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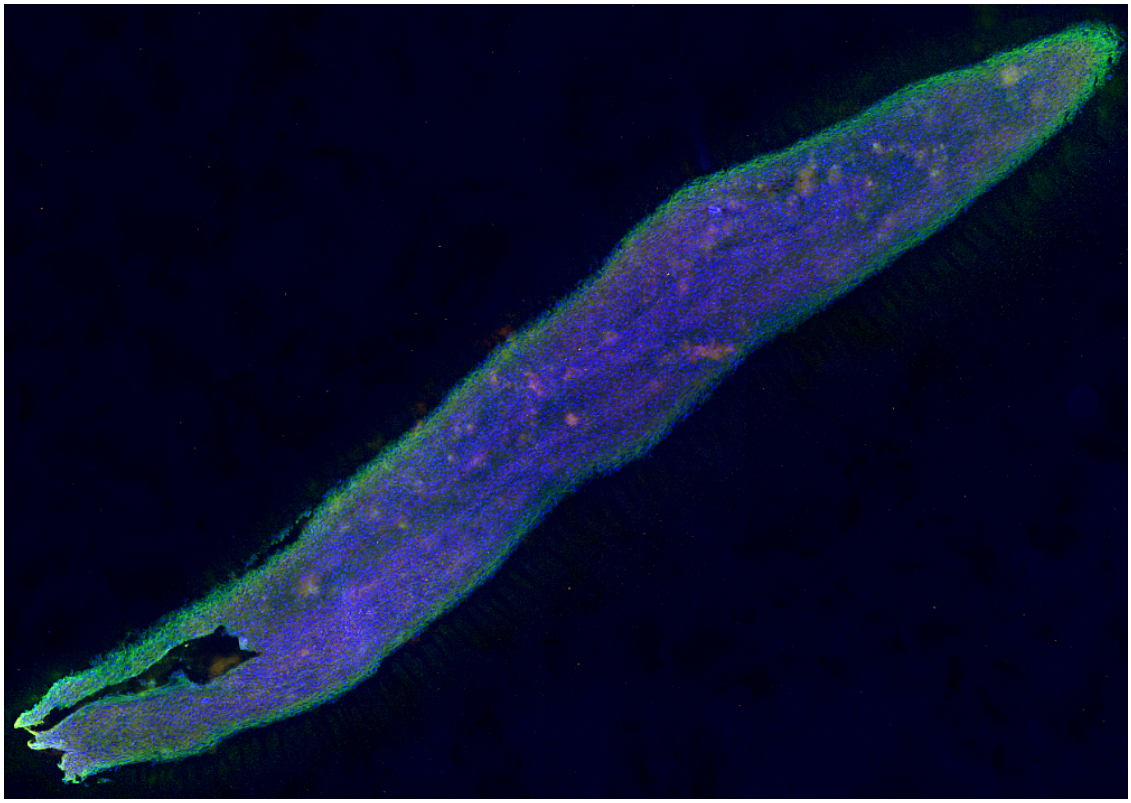

Tendon Cells from Younger and Older Patients Exhibit Similar Mechanical and Biological Properties in a 3D *In Vitro* Model

Master of Science thesis for the Biotechnology MSc degree

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Degree project report 2025

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Cover: Immunofluorescent staining of collagen type I, type III, and DAPI, in one of the tendon constructs formed by patient derived tendon cells in a 3D fibrin hydrogel *in vitro* model.

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Abstract

The Achilles tendon (AT) is the most frequently injured tendon in the human body, with incidence rates on the rise, particularly among older individuals. The healing capacity also decline with age based on functional outcomes post an Achilles tendon rupture. However, the cellular mechanisms behind this remains largely unknown as there is a limited number of studies investigating primary human tendon cells. The aim of this study was therefore to investigate mechanical and biological differences in new tendon tissue formation between young (n=6, mean age=40.5 years, range=21-53 years) and old (n=7, mean age=70, range=57-76 years) patients with chronic Achilles tendon ruptures. To achieve this, primary human cells extracted from tendon tissue were cultured in a 3D fibrin hydrogel model to form tendon constructs. Pull-to-failure tests were performed to determine the mechanical properties, including the maximum force, stress, and stiffness, as well as Young's modulus. The results show that there is no significant difference between the two groups in any of the measured parameters. These findings imply that neither the structural integrity nor the quality of the newly formed tendon tissue are affected by aging. The biological characteristics were assessed through cell health assays, histology, migration assay, and gene expression analysis. By examining metabolic cell activity and cytotoxicity it could be concluded that there was no change in cell health with age. Histology revealed no differences in cell density, circularity, eccentricity, or proteoglycan content nor in collagen type I and type III ratio. The migration assay showed that no difference could be found in cell motility with age. Analysis of the gene expression showed significant difference in two of the studied genes, namely DCN and S100A4, at one time-point of collection. DCN encodes for the proteoglycan decorin that is involved in matrix assembly and collagen fibril diameter regulation whilst S100A4 encodes for calcium binding protein A4 which is a marker for cells involved in fibrotic healing. The compiled results from the biological tests indicate that cells extracted from healing tissue does not seem to behave differently due to increasing age of the patients, except for the gene expression of two of the investigated genes. The mechanical and biological test results indicate that the capacity to repair and form new tissue does not decline with age, which is an encouraging outcome from a patient perspective. To the best of our knowledge, this is the first study to investigate age-related differences in healing Achilles tendons using patient derived primary human cells in an *in vitro* model, providing novel insights into Achilles tendon healing capacity as a function of age.

Keywords: Achilles tendon rupture, healing, aging, 3D hydrogel model, primary human cells, mechanical properties, gene expression, migration, histology.

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Josefin Ewerman and Ebba Danielsson, Gothenburg, June 2025

List of Acronyms

Below is the list the of acronyms that have been used throughout this thesis listed in alphabetical order:

AGE	Advanced Glycation Endproduct
AT	Achilles Tendon
ATR	Achilles Tendon Rupture
BrdU	Bromodeoxiuridine
BCP	Bromo-3-Chloropentane
CCK-8	Cell Counting Kit-8
CI	Confidence Interval
CSA	Cross-Sectional Area
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extra Cellular Matrix
EdU	5-Ethynyl-2'-deoxiuridine
FA	Focal Adhesion
FBS	Fetal Bovine Serum
FDL	Flexor Digitorum Longus
GAG	Glucosaminoglycan
GP	Glycoprotein
H&E	Hematoxylin and Eosin
LDH	Lactase Dehydrogenase
LOX	Lysyl Oxidase
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PBTD	PBS with 0.1% Tween and 1%DMSO
PG	Proteoglycan
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SLRP	Small Leucine Rich Proteoglycan
SD	Standard Deviation
TNMD	Tenomodulin
TSPC	Tendon Stem/Progenitor Cell
TSP	Thrombospondin
WHA	Wound Healing Assay

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1
Introduction

Despite the Achilles tendon being the strongest tendon in the body, it is also the most commonly injured. Only in Sweden, the incidence of Achilles tendon ruptures (ATRs) has been seen to increase by 45% between the years of 2002 and 2021 to 41.7 per 100 000 people [1]. At the same time, a decrease in surgical intervention could be observed up until 2017 when the proportion of patients treated surgically stabilized at around 14-15% following an ATR. However the time between diagnosis and surgery continuously increased, which often result in negative outcomes for patients and postponed recovery. It has also been observed that the median age at which patients suffer from ATRs has increased from 44 to 50 years between 2002 and 2021. Even though the exact reasons for this is not entirely understood, it is believed to partly be due to older individuals leading more active lifestyles [2].

As life expectancy rise, there is also a growing expectation to maintain an active lifestyle, which places pressure on healthcare systems to adapt [3]. The reported decreased functional outcomes with age following injury is one such challenge that the healthcare system faces [4]. It is therefore important to further research how aging affects the healing process of Achilles tendons (AT) in order to gain a deeper understanding and thereby optimize patient specific treatments.

1.1 Purpose

The purpose of the project was to examine cellular differences between young and old patient with ruptured Achilles tendons, where the young group consisted of patients younger than 55 and the old group of patients older than 55. This was a blinded study, meaning that which group each sample belonged to remained unknown up until the moment when statistical analysis was performed. The approach to study age-related differences at a cellular level was to extract cells from biopsies taken from patients undergoing surgery following a chronic Achilles tendon rupture and seed them in an *in vitro* 3D fibrin hydrogel model to form tendon constructs. These constructs were formed under circumstances that aimed to mimic new tendon tissue formation, making them suitable as an *in vitro* model of the tendon healing.

This model was utilized to address the two main objectives of this project. The first objective was to study differences in mechanical properties of tendon constructs, more specifically newly formed tendon tissue produced by primary human tendon cells from young and old patients. The second objective was to investigate if there

were biological differences between the cells from young and old patients. More specifically to examine whether the phenotype of the primary human tendon cells, influencing both the composition of the extracellular matrix and the health of the cells, differed between younger and older adults. Furthermore, to assess how these might have had an impact on the healing capacity following Achilles tendon rupture.

The hypothesis for the first objective was that the older patient group would exhibit impaired mechanical properties compared to the younger patient group which would reflect what has previously been observed *in vivo* in intact human ATs [5]. The hypothesis for the second objective differed depending on which biological property that was being studied but in general, the old patient group was expected to show impaired cellular response in the formation of new tendon tissue. For example, the cell proliferation was expected to be greater, at least during the initial phase of culturing in the 3D hydrogel model, for the younger patient group, similarly to what has been shown in previous studies [6], [7]. The migration of the cells have also been shown, in those same studies, to be faster in younger individuals which is why it was expected that the cells exhibit a faster migration in the younger patient group. When it comes to the collagen content, the hypothesis was that less collagen would be observed in the older patient group in the gene expression analysis [8].

1.2 Aims

The aim of this study was to investigate whether differences in functional outcomes post-injury, that can be seen between older and younger patients, could be linked to age-related differences at a cellular level, both in terms of mechanical and biological properties. To investigate this the 3D fibrin hydrogel model as previously mentioned, was utilized to mimic conditions that the cells experiences during new tendon tissue formation.

1.3 Division of Work

This study has been conducted by two students, in which the work load has been divided equally. Since the educational background is more or less the same for both parties, there was not an obvious way to divide the work. Another reason for sharing the practical laboratory work evenly was because both wanted to learn and gain experience in all techniques included in the project. Therefore, the division has been more in terms of responsibility of different areas in regards to data analysis. We did however have specific roles in the mechanical tests that were performed to minimize differences between runs. Here, Josefin had the responsibility of assembling the tendon construct packages with sandpaper, and Ebba had the responsibility of mounting the packages in the machine.

In terms of different roles in the data analysis, the histological analysis in QuPath was mainly done by Ebba, whilst the analysis of RT-qPCR data in Bio-Rad CFX Maestro was mainly performed by Josefin. Measurements of contraction, cross-

sectional area, and migration in the wound healing assay was always done by both parties separately and then compiled together to minimize variations due to measurement error.

The writing of this report has been divided equally between both students. Different sections have been written separately, but the text has been processed and worked through in close collaboration. The work of this thesis has meant an extensive amount of literature studies, both in terms of writing each section but also to learn and keep up with the latest research within the field. This included individual literature search and also regular journal clubs within the research group to increase the effectivity of gathering relevant information. Lastly, the planning of the work necessary for the completion of this Master's thesis has been done in close collaboration with the supervisors. A comprehensive plan for the entirety of this project was made at the start and weekly meetings have been held to follow up on the progress and to assess if changes had to be made.

2
Theory

The information presented in the theory chapter covers the necessary material to gain an understanding of the project. This includes the anatomy and biological function of the Achilles tendon, as well as the known effects that increasing age has on the tissue. Since this project focuses on the tissue post-Achilles tendon rupture, the healing process of tendons is also covered in this chapter. Additionally, different models for studying tendons are described, including different aspects that need to be taken into consideration depending on the kind of study that is being carried out. Lastly, the theoretical background for the different types of analytical methods is covered. This includes one section each for the mechanical and biological tests, as well as a section covering the basics of culturing mammalian cells.

2.1 Anatomy and Biological Function of Tendons

Tendons are fibrous tissue that connect muscle to bone in the body, where the main function is to transfer forces generated by muscles to the skeleton which facilitates movement around the joint [9]. The Achilles tendon (AT) is the thickest tendon in the human body and during these movements it can be subjected to tensile loads up to ten times the body's weight [9]. The AT attaches the lateral gastrocnemius, medial gastrocnemius and soleus muscles in the calf to the insertion point in the calcaneus, also known as the heel bone [10], as seen in figure 1. The average length of the AT is 15 cm and the average minimum width is approximately 1.8 cm.

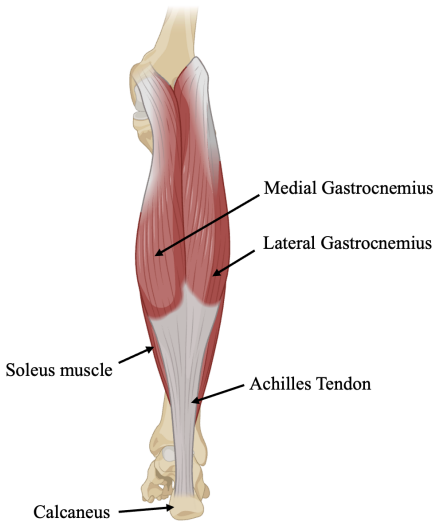


Figure 1: *The macroscopic anatomy of the Achilles tendon, including the surrounding muscles and bone that the AT attaches to (created in biorender.com).*

The strength of the tendon is often attributed to its size as well as the large amount of collagen that is within the tissue and the hierarchical structure of it [11], which can be seen in figure 2. The collagen is organized into fibrils, fibers, and fascicles, in a parallel structure and each subunit are surrounded by a layer of loose connective tissue called endotenon [10]. The entire tendon is then surrounded by an epitenon layer which, with a thin layer of fluid in between, is enveloped in the paratenon, reducing the friction during movement.

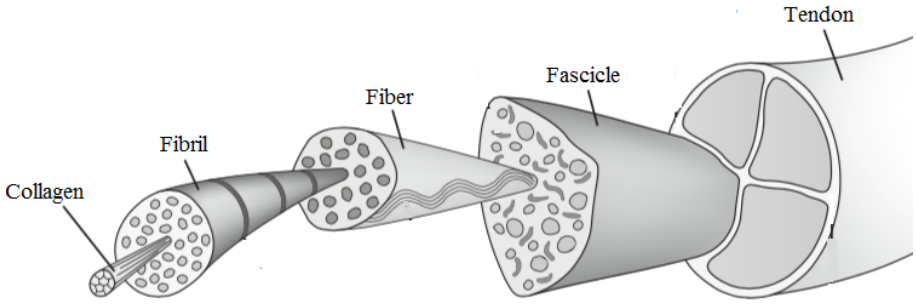


Figure 2: *The hierarchical structure of collagen in tendons (Blumpkin999, CC0, via Wikimedia Commons).*

The extracellular matrix within tendons mainly consist of collagen and the most prevalent type in the tissue is collagen type I which provides strength to the tendon. Collagen type III and type V, while not as abundant as collagen type I, are also important components of the tendon tissue as they help in creating a collagen network and in the regulation of the collagen fibril diameter [12], [13]. Another important component of the ECM is the elastin which contributes to elasticity and allows for rapid propulsion [9]. Collagen and elastin make up approximately 70% and 2%, respectively, of the dry weight of tendons [10]. Tendons also consist of proteoglycans (PGs), which include fibromodulin, decorin, biglycan and lumican, all having

the main function to lubricate the bundles of collagen fibers. Decorin and biglycan have been shown to bind to regions of collagen fibrils exposed to compression, indicating that they have a role in managing compressive forces [14], [15]. Glycoproteins (GPs), namely tenomodulin (TNMD), tenascin C and thrombospondins (TSPs) among others also make up a part of the ECM in tendons and are important for tendon stem/progenitor cell (TSPC) proliferation, management of compressive forces and collagen fibrillogenesis [16], [17].

There is a relatively low abundance of cells in tendon tissue and they are sparsely distributed in the dense ECM. The cellular content mainly consists of tenocytes, a specialized type of fibroblast, which can be further divided into at least five different subpopulations based on phenotypic markers [18]. Besides these different subclasses of tenocytes, there are also TSPCs, endothelial cells, T-cells, and monocytes present in tendon tissue.

