this ratio does result in impaired tendon healing as postulated.

Tissue work would be well-served to utilize blood samples as well, allowing for collection of A1C levels in addition to other blood-bound biomarkers. This may allow for a more solid proof of concept before extrapolating results to risk stratification by comorbidity.

Future in vitro studies should directly treat cells with AGEs to establish better control over AGE levels and assess AGE-dependent responding of associated molecules. In vitro studies should also utilize human tenocytes to generalize across species and to patients in need. Further,
subsequent research ought to conduct many experimental repeats in order to reduce variance in findings. This is true for tissue IF, MTT, cell IF, and PCR.

Further, as this work considers the activities of a healing tendon, future work should consider the activity of tenoblasts rather than fully formed tenocytes to discern the effects on these immature cells. Similarly, stem cells warrant investigation in this process as well as tenomodulin and other matrices. To more directly assess the activity of matrix metalloproteinases 2 and 9, zymography would be a useful tool.

Overall, these findings draw attention to the connections between AGEs, MMP2, Collagen 1:3 ratio, and rotator cuff tendon healing. As a positive pilot study, much work may be directed towards these particular molecules for conclusively elucidating the mechanism by which rotator cuff injury is sustained in diabetic and smoking populations. Clinicians may be well-served to share these associations with patients as a means of reducing smoking. Smoking has been well-demonstrated to have a plethora of negative effects and adding orthopedic effects to the mix may be impactful on patients. Extrapolating the harm of smoking from the direct association with lungs to other body systems such as tendon may spark change in those who are considering smoking or who currently engage in this behavior.
References