Another important component in tendon tissue are the cross-links between the longitudinally located collagen molecules [14]. There are two main types of cross-links, enzymatic and non-enzymatic, which both help to stabilize the collagen fibrils and improve the overall strength of the tendon. The enzymatic cross-links are formed due to enzymatic activity of the lysyl oxidase (LOX) [19]. The LOX enzyme acts on the ends of the collagen type I molecules, which in turn reacts with neighboring collagen molecules and produce cross-links. The non-enzymatic cross-links are mainly created through the Maillard reaction, in which a reducing carbohydrates reacts with amino acids, in this case within the collagen molecule, which then results in the formation of advanced glycation endproducts between two collagen molecules (AGEs).

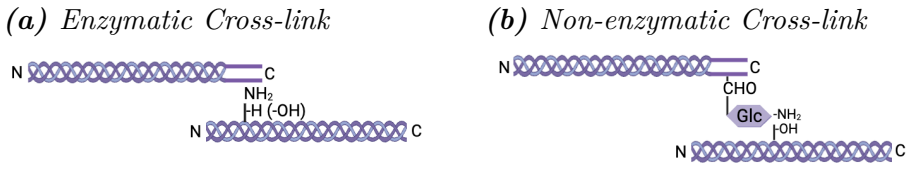


Figure 3: *Illustrations of, (a) an enzymatic cross-link, mediated by the LOX enxyme, and (b) a non-enzymatic cross-link formed from the creation of AGEs (created in biorender.com).*

2.2 The Effect of Aging

As previously mentioned, aging has been shown to be a factor influencing the prevalence of tendon injuries [1]. This can partly be explained by age-related changes in the composition and structure of the ECM, such as the composition of proteins and diameter of collagen fibrils, as well as the turnover rate within the tissue [8]. Additionally, cellular changes occur, including a decline in cell density and a gradual decrease in the metabolic activity of the remaining cells. Many of the structural and

compositional changes are well characterized and studied in animal models but lack similar evidence in human studies [14]. Moreover, cell proliferation and migration decrease with age, while apoptosis and senescence have been shown to increase [6],[7]. The decrease in cell migration is hypothesized to be caused by a re-localization of the focal adhesion (FA) proteins, from the cell surface to internal parts of the cell, impacting their ability to migrate and thus delaying the healing process [20], which will be discussed in more detail in the next section.

Another biological property that changes with age is the accumulation of AGEs, which both have biological and mechanical implications [19]. In a previous *in vitro* study it has been shown that increasing concentrations of AGEs in culture media impairs cell proliferation, electron transfer and ATP production [21]. Mechanically the accumulation of AGEs has however been shown, *in vitro*, to improve the strength of the tendon, while also reducing the viscoelasticity by inhibiting the sliding in between collagen fibrils as well as in between collagen fibers [22].

Morphological changes of the cells found in Achilles tendon tissue have also been reported during aging, where a more rounded cell shape has been found in younger individuals compared to older individuals [23]. However, these differences in morphology was observed in rabbits which could indicate that they may be more associated with degree of maturation rather than aging since the short lifespan of rabbits makes it difficult to differentiate between the two. The overall cellular and ECM changes that occur during aging in the is shown in the simplified illustrations displayed in figure 4.

(a) *Young ECM* ***(b)*** *Old ECM*

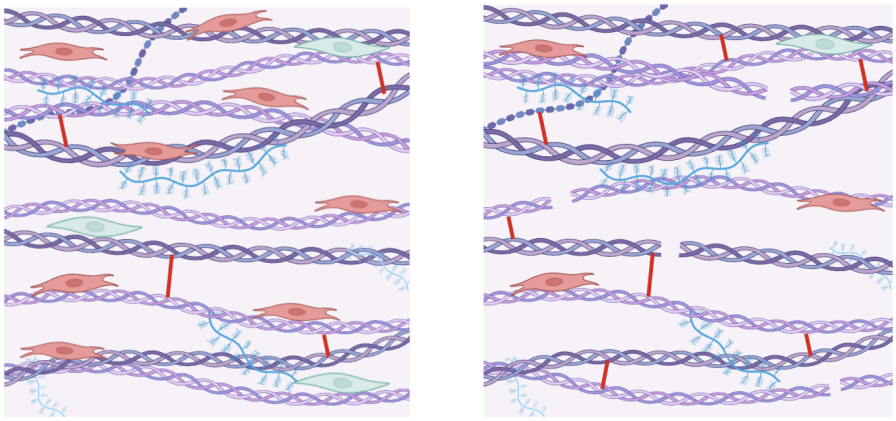


Figure 4: *Illustrations of the cells and ECM in (a) a young individual, and (b) an old individual. These simplified illustrations show changes linked to aging in cellular density, collagen fibrils, AGE accumulation (red lines), and cellular morphology (created in biorender.com).*

The mechanical properties are also important parameters to assess when evaluating the effect of aging on the overall quality of tendon tissue [8]. These parameters are among the most studied in tendon research, but there is still a lack of consensus due to inconsistent results. Some of the inconsistency in results could be explained by differences in study design, e.g. if the study was *in vitro*, *in vivo*, or *ex vivo* and if it was performed utilizing human or animal models. However, when looking at studies done on human ATs it has generally been observed that both the structural properties of the Achilles tendon as a whole as well as the material properties of the Achilles tendon tissue, i.e. independent of the length and CSA, decrease with increasing age [5],[7],[24],[25],[26].

2.3 Achilles Tendon Rupture and Healing

Although the Achilles tendon is the strongest tendon in the body, it is also the most commonly injured. As previously mentioned one factor that greatly influences the likelihood of a patient injury is their age [27]. With an increasing age the mechanical properties change, making the Achilles tendon less resilient to the forces it is subjected to. The change of mechanical properties throughout the lifespan could be explained by a limited tissue turnover and regeneration in adult life [28]. These changes might make the AT more susceptible to injuries which are most common in high intensity sports such as football, racket games or basketball [29], and the rupture is often described as a "snap" sound that is easy to distinguish [30]. The acute rupture can however also be more subtle leading to the injury either being missed or misdiagnosed which, after 4 weeks, leads to the injury being reclassified as a chronic Achilles tendon rupture [31]. Chronic ruptures are much harder to treat and surgically correct. It is thus beneficial to correctly diagnose and treat this type of injury immediately.

Current treatment methods mainly include surgery which tend to be more commonly used in younger and more active patients and non-surgical treatment which is more common among older patients [29]. However, different clinics have different policies and the view on surgical intervention vary greatly. This difference in treatment is mainly based on the belief that surgery reduces the risk of re-rupture making it the preferred treatment for younger patients [32]. Surgery does however increase the risk of complications, making a non-surgical approach an option for less active and older patients. The surgery is generally suture based, meaning that both the type of sutures used and the technique of the surgeon also influence the risk of re-rupture [27]. Surgeries following chronic Achilles tendon ruptures are typically more complicated as they often also entail the removal of excessive scar tissue that have formed around the site of injury [30]. Patients that either underwent surgery or received a non-surgical treatment are typically placed in a cast that ensures immobilization for the first couple of weeks but, is then replaced with a functional brace that allow some mobility [29]. The reason behind using a brace instead of a rigid cast during the later parts of the healing process is to minimize the risk of

blood clots and promote healing through increased perfusion and to elevate the comfort of the patient [33].

The healing process can be, as with all other tissues, divided into three phases, inflammation, proliferation and remodeling [27]. During the inflammation phase immune cells such as neutrophils and macrophages are recruited and cytokines such as interleukins, tumor necrosis factors and growth factors are secreted to recruit even more immune cells. The immune cells also promote TSPC proliferation, the secretion of ECM components and angiogenesis which then lead in to the next phase, proliferation. The change of healing phase reduces the inflammatory response and leads to an increased thickness of the cell layer at the site of injury and even more production of ECM components. The last phase, the remodeling phase, is when the ECM becomes more structured and collagen type III, which is very abundant during the healing process, is instead largely replaced with collagen type I providing the strength that characterizes the Achilles tendon [12].

2.4 Different Models for Studying Tendons

Many different methods can be used to study both the mechanical and the biological properties of the Achilles tendon and they all have different advantages and disadvantages. Some of the main methods for studying the different tendon properties are *in vivo* in either humans or model animals, and *in vitro* using either cells from biopsies, cell lines or from a model animal [14]. Studying the mechanical properties of the tendon more closely became possible first with the invention of ultrasound-based methods, which has since then helped in providing much new insight into the function of the tendon. However, studying mechanical properties within the human body also have some disadvantages as the obtained results are not only dependent on the properties of the studied Achilles tendon, but also greatly influenced by the surrounding muscles [5]. It is also not possible to do tests like pull to failure in patients which provides valuable information such as the maximal stress and force the tendon can endure before breaking.

Another problem that arises with *in vivo* studies in animal models is that it sometimes can be difficult to differentiate between maturation and aging of the tendon as many of the animals used have a relatively short lifespan [14]. Moreover, studies in patients and animals offers limited possibilities to combine investigation of the mechanical properties with analysis at the cellular level. It would therefore be beneficial to investigate an alternative approach to these types of studies by utilizing an *in vitro* model where the external factors are minimized while still the native environment of the tendon is mimicked as closely as possible. Moreover, such a model can also be beneficial to isolate different factors that are of interest.

This type of model can have many different designs depending on the aim of the study. The model used in this study is an *in vitro* 3D fibrin hydrogel model, which has been utilized in previous studies [34], [35]. In this model, cells extracted from tendon biopsies are seeded in a fibrin hydrogel created by the combination of fib-

rinogen and thrombin, which digest the fibrinogen into smaller fibrin fibers that act as a hydrogel and a matrix where the cells reside [36]. To ensure that the gel is not digested by the cells the inhibitor of proteolytic enzymes, aprotinin, is also added during seeding [37]. The gel is seeded on top of a silicone layer and in between two sutures, guiding the cells into aligning in a parallel orientation. The cells also exert tension on the sutures, causing the gel to contract when loosened from the edges from the well and form a tendon construct [38], as can be seen in figure 5. This means that the cells themselves do not migrate to form the construct but rather that the tension generated between the cells and the sutures is the main reason behind the contraction process. The cells are then also supplied with ascorbic acid and L-proline in addition to the essential nutrients to ensure efficient collagen production which is essential when trying to mimic the native environment within the tendon [39], [40].

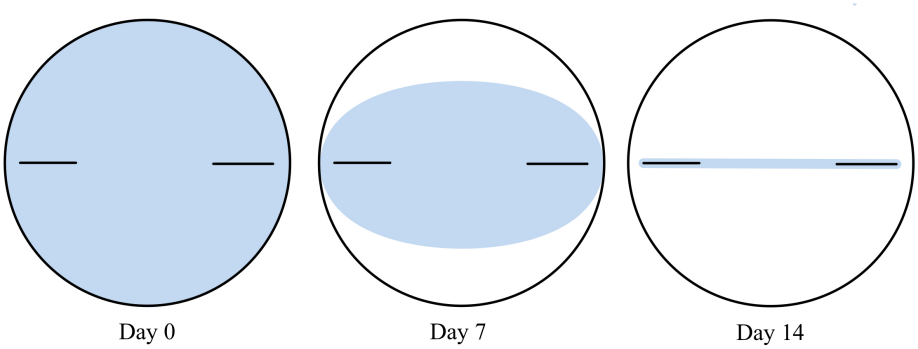


Figure 5: *Illustration of the tendon construct formation where the black circle represents the well, the black lines represents the suture threads and the blue circle represents the fibrin hydrogel that contracts over time.*

Another factor that further helps in mimicking the native environment is that the cells used are taken from biopsies obtained from patients undergoing surgery following a chronic Achilles tendon rupture, meaning that the cell population is very similar to the native one. This introduces some variation between patient samples as the biopsy could be representative of different parts of the tendon and that patients have an innate variation in tenocyte population regardless of the location of the rupture. However, cells extracted from a biopsy is still preferable to using cell lines as these cells closer resemble the native environment.

2.5 Cell Culturing

Cells from biopsies used to create the tendon constructs described previously need to be isolated and cultured before they can be used to form the actual constructs. When it comes to cells in tendons, collagenases are often used to break down the large amount of collagen in the tendon tissue to be able to obtain the cells [41]. This is followed by culturing the extracted cells in special culture flasks with a coating on the bottom where cells can attach, allowing for stem cells and fibroblasts to

proliferate. During proliferation the cells need to be fed regularly with a medium containing all the necessary nutrients and growth factors. Since it is hard to design a media containing everything the cells need, a commonly used additive to the well defined basal media is fetal bovine serum (FBS) which is a great source of both proteins and growth factors [42]. An alternative would be to use human serum to closer mimic the natural environment, but since FBS have been successfully used in studies utilizing a similar model, it motivates the choice of using it in this study as well [35]. The addition of FBS is somewhat problematic though as there are batch variations and it is not well defined, which could influence how well the cells grow and how well the constructs form. To minimize these differences it is important to try using serum from the same batch if possible and the same brand if not.
After letting the cells grow for a while they start populating more and more of the surface, until they reach confluency [43]. Different cells react differently to reaching confluency. Many experience morphological and behavioral differences which is why it can be beneficial to divide the cells into new culture flasks when they reach confluency, in a process called passaging. This process is also used to determine the age of the culture as the cells loose more and more of their native characteristics and replicative potential with each passage. This means that after a certain amount of passages the cells can no longer be used, but when the cells reach that limit depend on the study design.

Another important aspect in cell culturing is keeping everything sterile to minimize the risk of contamination [44]. As mammalian cells grow very slowly they are easily outcompeted if a fast growing bacteria or fungi were to enter the culture flask. This means that it is very important to keep the right conditions for optimal growth while also ensuring that everything is sterile. The risk of contamination can be further reduced by the addition of antibiotics in the culture media.

2.6 Mechanical Testing

The tendon construct model can as described above be beneficial when it comes to studying the mechanical properties. Even though the data obtained cannot be compared to data collected from studies done *in vivo*, it provides valuable information when, for example, comparing two groups of different age, as is done in this study. Two parameters that are of great interest when it comes to mechanical properties are stiffness and Young's modulus of the tendon [45]. The stiffness provide information of the mechanical properties of the whole tendon as it is dependent on the cross sectional area (CSA), whereas the Young's modulus provide more material specific information as it is independent of the cross sectional area. Stiffness is in other words dependent on both the amount and quality of the collagen fibers whilst the Young's modulus only is dependent on the quality of the collagen fibers. This means that a tendon with many weak collagen fibers could have the same stiffness as a tendon with few strong collagen fibers, but the Young's modulus would be much higher in the second tendon compared to the first.

Young's modulus is determined by finding the peak slope of the stress-strain curve, where the stress (σ) can be calculated by dividing the normal force with the CSA, as seen in equation 1, and the strain (ϵ) can be calculated by dividing the elongation with the original length, as seen in equation 2.

$$\sigma = \frac{F_n}{A} \tag{1}$$

$$\epsilon = \frac{\Delta L}{L_0} \tag{2}$$

By combining these two equations, one can obtain an equation that describes the Young's modulus as seen in equation 3.

$$E = \frac{\Delta\sigma}{\Delta\epsilon} = \frac{F_n L_0}{A\Delta L} \tag{3}$$

Stiffness can then either be described using the Young's modulus as in equation 4

$$k = E \times \frac{A}{L_0} \tag{4}$$

or by simplifying the expression with the definition of the Young's modulus as described in equation 5. This equation also shows that the stiffness can also be described as the peak slope of the force-displacement curve.

$$k = \frac{F_n L_0}{A\Delta L} \times \frac{A}{L_0} = \frac{F_n}{\Delta L} \tag{5}$$

2.7 Biological testing

The theoretical background for the different methods relevant for testing the biological properties investigated in this study is presented in the following sections. Most of the methods are performed on the 3D fibrin hydrogel model, with the exception for the migration assay where the extracted cells are seeded directly in 6 well plates.

2.7.1 Cell Health Assays

Cell health can be evaluated through metabolic activity using a Cell Counting Kit-8 (CCK-8) assay, which is a colorimetric assay that quantifies the metabolic activity of the cells [46]. The CCK-8 assay utilizes the active dehydrogenase enzymes present in viable cells and the reagent WST-8 [2- (2- methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5- (2,4-disulfophenyl)2H- tetrazolium, monosodium salt], which is a water-soluble tetrazolium salt [47]. When WST-8 is added, it enters the cells and the dehydrogenases reduce the WST-8 to WST-8 formazan, causing a change in color that can be quantified by measuring the absorbance. The absorbance can then be used to assess the metabolic activity of the cells. The CCK-8 assay offers a lot of advantages compared to other methods investigating similar properties, one of them

being that it has a very low toxicity, which allow for other assays to be performed on the same cells after the CCK-8 assay is completed.

Another method that can be used to assess cell health is a lactate dehydrogenase (LDH) assay that instead evaluates the cytotoxicity [48]. Lactate dehydrogenase is an enzyme that is only present in the cytosol of cells meaning that it can only be detected in the media after cell death and the release of cytosolic compounds. This can be utilized to measure cytotoxicity directly but it also gives an indication as to the overall health of the cells. The assay works by utilizing the enzymatic activity of LDH which catalyzes the conversion of lactate into pyruvate while also reducing NAD^+ into NADH [49]. The NADH can then convert a tetrazolium salt into a coloured formazan that can be detected and thus, the LDH activity can be measured and the cytotoxicity determined.

2.7.2 Gene Expression Analysis

Gene expression analysis is often performed to investigate which genes that are up- and downregulated under specific circumstances and, is in this study used to investigate age related differences associated with the healing process in the artificial Achilles tendon constructs created by utilizing the 3D hydrogel model described in section 2.4. The genes that are of interest in this study mainly include genes that encodes for different types of matrix associated proteins such as collagen and proteoglycans as they play an important role in regaining the function of the Achilles tendon following an injury. Tenocyte-specific markers are also important to study as their presence indicate that the correct type of cells have been isolated and used in the analysis, as well as to investigate the differentiation of the cells. Finally, other genes related to cell shape and migration capacity are also interesting to include as they provide insights into the overall phenotype of the cells. The specific genes investigated and their function are presented in more detail in table 1 below.

Table 1: *Description of the genes investigated in the gene expression analysis.*

Gene	Function
COL1A1	Encodes for collagen type I which is an important for the transfer of mechanical forces within the tendon [9].
COL3A1	Encodes for collagen type III which is typically the first type of collagen produced during the healing process and is largely replaced with collagen type I during the remodeling phase [12].
COL5A1	Encodes for collagen type V which regulates the collagen fibril diameter [13].
MMP-1	Encodes for an enzyme that degrades collagen [27].
MMP-13	Encodes for an enzyme that degrades collagen [27].
FMOD	Encodes for fibromodulin which is a class II small leucine rich proteoglycan (SLRP) that is known to regulate collagen fibril diameter and overall tendon structure and function [50].
DCN	Encodes for decorin which is a class I SLRP that is known to have similar functions as the class II SLRPs within the tendon [50].
BGN	Encodes for biglycan which is a class II SLRP and therefore have the same function as decorin [50].
ACTA2	Encodes for different actin proteins and is known as a marker of the healing tendon, especially in cells with similar features as smooth muscle cells [51].
S100A4	Encodes for calcium binding protein A4 and is associated with fibrotic tendon healing [51].
SCX	Encodes for scleraxis that is a tenocyte specific marker [51].
SPARC	Encodes for secreted protein acidic and rich in cysteine protein which has been shown to influence the mechanical load the tendon can withstand [52].
EZR	Encodes for ezrin which is an important protein in cell adhesion and motility [53]

One way the gene expression can be measured is through quantitative reverse transcription polymerase chain reaction, often referred to as RT-qPCR, which is a common method used to detect and quantify RNA [54]. Briefly put, the method involves reverse transcribing the RNA into cDNA, that is then amplified using PCR. During the amplification, the amount of the gene of interest is measured using fluorescence and can thus be quantified and the gene expression can be measured.

2.7.3 Histological Analysis

The morphology of the cells is another important factor to study when investigating the biological properties. Hematoxylin and eosin, commonly referred to as H&E stains, are two frequently used stains used to visualize the morphology of the cells as they stain different molecules in the cell respectively [55]. Hematoxylin stains nucleic acids in a blue-purple color and eosin stains proteins in a pink color, giving the nuclei a blue-purple color and the cytoplasm and ECM a pink color with different intensity depending on the amount of protein in a given area. Since only the nuclei is distinguishable using this type of stain it is not possible to measure the actual cell shape. However, softwares such as QuPath, uses the shape of the nuclei to estimate the cell shape and this estimation is often the one used in downstream analysis.

The morphological aspects that can be investigated through H&E staining is the shape of the nucleus which previously have been shown to vary with age and following injury [56]. Cells from older patients have a more homogeneous and elongated nuclei shape, but following injury the nuclei tend to become more rounded and the population more heterogeneous. Two parameters that are often used when describing the shape of the nuclei are circularity and eccentricity [57]. The circularity is described in equation 6,

$$Circularity = \frac{4\pi Area}{Perimeter^2} \tag{6}$$

and the eccentricity is described in equation 7,

$$Eccentricity = \frac{Max\ caliper\ -\ Min\ caliper}{2} \tag{7}$$

In these equations it can be seen that circularity considers the shape of the entire cell, whereas the eccentricity only considers the extreme points and does not provide any information about the overall shape of the cell. Illustrations of the what is measured when calculating the cell circularity and eccentricity can be seen in figure 6. A cell circularity of one means that the cell is a perfect circle and an eccentricity of one indicates that the cell is a perfect ellipse.

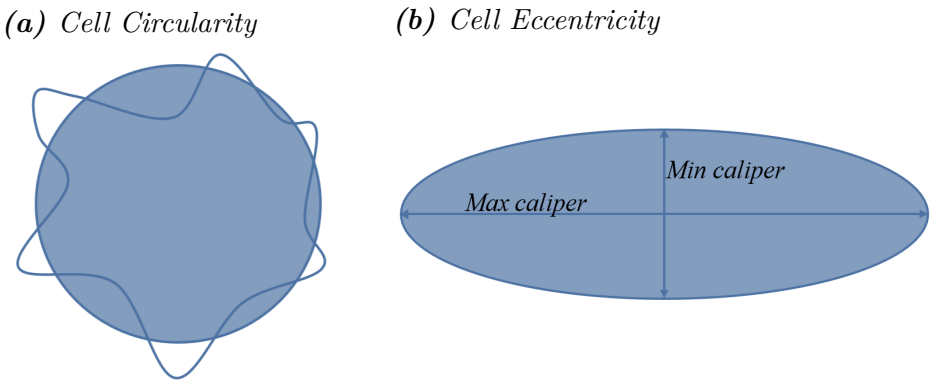


Figure 6: *Illustrations of the (a) cell circularity, which measures how closely the cells resembles a circle, and (b) cell eccentricity, which measures how elliptical the cells are.*

Another type of staining that is commonly used is Alcian blue that stains glucosaminoglycans (GAGs) and can thus be used to detect regions rich in PGs [58]. It has previously been shown that the gene expression of proteoglycan 4 increased with age in rats [59].

Immunofluorescence is another histological method used to visualize the morphology of the cells, as well as expression and localization of specific proteins that instead of stains uses labeled antibodies that bind to specific antigens [60]. The antigens can either be specific to a certain organelle or a type of protein present within the cell, on the cell membrane or in the ECM. A protein that is especially relevant to study when investigating tendons is collagen, and more specifically collagen type I and III [14]. By studying the ratio of collagen type I and III in the ECM, valuable information about the maturation of the tendon, as well as at what stage within the healing process the tendon presently is in, can be obtained. With aging the healing process is impaired and it can thus be assumed that aged tendons would have a different collagen type I and type III ratio compared to younger tendons, more specifically a higher collagen type III content in relation to type I [6], [7], [61].

2.7.4 Migration Assay

Cell migration is an important property in wound healing and can be studied using a migration assay. The most common way to study this is in a 2D assay where the cells are grown in a monolayer which makes their migration easy to study but gives a very simplified image of what is actually happening *in vivo* [62]. More complex 3D models have therefore been developed to better illustrate *in vivo* conditions, which is a valuable tool in studies where the mechanisms of the healing process are investigated. However, a 3D model can be unnecessarily complex in a comparative study such as the one described in this report, as the cell migration is only one of many factors studied.

One type of 2D model often used is the wound healing assay (WHA) in which a gap is created in a cell monolayer, either mechanically, chemically or thermally but they all ultimately result in a cell free area where migration later can be studied [63]. Creating the wound mechanically with a pipette tip or any other suitable lab equipment is a very common method to use as it is both easy and inexpensive. The downside of this method though, is that it can be hard to create reproducible results as the width and shape of the gap is dependent on how steadily and at what angle the instrument is scraped through the cell culture. Pressure of the tip against the cell culture could also affect the migration of the cells after the gap is created, as if too much pressure is applied this could not only damage the cells but also the coating of the well which the cells are dependent on during migration [62]. To mitigate some of the downsides with the poor reproducibility, methods have been developed where several wells are scraped at the same time [63].

Another method that is slightly more complex but produces more reproducible results is the transwell migration assay [64]. In this assay the cells are seeded on a membrane with pores that the cells can migrate through, which they are encouraged to do through a chemotactic agent [65]. The agent used is often serum which the cells need in order to proliferate and be healthy. When the cells have migrated they are stained and the amount of cells that have migrated during a set period of time can easily be quantified to determine the efficiency of cell migration.

3
Methods

Several different methods have been utilized in this project to achieve the stated aims. The first aim of the project involves mechanical testing of the artificial tendons constructs which were formed through utilization of the 3D fibrin hydrogel model, to investigate age-related differences. During the formation of the constructs, the contraction process of the gels were also evaluated. The second aim of the project was also to investigate eventual age-related differences, but instead evaluating biological properties. The methods used include CCK-8 and LDH assays to study cell health, RT-qPCR to study gene expression, a wound healing assay to study cell migration and finally histology to study cell morphology and density together with collagen type I and type III ratio.

All of the listed methods, except for the wound healing assay, were conducted on the tendon constructs. The wound healing assay was instead performed on cells grown in a 6-well plate, forming a monolayer of cells. These methods were performed using cells from 6 patients younger than 55 and 7 patients older than 55.

3.1 Extraction and Culturing of Primary Tendon Cells

The cells were extracted from biopsies collected from patients undergoing surgery following a chronic Achilles tendon rupture in accordance with the protocol seen in Appendix A.1. Briefly, the biopsy tissue was first mechanically cut into smaller pieces using a scalpel while also removing visibly vascularised and fatty tissue to ensure that most of the cells extracted are tenocytes. This was then followed by incubation for approximately 24 h in Dulbecco's Modified Eagle Medium (DMEM/F-12) containing 20% FBS and 1 mg/mL collagenase type II, which further digested the remaining tissue. The next day, the tubes were centrifuged and the cell pellet was isolated and resuspended in culture medium consisting of DMEM/F-12 supplemented with 10% FBS and 1% Pen/Strep before the cells were seeded out in a culture flask. The cells were cultured to reach confluence, and the medium was changed every 4-5 days, before conducting any further experiments with the cells.

If the cells reached confluence and were not to be used in any immediate experiments, they were frozen in accordance to the freezing protocol found in A.3. Shortly put, this process entailed washing the cells with phosphate buffered saline (PBS), to remove FBS which inhibits trypsin, before trypsinating them to detach the cells

from the culture flask surface. Thereafter, the trypsin was deactivated with culture medium containing 10% FBS. The cells were in contact with the trypsin for approximately 5-7 minutes and it was important to restrict the time since active trypsin can harm the cells if left for too long. After trypsination, the cells were centrifuged and the cell pellet was then resuspended in freezing medium containing DMEM/F12, 20% FBS, and 10% dimethyl sulfoxide (DMSO). The purpose of the DMSO is to protect the cells during freezing. The suspension was thereafter transferred to a cryo tube and placed in a cell freezing container which ensures that the cells are frozen by 1°C per minute. The cell freezing container was then placed in a -80°C freezer for 2-5 days before being transferred to a -150°C freezer for long term storage. When the time came to use cells that were stored in the freezer in experiments, they were taken up and thawed as described in the thawing protocol found in A.3. Which briefly entailed thawing the cells using warm culture media before centrifugation. The pellet was then resuspended in culture media and the cells seeded in a culture flask.

If there was a need to obtain more cells, the cells were instead passaged when confluence was reached. The cells used for creating the tendon constructs had a passage number between P_0-P_3, whilst the cells used for the wound healing assay had a passage number between P_2-P_5. This process is fairly similar to the freezing of cells, with the difference being that after the centrifugation step the cell pellet was instead resuspended in culture medium and seeded out into new culture flasks. The procedure used can be seen in the passaging protocol found in A.3.

3.2 Formation of Artificial Tendon Constructs

The details of tendon construct formation can be seen in the protocol in Appendix A.4. In brief, the 6-well plates were sterilized by submersion in ethanol and then the ethanol was aspirated before letting the plates dry completely. The plates were then washed with PBS twice. The cells used for the tendon constructs were prepared by washing with PBS, followed by trypsinization to loosen the cells from the cell culturing flask. The cells were then counted and diluted to reach a concentration of 333 333 cells/mL. From this solution, 600 μl was transferred to 6 1.5 mL tubes to achieve a cell density of 200 000 cells/well and 10 μl of aprotinin (10 mg/mL) was added to each tube and mixed together by pipetting up and down. Thereafter, 200 μl fibrinogen (20 mg/mL) and 4 μl thrombin (200 U/mL) were added to one tube at a time containing the cell and aprotinin mixture, followed by immediate mixing by pipetting up and down before the entire volume was transferred to one of the wells on the 6-well plate.

Which well each sample was transferred to is illustrated in figure 7, where the letters represents the patient, and the number represent which day after plating the specific experiment was conducted.

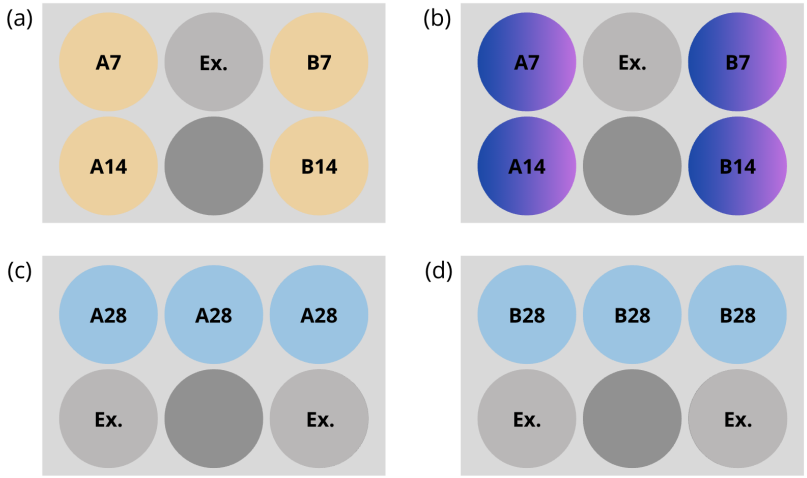


Figure 7: *Illustration of the plate design for two patient samples, where each letter represents a patient, and the number represent which day after plating each method was utilized. Each 6-well plate is designated for different types of methods, more specifically, (a) for CCK-8 assay, (b) for RT-qPCR and histology, (c) and (d) for mechanical testing. The light grey wells labeled Ex. are extra constructs and the dark grey wells are unused.*

The plate was then placed in the incubator for 1 hour before construct medium consisting of DMEM/F-12, + 10% FBS, + 1% Pen/Strep, + 1% 200 μM Ascorbic acid, + 1% 50 μM L-Proline was added to each well. When the plate had been incubated for 48 hours, the edges of the gels were loosened from the walls of the wells, allowing for each gel to contract into and form a tendon construct. This process was monitored through microscope imaging, using a Nikon stereo microscope and a Dino-Eye to capture the images, at specific time-points between day 7 and 19 in three wells for each patient sample. ImageJ was then utilized to measure the width of the gels over time. All the wells that were monitored was measured twice by two independent evaluators and normalized against the width of the well using ImageJ. The normalization and measurements of the width of the gels are displayed in figure 8. The mean value of the four measurements performed for each well and time-point was then used in the downstream analysis.

(a) *Normalization* ***(b)*** *Measurement*

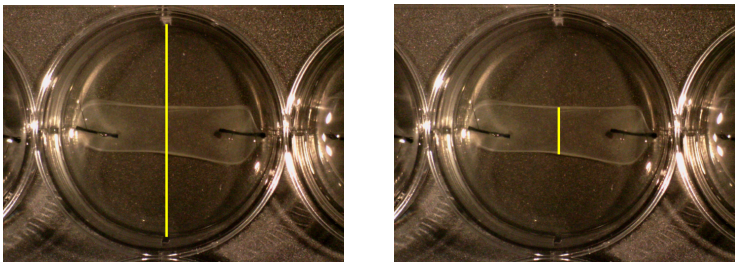


Figure 8: *Representative images of the (a) normalization against the well with a known width of 3.5 cm, and (b) measurement of the width of the gel in ImageJ.*

3.3 Mechanical Testing

The mechanical test for each patient was performed in triplicates, as can be seen from the plate design presented in figure 7. Mean values of the triplicates were then used in the downstream analysis. The machine that was used for testing the mechanical properties of the constructs was the UniVerts S - Universal Uniaxial Mechanical Tester (UV-200-02) from World Precision Instruments. The machine was new to the institution and thereby new protocols and routines had to be tested and established for this study.

The development of these protocols have primarily been done by conducting experiments on cells extracted from semitendinosus tendons, which are not included in the final study. This process included many different steps in order to ensure the most reliable and accurate results. The first step in the method development of the mechanical tests was to set-up the program and determine the different parameters used. One of the parameters, the speed at which the clamps were pulled apart, had already been optimized in a previous study [35], but other parameters, such as the pre-load, which is the force applied from the very beginning, and the distance between the clamps at the start of the test had to be determined. After some trial and error it was determined that no pre-load should be used due to the low forces being recorded and that only the elongation past a threshold of 0.025 N should be included in the downstream analysis. The reason for this was that force fluctuations was very common in the trial runs prior to reaching this threshold, meaning that the machine essentially did not exert any force on the construct during the first period. The distance between the clamps was determined to best be kept at 8 mm, even though the constructs in theory should be 15 mm, due to variations during mounting and some shrinking during the mounting process. The risk of injuring or stretching the tendon constructs before the test began were therefore minimized by staring at a relatively short clamp-to-clamp distance. Some adjustments to the hardware was also done and included replacement of the original clamps to smaller and lighter ones and changing to a more sensitive load-cell that could record smaller forces. Finally, a mounting protocol was developed to prevent the tendon construct from breaking at the contact point with the clamps or to slide out of the clamps. Therefore, several different types of sandpaper were tested as well as different ways to assemble the whole package before mounting it in the machine. The goal was to have the breaking point somewhere in the middle to ensure that the resulting data was representative of the tendon construct and was not influenced by technical errors caused by faulty mounting. The whole process of the mechanical tests is briefly described below, and in more detail in Appendix A.5.

The mechanical tests on the Achilles tendon constructs was performed 28 days after cell seeding, in accordance to protocol A.5 found in Appendix A. Briefly, the constructs were mounted on sandpaper using super glue and kept hydrated by having wet gauze underneath, illustrated in figure 26, in Appendix A. It was then immediately fastened into the clamps of the testing machine submerged in a PBS bath. The tendon constructs were then exposed to tensile forces until failure, meaning

that they were attached to the machine at each end and stretched out at 4 mm/min until their breaking point. This process is shown in figure 9, where representative images of the construct at the start of the test, when it is stretched out and when it ruptures are shown. Images were taken continuously during the entire run, which were used for the measurement of the cross sectional area. The cross-sectional area (CSA) was measured in ImageJ by recording the area of the entire construct and dividing it with the length of the construct to obtain the diameter and thus being able to calculate the CSA. This data along with the recorded force were then used to determine both Young's modulus and the stiffness as described in section 2.6.

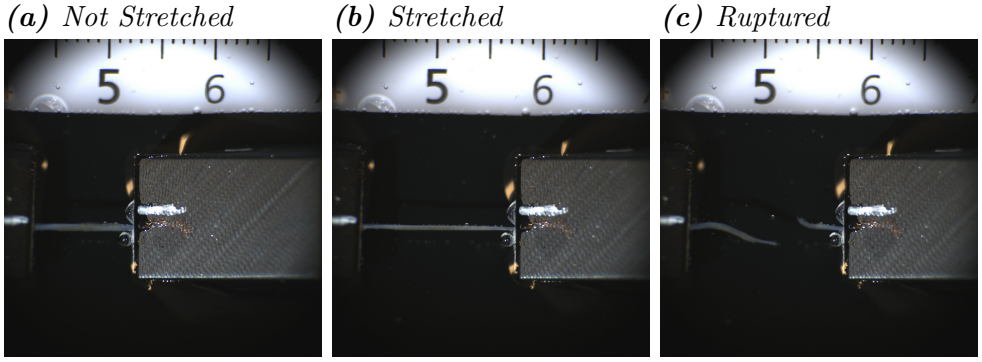


Figure 9: *Representative images of a tendon construct during a pull-to-failure test, where the construct is (a) not stretched, (b) stretched, and (c) ruptured.*

3.4 Cell Health Assays

To examine the cell metabolic activity, a CCK-8 (Dojindo Laboratories) assay has been performed at both day 7 and day 14 for each of the donor sample. The procedure was conducted in accordance to the CCK-8 protocol found in A.6 and briefly consisted of mixing 1 part of CCK-8 solution with 10 parts of construct medium and adding it to each well that were being examined, before placing them in the incubator. The absorbance was then measured at 450 nm after 1 hour of incubation and the metabolic activity of the cells in the solution could thereby be assessed.

Another method used to asses the cell health was the LDH assay, which was performed in accordance to the protocol found in A.6. An LDH assay kit (abcam) was used, which briefly consisted of the preparation of a standard curve by diluting six samples of NADH to final concentrations ranging from 0-12.5 nmol/well with LDH Assay Buffer on a 96-well plate. Of each sample 50 μL was added to separate wells before Substrate Mix also was added to all wells to a final volume of 100 μL. Each sample, including the standard curve samples, were done in duplicates and a mean value of these were then used in the downstream analysis. Absorbance was measured every two minutes at 450 nm to find an optimal time-point when all the samples were within the range of the standard curve. Finally, the standard curve was used to calculate the LDH activity in each sample.

3.5 Gene Expression Analysis

Gene expression analysis was performed to provide information about which genes were upregulated and downregulated during during new tissue formation in regards to aging in the tendon constructs. The procedure is described in detail in protocol A.7, but in brief involves the collection of half the gel from the well destined for both gene expression analysis and histological analysis, at both day 7 and day 14 after seeding, in a tube with stainless steel beads and silicon carbide beads. These tubes were then placed in a bead beater (MP, FastPrep-24 Classic) to break down the tissue and snap frozen in liquid nitrogen in preparation for the following extraction and analysis. When all the samples had been collected the tubes were thawed and 1-Bromo-3-chloropropane (BCP) was added, before centrifuging the tubes to ensure phase separation. The phase containing the RNA was then transferred to another tube and glycogen was added, to make the RNA pellet visible, before beginning the stepwise cleaning process of the RNA using different concentrations of ethanol. Finally the pellet was dried and then resuspended in RNAase free water before the concentration was measured using a NanoDrop spectrometer. The next step of this process was to reverse transcribe the RNA into cDNA which was done using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems and a T100 Thermal Cycler from Bio-Rad. To function as a standard curve for the subsequent gene expression analysis samples containing universal human reference RNA was also included and reverse transcribed into cDNA.

The cDNA was then used in the gene expression analysis where pre-designed TaqMan primers for each gene described in table 1 along with housekeeping genes were used. The housekeeping genes were included to correct for possible differences in starting cDNA concentration of each sample. To make quantification of the gene expression possible a dilution series of the cDNA obtained from reverse transcribing universal human reference RNA was also included for each gene and worked as a standard curve in the analysis. The machine used for the RT-qPCR was the CFX Opus 96 Real-Time PCR System from Bio-Rad. Due to some issues with the housekeeping genes being differently expressed in the different patient groups and at the different time-points in which the sample was collected, six different genes were tested in total. From these, TBP and B2M were the only reference genes that did not show significant differences, neither between patient groups nor time-points, and their geometric mean was therefore used for normalization in the following analysis. The geometric mean was used to reduce possible influence from outliers and large variation within the reference gene data, which was also the reason behind logarithmically transforming all the gene expression data prior to analysis.

3.6 Histological Analysis

Samples for histological analysis were collected at day 7, day 14 and day 28 after seeding, meaning that the whole process from the cells simply proliferating within the fibrin hydrogel to them forming constructs can be visualized in the subsequent

staining. The samples from days 7 and 14 were used for both histological and gene expression analyses, requiring the gels to be cut in half for different downstream purposes. In contrast, entire constructs were collected for day 28 samples. This process is described in more detail in protocol A.8, but briefly includes submersion of the sample in an optimal cutting temperature (OCT) embedding matrix (CellPath) and freezing in liquid nitrogen. The samples were then cut into 8 µm thin frozen sections using a cryostat (CryoStar NX70, epredia) and transferred to glass microscope slides by gently pressing the glass onto the frozen section.

After cryosectioning, the different slides from each sample were stained with H&E to study cell morphology and cell density, Alcian blue to study PG content, and antibodies for collagen type I and type III to determine their ratio. Protocols for these processes can be found in section A.8 of Appendix A, but in brief the H&E staining entailed fixation of the constructs in 4% formaldehyde before washing them in tap water and staining with Harris hematoxylin. The slides were then washed again before being differentiated in acid alcohol and counterstained with Eosin. Excess eosin stain was then washed away in MQ water and the constructs were dehydrated in increasing concentrations of ethanol and finally xylene before being mounted in Pertex. After mounting the slides were scanned and analyzed using QuPath, where the cell detection tool in the software was used to determine the cell density, circularity and eccentricity.

The process of staining the constructs with Alcian blue started similarly to H&E staining with fixation in 4% formaldehyde before rinsing the samples in tap water. They were then incubated in 3% acetic acid and stained with Alcian blue solution before being rinsed in tap water again. The samples were then counterstained with Nuclear Fast Red solution that was rinsed off in tap water before the constructs were dehydrated, mounted, scanned and was also analyzed using Qupath, but instead by creating a threshold for area positively stained for proteoglycans. This made it possible to calculate a percentage of positively stained area versus the area of the entire construct.

Samples used for immunofluorescence, and more specifically detection of collagen type I and type III, were instead fixed in -20 ∘C methanol before an ImmEdge pen was used to create a hydrophobic barrier around the construct. The slides were then rehydrated in PBS with 0.1% Tween and 1%DMSO added (PBTD) to it, before blocking with 5% normal goat serum and subsequent application of the primary antibodies. After overnight incubation at 4 °C the slides were washed in PBTD before incubation with the fluorophore-conjugated secondary antibodies and counterstaining with DAPI. The slides were then finally washed in PBTD and mounted using ProLong Gold Antifade Mountant before being scanned and then analyzed using QuPath. The analysis in QuPath involved creating a threshold for both collagen type I and collagen type III which allowed for measurements of the positively stained area for both proteins. Then a Col I/Col III ratio could be calculated.

3.7 Wound Healing Assay

In this project, the 2D model described in section 2.7.4, which entails the creation of a wound with a pipette tip, have been used and optimized by seeding different cell concentrations, finding the optimal pipette tip size for the given cell concentration and finally determining the time-frame required to obtain a representative image of the wound closure process. Cells from all donors were cultured in triplicates with two scratches made in each well to account for possible variation. The area with the most even scratch was then used in the downstream analysis. In brief, this method thus involved seeding cells from each patient in a 6-well plate at a concentration of 300 000 cells per well using DMEM/F-12 supplemented with 10% FBS and 1% Pen/Strep. After 40 hours two scratches were made with a 200 μL pipette tip in each well and the area of the scratch was monitored every two hours at first and then every hour when the gap began to shrink more rapidly. The area of the gap was then measured twice by two independent evaluators in ImageJ using the freehand selections tool, and a mean value of these measurements was used in the analysis of the results. This process is described in more detail in protocol A.9.

3.8 Data Processing

The data processing included comparisons between the results obtained from the two patient groups. Data obtained from the different methods used during this study includes both pictures that needed interpretation and more concrete numbers that were analyzed statistically. To eliminate any possible bias, the analysis of the data have been conducted without knowing which group each sample is a part of. This was especially important during the interpretation of pictures obtained from the wound healing assay, as it is easy to form an unconscious bias of what is supposed to be seen in the pictures. The measurements made from these pictures were also repeated a total of four times, twice by each evaluator that manually draws the outline, reducing the likelihood of possible mistakes made during measurement influencing the resulting analysis. The final statistical analysis was then done after the patient groups had been revealed, as an actual statistical analysis would not be influenced by a potential unconscious bias in the same way as manual measurements can be.

All data, except the the one collected from gene expression analysis, were compared between the age groups using a two-sided Mann-Whitney U test, which is a non-parametric comparison between the median of the two groups, since the data was assumed to not be normally distributed. The Mann-Whitney U tests were performed and the data was visualized using the program GraphPad Prism. For the gene expression the data was first Log-10 transformed to reduce the effect of outliers before the Levene's test for equality of variance, in the program SPSS, was performed to investigate whether a parametric or non-parametric test should be used. After ensuring that equal variances could be assumed, a parametric two-sided unpaired t-test was used to analyze the gene expression data in SPSS and then visualized

using GraphPad Prism. Additionally, Spearman correlation analysis has been used to investigate whether the studied variables had a correlation with age.

3.9 Societal, Ethical and Ecological Aspects

The Achilles tendon is as previously mentioned the most commonly injured tendon in the human body and the incidence rate has increased during the last decades [30]. People also tend to exercise and participate in sports at an older age which thereby further increases the risk of injury [30]. Older people are not only at a greater risk of injury, chronic rupture is also more common within this patient group which have been shown to both cause greater pain and discomfort as well as reduced function in the healed Achilles tendon. The healing capacity of the aged tendon is also impacted, making the recovery following this type of injury more lengthy and more difficult [66], [67]. Therefore understanding the mechanisms behind impaired healing in aged tendons would greatly benefit not only the individual but also society as a whole, as a better understanding of the tendon biology could lead to improved treatment following an Achilles tendon rupture.

The design of this study does not only have societal implications that need to be considered, there are also ethical aspects. Biopsies have been collected from patients that have given their consent and approval has been given from Etikprövningsmyndigheten (case number: 2023-05442-01) to collect these samples, which is necessary when working with traceable human tissue [68]. The patients are anonymous and only their age is documented since that is the only factor which is being studied. The biopsy taken would also, otherwise be thrown away if not included in the study, meaning that the procedure of collecting the biopsies for this study had no impact on the patients and their healing outcome. This means that the amount of tissue collected from each patient could vary greatly depending on how long post injury the operation was performed and how much scar tissue had been produced.

Additionally, the use of FBS, which is a common component in mammalian cell culture medium, is another ethical aspect that needs to be considered. The use of FBS for scientific purposes has been up for debate since the process of obtaining it involves taking blood from bovine fetuses, which is believed to cause discomfort and ends in the fetus dying [69]. However, the use of FBS is the standard for *in vitro* studies of mammalian cells and as of now, the best alternative [42]. The antibodies used in the immunofluorescence staining also have ethical issues as they are developed in mice, goats or rabbits, but as this type of production still is by far the most common and since these antibodies are most commonly used and well studied, they were also used in this study [70].

Lastly the ecological aspects that need to be considered include the use of antibiotics in the culture media and the large use of disposable plastic and glass material. Both of these aspects are well established challenges within the scientific community that are hard to completely eliminate as sterility is of the outmost importance in

research. In this project, only a limited amount of antibiotics was used and when it is used, the waste is handled according to the regulations of the institute. Also the disposable plastic and glassware was used as sparingly as as possible, while still not compromising on sterility.

4

Results

The results are presented in subsections for each of the methods utilized. The aims of this study were to investigate whether healing outcomes following a chronic Achilles tendon rupture can be linked to age related differences in (1) mechanical properties, and (2) at a cellular level, when utilizing the 3D fibrin hydrogel model to form tendon constructs. The results are therefore presented in terms of the two patient groups, where the young group include 6 patients younger than 55 and the old group include 7 patients older than 55.

4.1 Contraction of Artificial Tendon Constructs

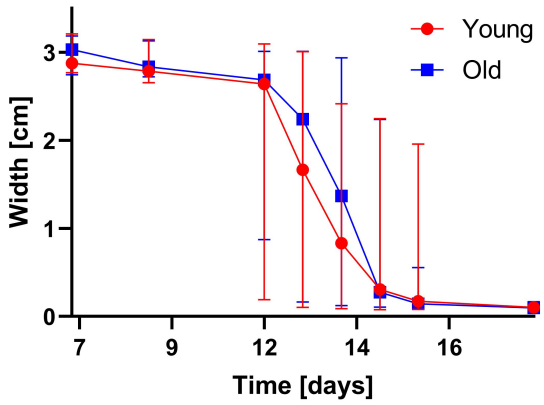
The contraction process is visualized below in figure 10, where representative images from gels formed by cells from one of the patient samples are displayed.

(a) *Day 7* ***(b)*** *Day 13* ***(c)*** *Day 19*

Figure 10: *Example of what the fibrin hydrogels look like as they gradually form tendon constructs. The width of the well is 3.5 cm and the distance between the sutures, and thereby also the length of the tendon construct, is approximately 1.5 cm. Pictured is one of the gels at (a) day 7, (b) day 13 and (c) day 19.*

The results of the contraction data for the two patient groups are presented in figure 11, where the width over time is shown in figure 11a and the contraction rate in figure 11b.

(a) Contraction



(b) Contraction Day

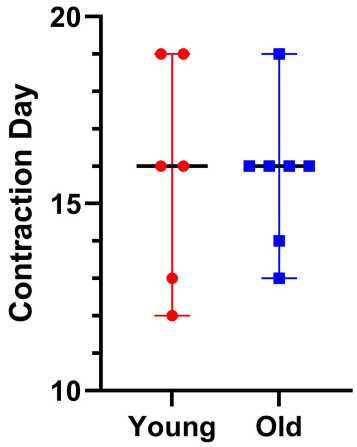


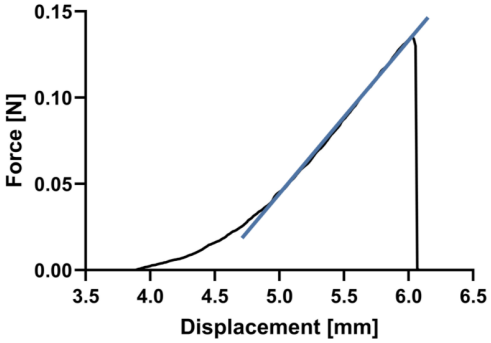
Figure 11: *Contraction of the fibrin hydrogels in the formation of tendon constructs. Shown are (a) the median width of the gels in both patient groups over time, and 95% confidence interval (CI), and (b) the day at which the constructs are fully contracted, including the median value and 95% CI.*

As can be seen in figure 11a, there were not any apparent visual difference in the contraction process of the gels. This was further validated in figure 11b, where no significant difference (p=0.999) was observed. The median contraction day of the young group in figure 11b was 16 days after seeding, with a range of 12-19 days. The median contraction day of the old group was also 16 days after seeding, with a range of 13-19 days. Correlation analysis between the day at which the constructs were fully contracted and age was also performed and is shown in figure 30 in Appendix B, where no correlation was found.

4.2 Mechanical Testing

To evaluate the mechanical properties of the tendon constructs, pull-to-failure tests were performed on triplicates of constructs for all patient donor samples and a mean of these triplicates was used for analysis. The Young's Modulus was calculated using the mechanical testing machine software and the stiffness was then calculated using the equations in section 2.6 and the obtained Young's modulus. An example of a force-displacement curve and a stress-strain curve created from data obtained from a mechanical test for one of the tendon construct is displayed in figure 12. From the force-displacement curve, shown in figure 12a, the max force is seen at the peak and the stiffness is calculated as the slope of the linear region within the curve. Similarly, the maximum stress can be seen at the peak in the stress-strain curve, displayed in figure 12b, where the Young's modulus also can be calculated as the slope within the linear region of the curve.

(a) *Force-Displacement Curve*



(b) *Stress-Strain Curve*

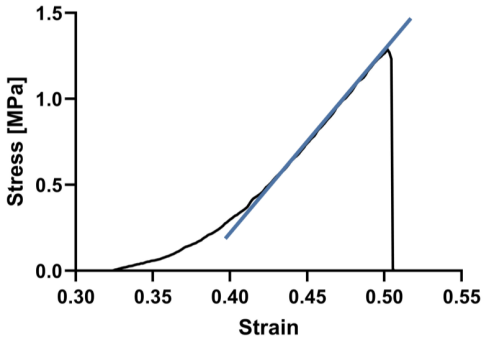


Figure 12: *Representative images of what the (a) Force-Displacement curve and the (b) Stress-Strain curve could look like. Shown in blue is also the slope within the linear region of the curve illustrating the stiffness and Young's modulus respectively.*

The resulting data from all of the performed mechanical tests can be seen illustrated in figure 13 where the maximum force, cross sectional area, maximum stress, maximum stiffness, and Young's modulus are shown.

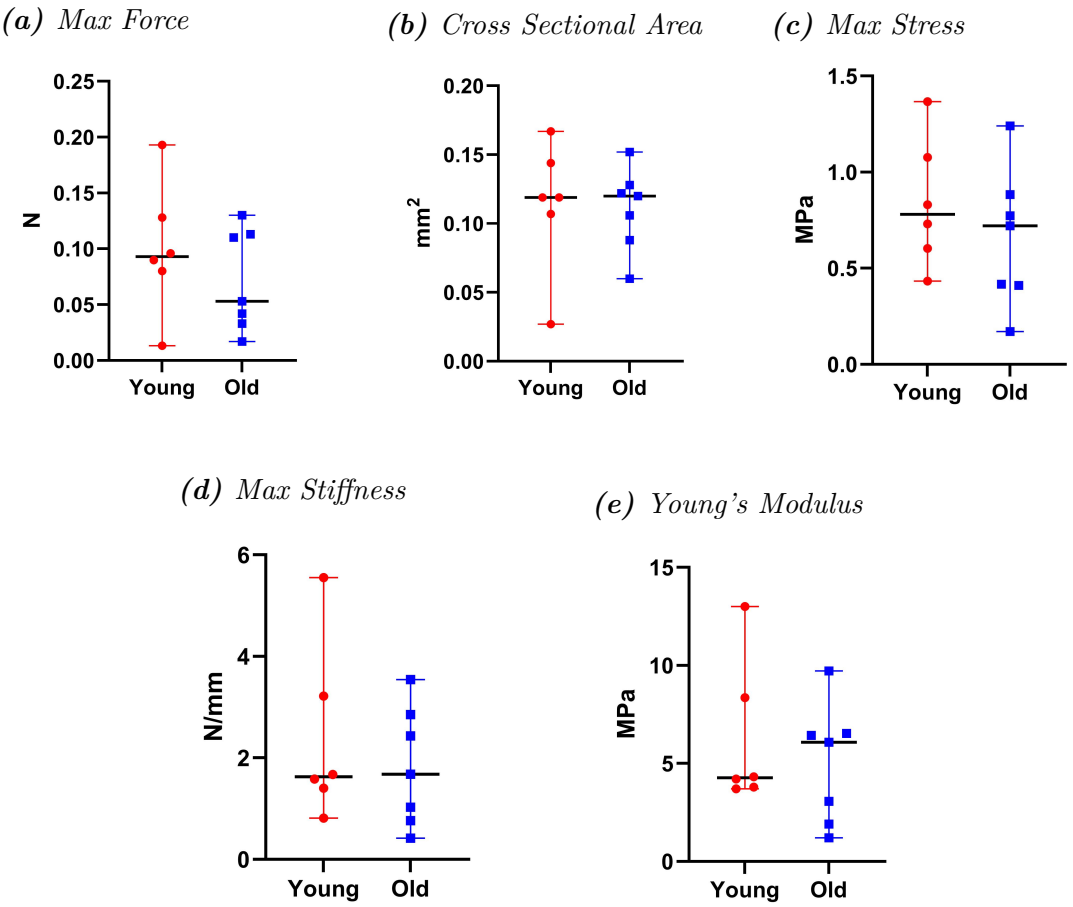


Figure 13: *Mechanical data of tendon constructs 28 days after seeding, including the median value for each test and the 95% CI. Displayed in (a) maximum force, (b) cross sectional area, (c) maximum stress, (d) maximum stiffness, and (e) Young's modulus.*

The median value of the maximum force in the young group was 0.093 N, with a range of 0.013-0.193 N and in the old group 0.053 N, with a range of 0.017-0.130 N, with no significant difference (p=0.628). The CSA for the young group had a median of 0.119 mm^2, with a range between 0.027-0.167 mm^2, and the old group had a median of 0.120 mm^2, with a range between 0.060-0.152 mm^2. There was no significant difference in CSA between the two groups (p=0.916). The median maximum stress for the younger group was 0.780 MPa, with a range of 0.433-1.367 MPa, and the median of the older group was 0.720 MPa, with a range of 0.170-1.240 MPa, which showed no significant difference (p=0.366). Maximum stiffness had a median value of 1.627 N/mm, with a range of 0.812-5.550, for the young group and a median value of 1.678 MPa, with a range of 0.418-3.541 MPa, for the old group, which means that there was no significant difference between the groups (p=0.731). Finally the median of the Young's modulus for the young group was 4.264 MPa, with a range of 3.710-13.010 MPa, and for the old group it was 6.080 MPa, with a range of 1.210-9.723 MPa, which means that there was no significant difference

(p=0.628). In conclusion, no significant differences were observed between the young and old patient groups for any of the mechanical properties evaluated. For all data there was a large variance, especially within the young group. Correlation analysis between each mechanical property and age are shown in figure 27 in Appendix B, where no correlations were observed.

4.3 Cell Health Assays

The resulting absorbance data for the CCK-8 assay, at day 7 and day 14, are displayed in figure 14a and 14b. Data displaying the enzymatic activity of LDH in the media, at day 7 and day 14, are presented in figures 14c and 14d.

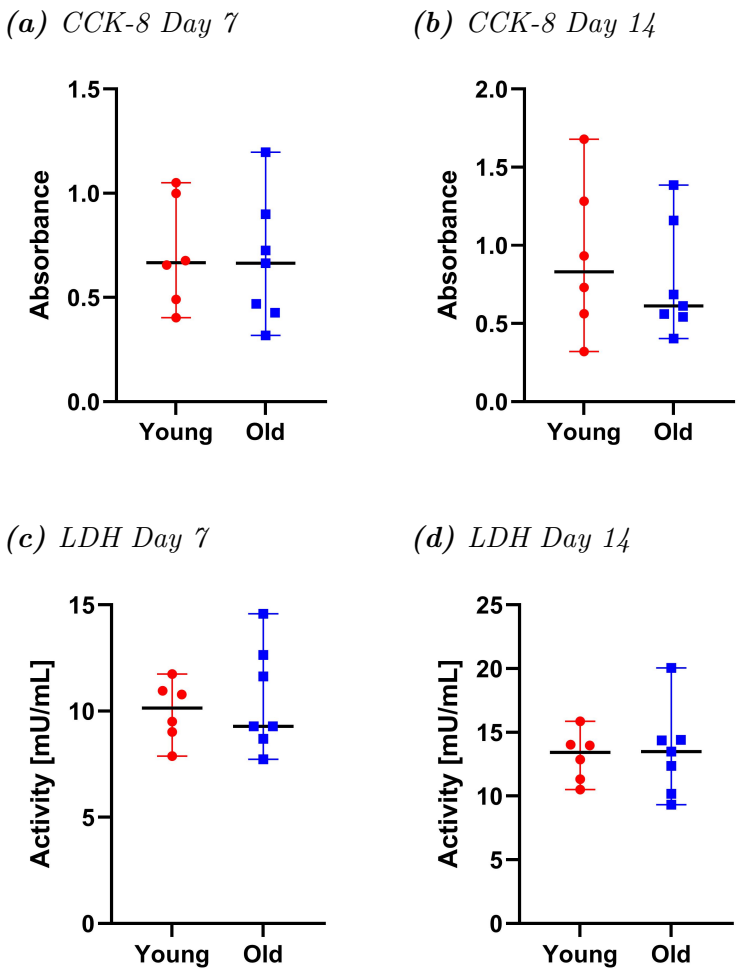


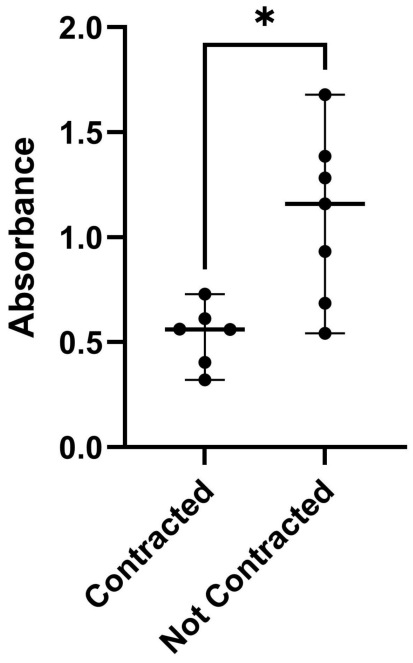
Figure 14: *Cell health assays including the median value and the 95% CI. Absorbance from the CCK-8 assay for both patient groups at (a) day 7 and (b) day 14 after seeding. Lactate dehydrogenase activity (mU/mL) from the LDH assay for both patient groups at (c) day 7 and (d) day 14 after seeding.*

For the CCK-8 assay, the median of the samples collected at day 7 in the young group was 0.667, with a range of 0.403-1.051, and in the old group 0.664, with a range of 0.319-1.196. For the second time point, at day 14, the median of the samples in the young group was 0.831, with a range of 0.321-1.679, and the median of the old group samples was 0.612, with a range of 0.404-1.385. There was no significant difference of the metabolic cell activity between the patient groups at either day 7 (p=0.836) or at day 14 (p=0.534).

In the LDH assay the median value of the samples at day 7 was 10.150 mU/mL, with a range of 7.878-11.740 mU/mL, for the young group and 9.277, with a range of 7.726-14.583 mU/mL, for the old group. At day 14 the young group had a median value of 13.420 mU/mL, with a range of 10.503-15.875 mU/mL, and the old group had a median value of 13.490 mU/mL, with a range of 9.332-20.052 mU/mL. There was no significant difference between the two patient groups at either day 7 (p=0.915) or day 14 (p>0.999).

Both CCK-8 and LDH data collected at day 14 was also analyzed in regards to contraction since some of the tendon constructs had fully contracted at this time point. The results are displayed in figure 15a and figure 15b.

(a) *CCK-8*



(b) *LDH*

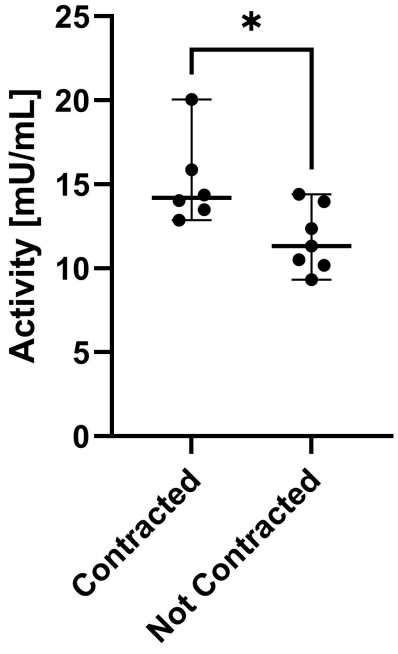


Figure 15: *Comparisons between contraction and the metabolic activity and cytotoxicity in the samples collected at day 14. (a) Displays the average absorption of the samples at day 14 divided into two groups, contracted and not contracted, based on microscopy pictures taken of the exact wells used in the CCK-8 assay. (b) Displays the activity of lactate dehydrogenase in the same way as the metabolic activity.*

Significant differences were observed between the contracted and the not contracted samples in both absorbance from the CCK-8 assay (p=0.022), as well as in the activity measured in the LDH assay (p=0.035).

4.4 Wound Healing Assay

In the wound healing assay each measurement was done manually and an example of the measurements is shown in figure 16, where the newly created wound is displayed in figure 16a and then the progression of the wound closing at first 8 hours after the wound was created in figure 16b and finally when it is closed to a degree that makes measurement impossible after 24 hours in figure 16c.

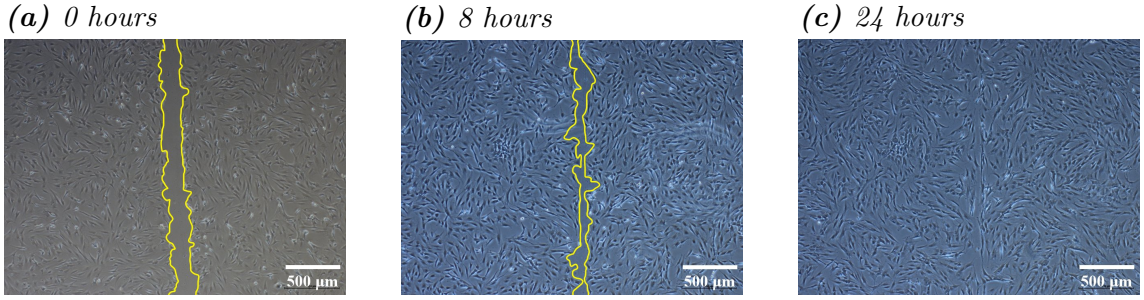


Figure 16: *Examples of images taken of the wound healing assay and how the measurements in ImageJ looked like in 4x magnification. Pictured above are cells from one representative patient sample at (a) 0h, (b) 8h and (c) 24h after the scratch was made.*

In the representative picture of the wound healing process above it can be seen that the cells has not entirely migrated and closed the wound after 8 hours, which is something that none of the samples did. The progress of wound closing was monitored and measured at regular time points from the time that the wound was created until 8 hours after the scratch was made. The results of the wound healing assay can be seen in figure 17, where the wound closure over time is displayed in figure 17a and the final wound closure at 8 hours in 17b.

(a) Wound Closure over Time *(b) Wound Closure at 8 Hours*

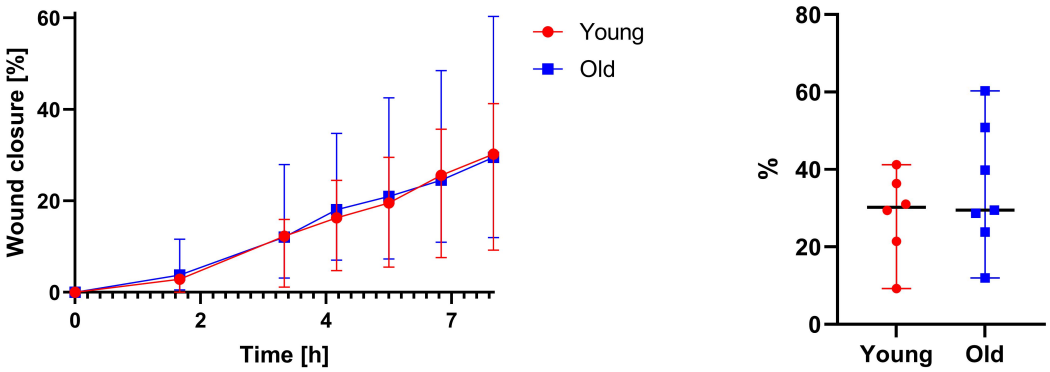


Figure 17: *Wound Healing Assay, where (a) shows the median value of the wound closure in percentage over time, with 95% CI, and (b) the wound closure in percentage at 8 hours after the scratch was made, including the median and 95% CI.*

In figure 17a it can be seen that the wound closure data closely resembled each other between the two patient groups. This was validated in figure 17b, where the median in the young group was 30.24%, with a range of 9.24-41.23%, and the median in the old group was 29.51%, with a range of 11.97-60.32%, which showed no significant difference (p=0.628). There was however a correlation between the original wound size and the wound closure (p=<0.0001), which can be seen in Appendix B, in figure 28.

4.5 Gene Expression Analysis

The mean of the normalized and logarithmically transformed data from the gene expression analysis, along with the SD, at day 7 is presented in table 2 and at day 14 in table 3. The presented p-values in both tables were calculated using an unpaired two-tailed Student's t-test.

Table 2: *Gene expression at day 7 after seeding. The data presented in the table is normalized to two reference genes, TBP and B2M, and logarithmically transformed.*

Genes	Group	Mean	SD	p-value
COL1A1	Young	1.853	0.228	0.761
	Old	1.820	0.149	
COL3A1	Young	3.609	0.186	0.996
	Old	3.608	0.207	
COL1A1/COL3A1	Young	-1.756	0.058	0.609
	Old	-1.788	0.140	
COL5A1	Young	1.203	0.157	0.957
	Old	1.198	0.152	
MMP-1	Young	-0.400	0.272	0.183
	Old	-0.211	0.208	
MMP-13	Young	0.532	0.558	0.922
	Old	0.505	0.443	
FMOD	Young	2.143	0.204	0.606
	Old	2.096	0.108	
DCN	Young	1.518	0.108	0.292
	Old	1.446	0.124	
BGN	Young	1.439	0.197	0.537
	Old	1.491	0.083	
ACTA2	Young	1.151	0.222	0.440
	Old	1.246	0.210	
S100A4	Young	0.627	0.122	**0.033***
	Old	0.466	0.115	
SCX	Young	0.233	0.404	0.329
	Old	0.049	0.240	
SPARC	Young	0.925	0.179	0.517
	Old	0.867	0.135	
EZR	Young	-0.179	0.130	0.059
	Old	-0.324	0.118	

Table 3: *Gene expression at day 14 after seeding. The data presented in the table is normalized to two reference genes, TBP and B2M, and logarithmically transformed.*

Genes	Group	Mean	SD	p-value
COL1A1	Young	1.980	0.145	0.860
	Old	1.994	0.133	
COL3A1	Young	3.664	0.176	0.349
	Old	3.748	0.135	
COL1A1/COL3A1	Young	-1.684	0.044	0.133
	Old	-1.755	0.098	
COL5A1	Young	1.222	0.181	0.307
	Old	1.309	0.108	
MMP-1	Young	-0.533	0.565	0.333
	Old	-0.204	0.599	
MMP-13	Young	1.289	0.382	0.880
	Old	1.326	0.466	
FMOD	Young	2.349	0.175	0.922
	Old	2.340	0.142	
DCN	Young	1.731	0.046	**0.027***
	Old	1.664	0.047	
BGN	Young	1.572	0.210	0.378
	Old	1.650	0.077	
ACTA2	Young	1.145	0.161	0.566
	Old	1.225	0.297	
S100A4	Young	0.130	0.128	0.550
	Old	0.056	0.266	
SCX	Young	0.436	0.405	0.447
	Old	0.613	0.402	
SPARC	Young	0.986	0.122	0.777
	Old	1.007	0.142	
EZR	Young	-0.491	0.081	0.730
	Old	-0.518	0.171	

The genes most relevant for new tissue formation are presented in figure 18. These include COL1A1 and COL3A1, that encodes for collagen type I and collagen type III, as well as the ratio between the two, indicating the progress of new tissue formation. Moreover, MMP-1 which encodes for an enzyme that degrades collagen is also presented.

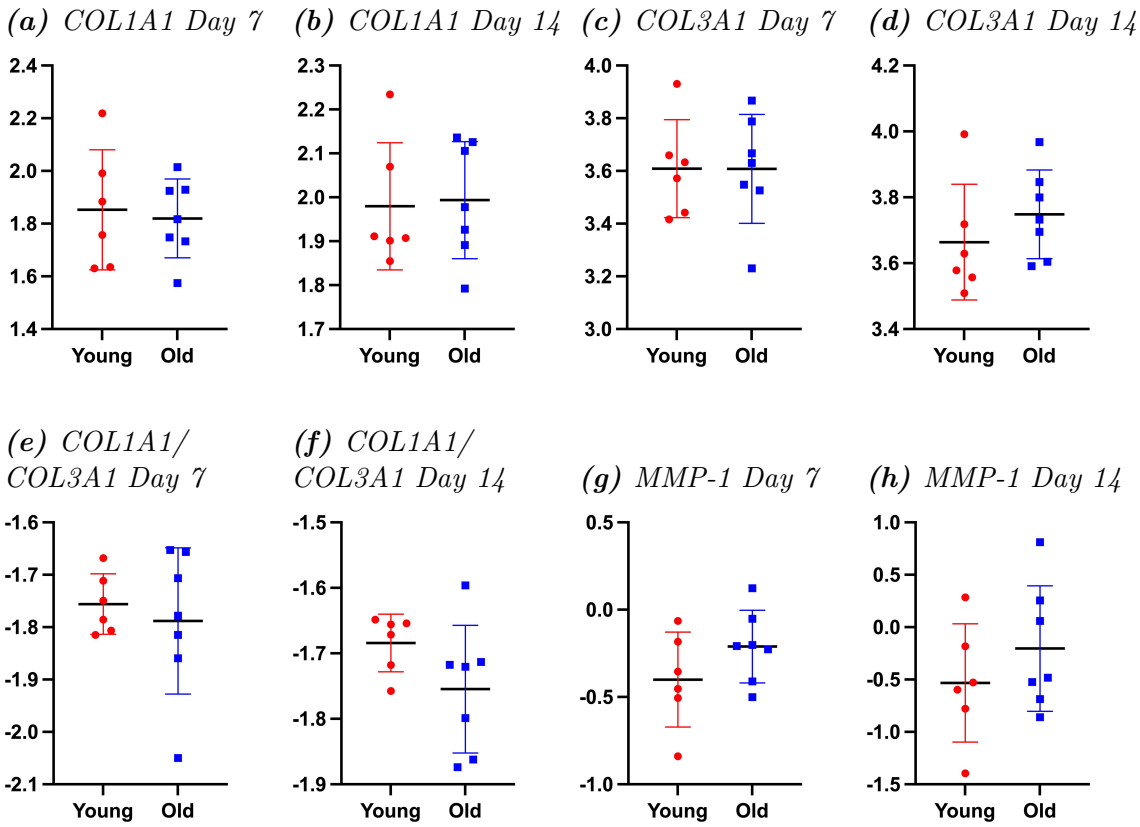


Figure 18: *Gene expression data, with the mean and SD, for (a) COL1A1 at day 7, (b) COL1A1 at day 14, (c) COL3A1 at day 7, (d) COL3A1 at day 14, (e) COL1A1 and COL3A1 ratio at day 7, (f) COL1A1 and COL3A1 ratio at day 14, (g) MMP-1 at day 7, and (h) MMP-1 at day 14.*

The genes that were differently expressed between the two age groups, DCN and S100A4, are displayed in figure 19, together with EZR that had a p-value of 0.059 at day 7 and SCX which is an important marker for tenocytes.

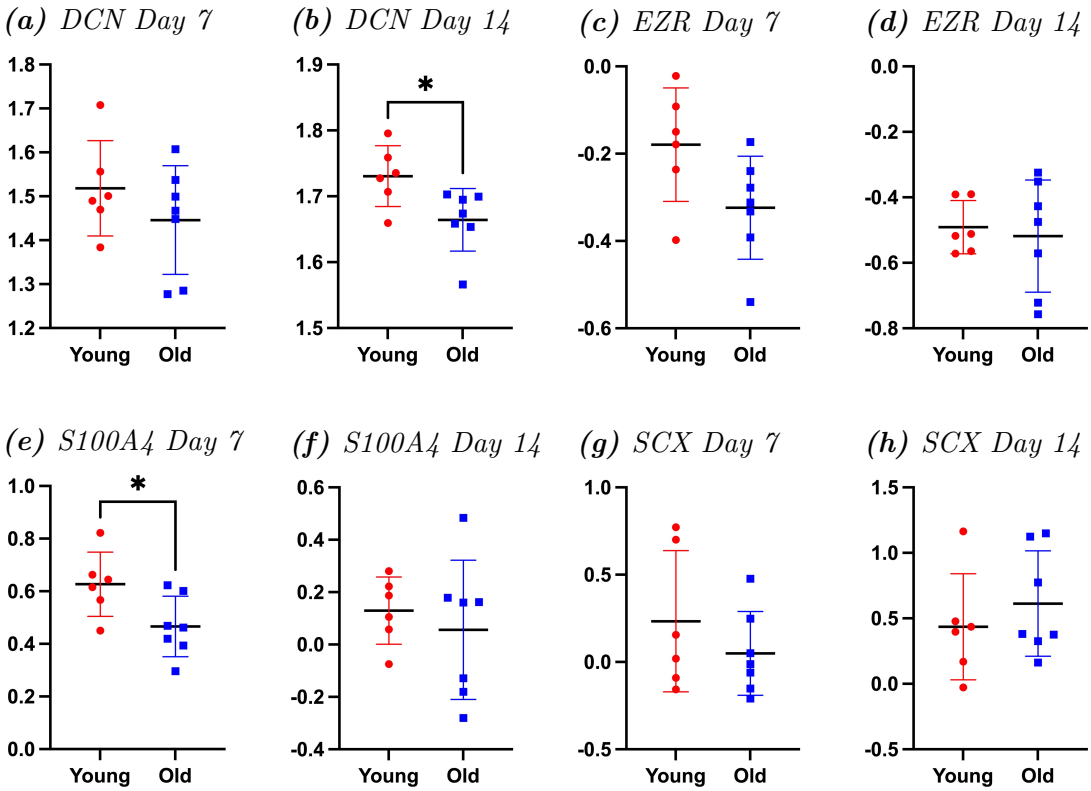


Figure 19: *Gene expression data, with the mean and SD, for (a) DCN at day 7, (b) DCN at day 14, (c) EZR ratio at day 7, (d) EZR ratio at day 14, (e) S100A4 at day 7, (f) S100A4 at day 14, (g) SCX at day 7, and (h) SCX at day 14.*

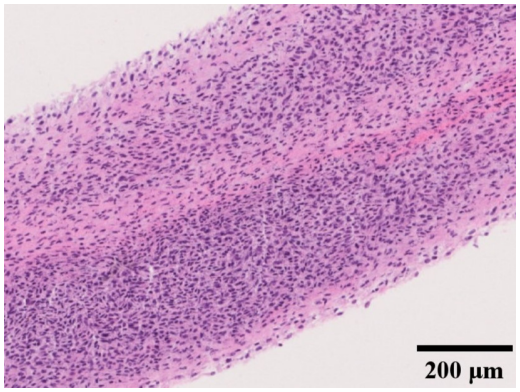
4.6 Histological Analysis

All samples collected at day 7, day 14 and day 28 have been stained for nuclear morphology and cell density using H&E and PG content using Alcian blue. However, only data collected at day 28 is presented in the following sections as the samples collected at the other time-points proved difficult to section and analyze in an equivalent manner. This was due to the fact that many of the constructs had not yet contracted and that the gels were divided in two, meaning that only a limited amount of tissue could be visualized on each slide. Due to the same reasons, collagen type I and type III staining was also only performed on samples collected at day 28 and thus is the only data presented below.

4.6.1 H&E Staining

Representative pictures of what the H&E staining looks like can be seen in figure 20, as well as how the Qupath software used has classified the cells.

(a) H&E Staining



(b) Nuclei Classification

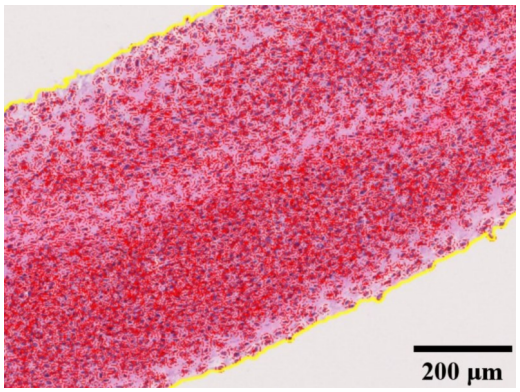
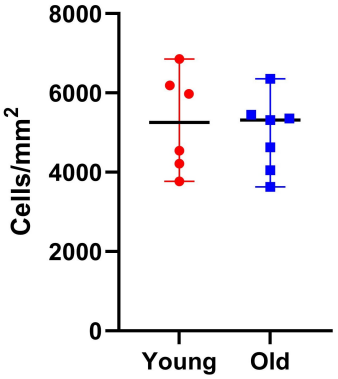


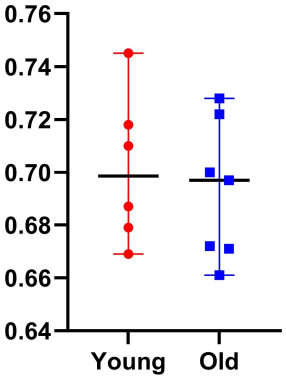
Figure 20: *Representative pictures of (a) H&E staining and (b) Qupath classification of cell nuclei.*

The results from the H&E staining can be seen in figure 21, including the cell density in figure 21a, the cell circularity in 21b, and the cell eccentricity in 21c.

(a) Cell Density



(b) Cell Circularity



(c) Cell Eccentricity

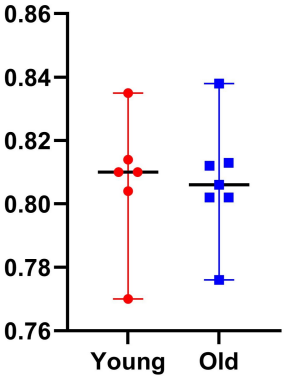


Figure 21: *H&E staining of tendon constructs 28 days after seeding with median and 95% CI also displayed in the graphs showing, (a) cell density (cells/mm^2), (b) cell circularity, and (c) cell eccentricity.*

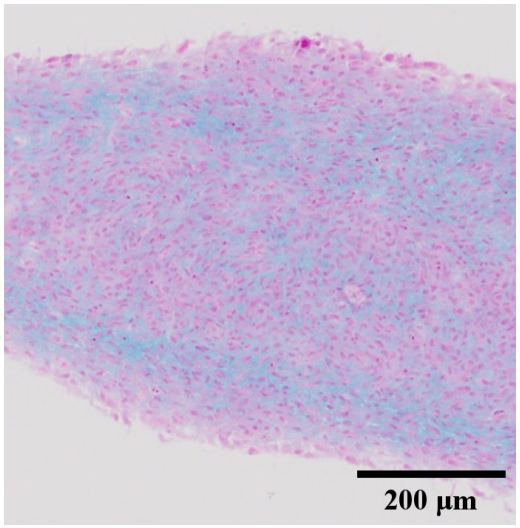
There was no significant difference between the two patient groups in cell density, with a median of 5260 cells/mm^2 for the young group and values ranging between 3768-6189 cells/mm^2 and a median of 5317 cells/mm^2 for the old group and values ranging between 3629-6355 cells/mm^2 (p=0.731). No significant difference was found in the cell circularity either, with a median of 0.699 for the young group and

values ranging between 0.669-0.745 and a median of 0.697 for the old group and values ranging between 0.661-0.728 (p=0.731). The cell eccentricity also showed no significant difference between the age groups, with a median of 0.809 for the young group and a range of 0.770-0.835 and a median of 0.806 for the old group and a range of 0.776-0.838 (p=0.809).

4.6.2 Alcian Blue Staining

Representative pictures of the Alcian blue staining can be seen in figure 22, together with how the threshold created in the Qupath software was used to measure the area positively stained for proteoglycans.

(a) *Alcian Blue Staining*



(b) *Proteoglycan Threshold*

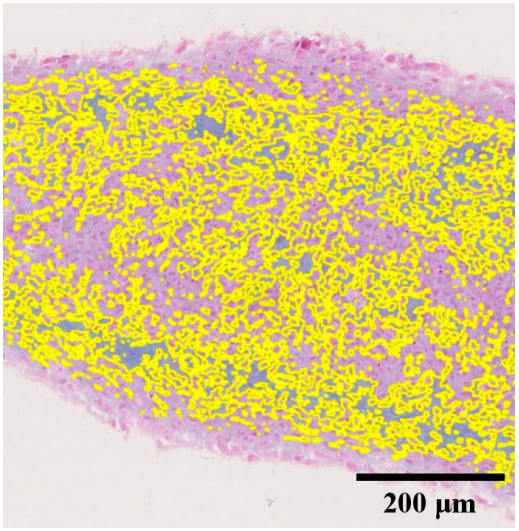


Figure 22: *Representative pictures of (a) Alcian blue staining and (b) Qupath threshold for area positively stained for proteoglycans.*

Results from the Alcian blue staining is presented in figure 23, as a percentage of the total tissue area.

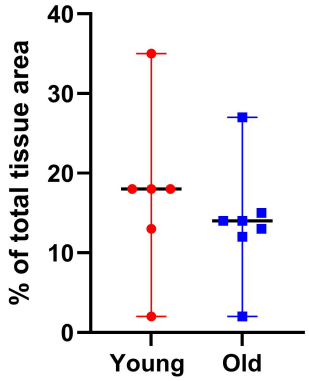


Figure 23: *Percentage of total tissue area stained with Alcian blue for both age groups at day 28 after seeding, including the median value and 95% CI.*

The area positively stained for proteoglycans in the young group had a median value of 18%, with values ranging between 2-35%, and the old group had a median value of 15%, with values ranging between 2-27%. No significant difference between the patient groups could be seen (p=0.342).

4.6.3 Immunofluorescence

The immunofluorescent staining included, as previously mentioned, DAPI to stain the nuclei as well as antibodies to stain collagen type I and type III. Representative images for all three are displayed in figure 24, showing the same location of the same sample but using different channels of the fluorescence microscope and picture editing to make the new tendon tissue structure more visible.

(a) *Collagen Type I* ***(b)*** *Collagen Type III* ***(c)*** *DAPI*

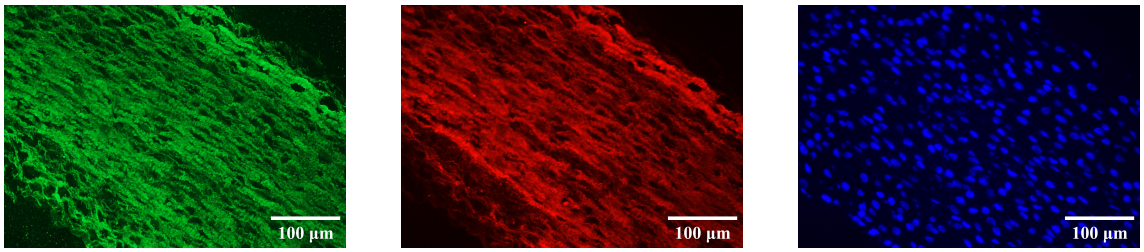


Figure 24: *Examples of the immunofluorescent staining at 20x magnification for one representative sample of (a) collagen type I, (b) collagen type III, and (c) nuclei stained with DAPI.*

The quantification from the immunofluorescent staining can be seen in figure 25, where it is displayed as the ratio between collagen type I and type III (Col I/Col III). Three samples, one young and two old, were excluded from the analysis due to errors during the staining procedure.

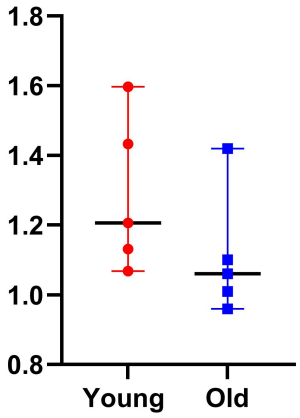


Figure 25: *Ratio between positively stained collagen type I and type III area calculated using a pixel intensity threshold, including median and 95% CI.*

The median value for the younger group was 1.207 with values ranging between 1.068-1.597 and the median value for the ratio in the older group was 1.057 with values ranging between 0.964-1.420. There was no significant difference in Col I/Col III ratio between the patient groups using the pixel threshold (p=0.095).

5
Discussion

The incidence of Achilles tendon ruptures and the median age at the time of injury has increased during the last decades [1]. At the same time functional outcome post injury have been shown to decline with age [4]. It is therefore of great importance to study age-related differences in the formation of new tendon tissue at a cellular level. The aim of this project was therefore to utilize primary human tendon cells in a 3D fibrin hydrogel *in vitro* model to investigate age-related mechanical and biological differences. The results obtained indicate that the mechanical properties of the tendon constructs were not influenced by age. Similar results were found in the majority of the biological properties of the cells in the tendon constructs, with the exception of gene expression of DCN and S100A4, where the younger group had a higher expression of both genes. The results are further discussed in the following sections.

5.1 Mechanical Test

We found no significant difference between the two patient groups in any of the mechanical properties (max force, CSA, max stress, max stiffness and Young's modulus) studied. Previous studies present conflicting results when it comes to the correlation between age and mechanical properties [71], [72], [73], [74]. The inconsistency between studies may, as previously mentioned, be attributed to differences in experimental setup, i.e. if it was performed *in vitro*, *in vivo*, or *ex vivo*, and which model organism that was utilized. Furthermore, there are studies investigating age-related differences in healthy Achilles tendons [71], [72], but the same coverage is lacking when it comes to assessment of differences in healing capabilities. These are important considerations when comparing and interpreting results across different studies. By the utilization of the 3D fibrin hydrogel model used in the present study, the cells' abilities to form new tissue following a chronic ATR is assessed, and more specifically how the mechanical properties may be influenced by the age of the individual from where the cells were obtained. It is also worth mentioning that although the mounting and loading of the tendon constructs were performed using the same protocol and was carried out by the same person, some variance could have occurred due to the difficulty of handling such small samples. For example, this could have caused some of the constructs to be harmed by either stretching or other types of damage prior to starting the test, which need to be taken into consideration when discussing the results. However, the constructs were handled very carefully and each

patient donor was evaluated in triplicates of tendon constructs to minimize getting faulty results caused by mishandling.

The maximum tensile force that the tendon constructs could withstand before breaking was one of the properties investigated, which had no significant difference between the two groups. These findings contrast a previous *in vivo* study that investigated age-related differences in intact Achilles tendons between young and elderly women [75]. They found that Achilles tendons in the younger group could exert higher tensile forces compared to the older group. However, compared to our study where tendon constructs were formed by cells extracted from tissue in the healing process, the study mentioned above investigated intact and healthy tendons. Additionally, the study was performed *in vivo* making direct comparisons difficult. In another study that instead investigated age-related differences in healing outcome between young and aged flexor digitorum longus (FDL) tendons in mice, it was found that the aged group had impaired results in the mechanical tests compared to the young group [76]. More specifically, they found a decrease in both max load and stiffness in aged tendons 14 days post-injury compared to the young group. However, this study was performed on mice that have very homogeneous genetic makeup and living conditions. They also divided them into age groups with a distinct age gap, 4 and 22 months old respectively, which makes possible differences more evident. Additionally, the tendon they studied is not the mice equivalent of the human Achilles tendon and the healing response can thereby differ. Lastly, animal studies are difficult to compare with as the difference in life-span of animals and humans may influence how aging affects tendons.

In rats it was shown that the group consisting of middle-aged rats had inferior mechanical properties in healing tendons, compared to both the younger and the older group [77]. The maximum stress in the injured region was reduced in the middle aged group and also the modulus showed a greater heterogeneity across the tendon in this group. This differs from the steady decrease in mechanical properties that have been seen in studies on intact human tendons [5]. The present study only included two groups, young and old, to study age-related differences. However, there was no correlation between any of the mechanical properties and age and no visual drop could be seen for middle-aged patients either.

From previous *in vivo* studies, the Achilles tendon CSA of healthy tendons has been shown to increase with age [5], [75], which is not seen in the results from this study. However, it is still unclear what the reason for the findings of an increased CSA with age in previous studies could be attributed to. It is argued that it could be due to an increase of extracellular water content as compensation for decreased loading of the tendon, which often is the case with rising age [78]. Regardless of the reasons, these studies have been performed *in vivo* whereas in this study, the cells are extracted and utilized in the 3D fibrin hydrogel model which has not been subjected to any loads. This means that the mentioned parameters are more or less removed and that the CSA of the tendon constructs formed could rather be attributed to e.g. the collagen production. The mRNA levels of collagen in aged versus young Achilles

tendon have previously been shown to be downregulated [8], which in the case of this study would imply that a smaller CSA could indicate an older age of the patient donor. However, the results presented in this study shows no significant difference in measured CSA of the tendon tissue formed from young versus old patients. Further, no correlation between CSA and increasing age was found in this study.

When it comes to age-related differences in stress, stiffness and Young's modulus of intact Achilles tendons, they have been shown to decline with age when comparing young adults with middle-aged *in vivo* [5]. Such a decline was not seen in this study. This indicate that neither the structural integrity of the entire tendon construct nor the quality seem to decline with the age of the patient from which the cells came from. However, the differences in results could be attributed to the fact that they investigated intact Achilles tendons, whereas this study focused on newly formed tendon tissue generated by cells extracted from a healing tendon.

To conclude, there is no consensus when it comes to age-related differences in mechanical properties of the Achilles tendon. Furthermore, there is a lack of studies made in humans, either *in vitro* or *in vivo*, investigating age-related differences in healing capacity between young and old patients. To the authors knowledge, this is the first study investigating the healing process following a chronic Achilles tendon rupture using human donor samples. The results indicates that there is no significant difference between the newly formed tendon tissue under non-loading conditions of the young and old patient group, in none of the mechanical properties investigated. Furthermore, these findings suggest that the poorer healing outcomes observed in older patients with chronic ATRs are not due to a reduced ability of the cells to produce repair tissue. The mechanical test results indicate that the capacity to form repair tissue does not decline with age, which is an encouraging outcome from a patient perspective. However, further research needs to be made, including larger patient groups to validate the results found in this study.

5.2 Biological Tests

In contrast to the specific effects of the tendon mechanical properties and the structural differences that arise with age, the cellular changes are well studied and in many ways well understood [6], [7], [79], [80], [20], [81], [82]. However, there are still gaps in the knowledge when it comes to changes in tenocytes and the effect these changes have on the structure and properties of the Achilles tendon. Overall cell health is a commonly studied biological property that can be investigated in many different ways. In this study it was done by looking at the metabolic activity and the cytotoxicity which revealed that there was no significant difference between the young and the old group in none of these parameters. Another biological property that, just as the cell health, is important to healing and have previously been shown to alter with age is cell migration [20]. However, in our study no significant difference between the two age groups was found. The same can be seen when assessing the day at which the constructs had fully contracted, where no significant difference could be observed either.

By the histological analysis of the tendon constructs both the nuclear morphology, cell density and the collagen type I and type III content was investigated. Neither the circularity, eccentricity nor the cell density was significantly different between the two age groups. The collagen type I and type III ratio also revealed no significant difference between the age groups in the immunofluorescence staining. Gene expression also revealed that there is no significant difference in the expression of COL1A1 and COL3A1, including the ratio between the two. The other genes investigated showed similar results with no difference in expression, except for DCN and S100A4. Below all of these results are discussed in more detail and also possible sources of error are listed for each method when relevant. However, there are also some potential sources of error that could influence the results obtained from all the biological methods listed below and these include pipetting errors and a lack of sterility when working in the cell lab. These could influence the results in many different ways but the effect of pipetting errors were minimized by including both biological and technical replicates and the risk of contamination was reduced by adding antibiotics to the growth medium and by always working according to guidelines and protocols to maintain sterility. Additionally, no contamination was observed in any of the cells handled in the lab over the duration of this study.

The contraction rate is mainly determined by the organization of the actin cytoskeleton as it is not actual migration that causes the gel to contract, but rather the cells themselves becoming stiffer and creating tension between the two sutures, forming a construct. Previously it has been shown that the actin content increase with age [79], but that it becomes less organized and the filaments become shorter [20]. Since the actin content increase with age it was expected that the contraction rate also would increase, however no significant difference between the two age groups was observed. One explanation behind this could be the use of different FBS batches throughout the study, which is known, from previous observations within the research group, to determine whether the gel can contract. Therefore, batches that had been previously tested were the only ones used in this study. Since it is known that the batch determines whether the hydrogel will contract, it is not unlikely that it also could influence how fast it does contract. However, what determines whether the gel contracts or not is unknown and is hard to define since the components of the FBS in itself is undefined. Because of this it would have been ideal to use the same batch for all the samples. However, due to the small volume of FBS in each batch, this was not possible but will be taken into account for future studies. This introduces variation between the samples which could mask potential differences between the two patient groups.

The metabolic activity was also similar in the two groups at both day 7 and 14. It was however observed that the variance within both patient groups increase in the samples collected at day 14 which was proven to be due to the variation in how contracted the samples are. The measured metabolic activity was significantly higher in the patient samples that had not yet contracted and formed constructs. In native tendon tissue, the cells relies on mechanical loading for diffusion of synovial

fluid to get nutrients and oxygen whereas the tendon cells in the model, which does not experience any mechanical loading, only relies on diffusion from the surrounding media. Therefore, the radius of the tendon constructs could influence how well nutrients are distributed to the cells residing in the middle of the constructs, and thus influence the metabolic activity of the cells. This could help in explaining the lack of significant difference found between the young and old patient group, at least at day 14, which was unexpected as previous studies have shown a reduced metabolic activity with age [79], [80]. These previous studies were however performed in rat tendon stem/progenitor cells (TSPCs) which ensures a much more homogeneous cell population and TSPCs could also be disproportionally effected by aging, making it easier to detect subtle differences. They were also performed on cells extracted from healthy tendons which is another factor that could explain why our findings differ from theirs. To truly investigate whether these differences also could be observed in human tendon cells extracted from healing tendon tissue it would therefore be necessary to find a way to account for the variation introduced by the differing contraction rate. However, since there was no significant difference even at day 7, where the contraction rate should have less impact, the lack of difference could also just reflect the actual conditions within the healing tendon. To further validate these findings other assays could also be included in future studies, e.g. EdU that instead specifically look at proliferating cells.

The other cell health assay that was performed, a lactate dehydrogenase assay that measures cytotoxicity, did not show any differences between the age-groups. However, it can be observed that the median activity for both groups increased at day 14 but, whether this is a significant difference is unknown as no statistical evaluation have been performed between the time-points. Since the LDH assay reports on the presence of the LDH enzyme, which is a cytosolic enzyme, in the media it indirectly reports on the level of cell death. This indicates that age does not influence cell death but, that the longer the cells reside within the model does. Cell death have previously been shown to increase with age in TSPCs [6], [7], which contradicts these findings. Both of these studies were performed *in vitro* using human TSPCs. The young samples were taken from completely healthy individuals and the old samples were taken from degenerated tissue. This means that it is quite difficult to compare the data presented in these studies to the data obtained in our study as it is healing tissue that is being studied and an exclusion criteria listed for this study is that the tendon should not show any signs of tendinopathy.

Cell migration is studied in the wound healing assay, by looking at how fast the cells migrate to close an artificially created wound. There was no significant difference in the migration rate between the young and old patient group, and no correlation could be found between the migration rate and age. This was unexpected as it has previously been well established that cell motility decrease with age [79], [20], [6], [7]. The motility of the cells is highly dependent on the actin cytoskeleton and its organization [81], which is a property that is also believed to change with age [20]. It has previously been shown that the actin cytoskeleton becomes less organized and that the filaments become shorter with age, which influences the function of

the FAs that are crucial for migration. Because of this extensive evidence it was believed that there would be a difference between the two groups. One possible explanation behind this lack of difference is that a lot of variation was introduced during the scratching. The original wound area varied greatly between samples and it was found that there is a correlation between the migration rate and the original area. This means that some of the potential differences with age could be masked by the variation introduced by using this method to measure migration rate. For future studies it would therefore be beneficial to look into using a different type of migration assay or to develop a way to standardize the created wound. The varying size of the original wound could however also be explained by inherent biological differences, including proliferation and adhesion. Slower proliferation would create a less confluent cell layer which could lead to an uneven wound that would be harder to accurately measure. The proliferation would however not influence the measured migration rate during the actual test as the migration is only monitored during eight hours which is much shorter than the doubling time of the cells. Weaker adhesion would instead cause more cells to detach during scratching, creating a bigger wound from the start and thus influencing the migration rate.

Cell morphology was also similar in the two groups in all of the analyzed parameters, circularity, cell density and eccentricity. In previous studies it has been shown that aged tenocytes generally have a more rounded shape [20], [6], [7]. It has however also been shown that stretching of the tissue causes remodeling of the nuclear shape [83], and since it is assumed that the shape of the cell is the same as the shape of the nuclei in this study, this could have a big influence on the results. Prior to freezing, the constructs were cut from the sutures which, when the tension is removed, causes the construct to contract. This would cause the nuclei to begin remodeling and thus could provide inaccurate results during staining as the cells might have already remodeled to adapt to the new environment. The same studies also showed that the cell density decrease with age [20], [6], [7], but none of them take healing into account which could have an impact on the cells' ability to proliferate.

The change in expression of collagen type I and collagen type III is an essential part of tissue remodeling following tendon injury [84]. The ratio of the two is hence an important factor to investigate and determine how well the tendon cells form new tendon tissue and may also indicate how far in the healing phase they have come. From a previous study, they used immunohistochemical staining to determine protein expression of collagen type I and type III in healing patellar tendons in young and aged rats. Their results revealed that the levels of collagen type I was the same for both young and old rats, but for collagen type III there was an increase in the younger group [85]. This implies that the young group would be expected to have a lower Col I/Col III ratio compared to the old group. However, this was not assessed in their study, making it difficult to draw conclusions. Due to the proved better functional outcomes in younger patients following an ATR [4], [86], we hypothesized that the cells from the younger group would have a faster conversion from containing more collagen type III to containing more collagen type I, leading to a higher Col I/Col III ratio in that group. However, analysis of the

ratio from the immunofluorescent staining revealed that there was no significant difference between the young and old patient groups (p=0.095). This indicates that there is no age-related difference in how fast cells convert collagen. But it is worth mentioning that possible time difference between injury and surgery for each patient also could influence how the cells behave. Additionally, surrounding cells, such as immune cells, can have an impact on ECM production in the native tissue which is eliminated in the use of the 3D fibrin hydrogel model and this could explain the lack of significant differences in terms of COL1A1, COL3A1, and COL5A1 expression as well as the Col I/Col III ratio. We also had less samples in these tests since three samples had to be excluded, which increases the risk of type 2 errors, meaning that there is a risk that there could have been a significant difference that was missed due to the low number of samples that were analyzed.

Gene expression analysis revealed that there was two significantly differently expressed genes, DCN at day 14 and S100A4 at day 7, and the rest showed no difference between the age groups. All of the studied genes are in different ways connected to new tendon tissue formation, meaning that even the genes that did not show a significant difference in expression also provide valuable insights. The three types of collagen being studied all have different roles in the tissue formation process, and their expression could thus be used to determine how far this process has progressed. Since there was no significant difference between the age groups of either COL1A1, COL3A1 or COL5A1 expression, the tissue formation seems to progress at a similar rate in both groups using this model, which was further validated from the results of the immunofluorescent staining in this study. This is contrary to what previous studies have shown where COL1A1 and COL3A1 have decreased in aging mice tendons [61], and the expression of COL5A1 have instead been shown to increase [13]. Both of these studies were done in healthy mice however, which does not accurately reflect the conditions in a healing tendon and could thus explain the lack of difference observed in this study. The up regulation of collagen production could therefore be similar in all ages during new tendon tissue formation, even though the levels within the healthy tendon have been shown to change with age. Another study found that this was partly true, as it showed that there was no significant difference in COL1A expression with age during healing [85]. However, no gene expression analysis was done for COL3A1 or COL5A1, making it hard to compare these results to the ones obtained in this study.

From the results it was observed that there was not a significant difference in gene expression of MMP-1 or MMP-13 between the patient groups. In a recently published study, differences in mRNA levels of human tendon cells following acute, short-term chronic, and long-term chronic ATRs were investigated [87]. Here they found significantly higher mRNA levels for MMP-1 and MMP-13 in the acute group compared to the long-term chronic group. This study did not include age as a factor, meaning it could be interesting to investigate age-related differences at the acute stage following an ATR. In a study that did investigate age-related differences in healing, they examined young and old rat Achilles tendons 2 and 4 weeks after injury through RT-qPCR and immunohistochemical staining. They found higher

protein expression levels of MMP-1 in young rats compared to old [85]. However, as previously mentioned, it can be difficult to make direct comparisons to age-related studies in animal models since differences could be due to maturation rather than aging.

The gene expression for the different SLRPs, FMOD, DCN and BGN, all having a role in matrix assembly and regulating collagen fibril diameter [50], revealed that the only one that exhibited a significant difference between the age groups was DCN at day 14 after seeding. Alcian blue staining, which reveales overall proteoglycan content, however showed that there was no significant difference between the age groups. Previously it has been shown that FMOD and BGN did not show an altered expression with age in rats [85], [88], which aligns with our results. Another study investigating human patellar tendons also revealed that the BGN expression was not altered with age, and the same applied to DCN [89]. Both the effect of BGN and DCN expression on healing have been investigated in mice where these genes have been knocked out and shown to have varying effects on healing [90], [91], [92]. Some of the studies showed improved healing in the DCN knockdown mice, while others showed improved healing in the BGN knockdown mice. An increase of FMOD have instead been shown to improve tendon healing in rats [93]. Neither of the previously mentioned studies have compared healing in younger and older animals and did not investigate whether the healing process had an effect on the gene expression which is what is being studied here. These studies however prove that SLRPs have an effect on healing and in this study it was found that the only differently expressed SLRP was DCN, implying that this gene would be interesting to investigate further in future studies. It is somewhat surprising though that this increased gene expression of DCN was seen in the younger patient group that is generally associated with better healing. However, this increase was only seen at day 14 after seeding, suggesting that DCN might have an important function during some parts of the new tissue formation process that need to be studied further.

The S100A4 gene encodes for a calcium binding protein which is involved in fibrotic tendon healing [51]. The ACTA2 gene encodes for actin proteins and is associated with cells exhibiting similar features as smooth muscle cells [51]. In a previous study performed in mice, it was observed that S100A4 knockdown led to improved tendon function post-injury and an increase of ACTA2 expression [94]. In another study, they further validated this by showing that inhibition of S100A4, through the use of a small hydrophobic drug, enhanced the healing in injured mice tendons [95]. The results from our study showed that the younger patient group had a significantly higher gene expression of S100A4 at day 7 after seeding compared to the older group ($p=0.033$). However, no significant difference was found in ACTA2 expression between the two groups. These results are contradictory to what was hypothesized and in regards to the shown effect of S100A4 and ACTA2 expression in the previously mentioned studies. However, the studies investigated the effect of S100A4 and ACTA2 in acute injuries and did not examine age-related differences which is the main focus of our study, making it hard to draw direct comparisons.

SCX is a well established tenocyte marker that has been shown to be expressed in certain tenocyte subpopulations [18]. It has been suggested that SCX positive cells have a bigger role in the development stage of the tendon rather than in the mature phase [96]. It has also been shown that SCX-lineage cells has a contributing role in adult tendon healing in mice [97]. On the contrary, the expression of SCX in tendon cells from injured Achilles tendons has been shown to have similar levels to tendon cells from healthy semitendinosus in humans [87], suggesting that it does not increase in ruptured tendon as a result of healing response. The same study utilized a similar model to the one used in this study, which simplifies comparisons. The results from the current study shows that there is no significant difference in gene expression of SCX between cells from newly formed tendon tissue in the young and old patient groups. This together indicate that gene expression of SCX does not change after injury and is not dependent on the age of the patient.

SPARC is a gene that have been linked to increased collagen fibril diameter, increased tendon thickness and overall better mechanical properties [52], [98]. This gene have previously also been shown to decrease in expression with age [99], which is contradictory to our results. Surprisingly, when studied in dermal wound healing, it had a negative effect on healing, and the mice that lacked SPARC expression healed faster [100]. Since there was no difference in expression between the young and old group, at either day, it can be assumed that SPARC does not influence tendon healing in the same way as it influenced dermal healing.

Analysis of the gene expression of EZR showed no significant difference at either day, but at day 7 the p-value was 0.059. EZR has not been studied in tendons before, but is known to regulate cell shape and work as a linker between the actin cytoskeleton and the plasma membrane [101]. Since the actin cytoskeleton is known to have an important role in cell motility, which is crucial for healing, the expression of EZR could possibly also influence new tendon tissue formation, making it interesting for our study. The gene expression analysis did not show any significant difference between the age groups, indicating that EZR expression does not influence the impaired healing seen with aging. However, it is still unknown whether EZR expression have an effect on overall AT healing.

5.3 Limitations

This project aimed to investigate age-related differences in tendon healing meaning that the sex of the patient have not been considered when interpreting the final results. Since sex is a factor that have previously been shown to influence the mechanical properties of the AT, it could introduce some variations in the results [102]. However, since this study only looks at newly formed tendon tissue in an *in vitro* model, and thus eliminates many of the systemic effects that sex could have on the body, these variations should be minimized. Further limitations concern both the time frame and the biological variation between biopsies collected from patients. The project have been conducted between September of 2024 and June

of 2025 which have limited the size of the study, both in terms of the amount of samples that could be included and the amount of methods that have been used to investigate differences between the two patient groups. Due to inherent biological variation between the samples a larger amount of samples are always preferable, but unfortunately not feasible given the scope of the study. The limited amount of biopsies also influenced the choice of age groups. Under ideal circumstances two age groups with a few years separating the groups would have been chosen but this was not possible, leading to the division of patients below the age of 55 belonging to the younger group and patients above the age of 55 belonging to the old group. This could make it harder to find differences between the two groups but was, as previously mentioned, out of our control. However, no correlation was found with age in any of the tested parameters, implicating that the division of the age groups probably did not affect the results.

In this study, biopsies have been collected from patients undergoing surgery following a chronic Achilles tendon rupture. The rupture is defined as chronic if the injury has not been treated within 4 weeks [31], however many patients might wait longer to seek out treatment or their rupture might have been missed during examination. This means that the biopsies have been collected at different time-points post-injury, which influences at what stage of the healing process the cells are in when being extracted. Depending on at what stage the cells are, they will have different phenotypes, which could influence how they behave within the model. Another factor that also could have an impact on the cell behavior is the lifestyle of the patient. Since all patient data except for age are unknown, factors such as lifestyle of the patient have not been considered during the data analysis.

Lastly using primary cell cultures rather than an established cell line makes this study less reproducible and more patient specific, meaning that the difference between each sample will be greater. However, this also means that the Achilles tendon constructs more closely resembles the variation seen in the general population. Culturing the cells in an *in vitro* model may still favor certain tenocyte subpopulations though, which means that it may not mimic the native tissue as closely as intended. Moreover, certain cell types that are more affected by age may be lost, which could mean that possible age-related differences that are present in the native tissue are missed in the use of the model. But the lack of differences seen in this study could also be explained by the cells from young and old patients behaving similarly when activated as a response to injury in contrast to what have been observed in healthy tendons. This would mean that tendon cells from older patients exhibit similar properties to the ones from younger patients in their activated stage.

5.4 Future Studies

The results of this study imply that there is no major difference in younger and older cells' ability to form new tendon tissue following injury when seeding them in a 3D *in vitro* model. Future studies are, as always, needed to validate the correctness of the results. In this project it would include extending the patient groups and the

methods used to investigate the possible differences between the patient groups. It would be beneficial to have larger patient groups and also be able to have an age gap to distinguish them to increase the chance of finding significant differences in regards to age. Given the time period, this project have included quite a lot of methods but recommendations for future studies would be to both investigate even more properties and to use alternative methods to investigate already covered properties. For instance, cell migration should be assessed by using a more quantitative method compared to the 2D wound healing assay that was used in this study. The method of using a pipette tip to create scratches of similar size of cell free areas proved to be difficult and it was seen that the size of the original wound had a correlation with the migration rate of the cells. It was therefore difficult to draw any conclusions about differences in the migratory ability between young and old cells. An alternative method for future research could be the transwell migration assay, which is more reproducible since the risk of variation between individual experiments would be reduced. In combination with further assessing the cell migration, it would also be interesting to investigate the localization of focal adhesion proteins through immunofluorescent staining which, due to the time constraint, had to be excluded from this study. Another method that had to be excluded because of this was the EdU assay, which is an assay used to specifically look at proliferating cells. This assay would have been a good addition to further validate the lack of differences seen in the results obtained from the two cell health assays that were conducted in this study. The determined time set for the project also influenced the amount of data processing that could be carried out, and recommendations for future studies would therefore include expanding the correlation analysis. In terms of this project, it would mean investigating possible correlations between the Col I/Col III ratios and the assessed mechanical properties as well as between the CSAs of the tendon constructs and the two cell health assays that were performed.

Future research should also include further development of the model used in this project to better replicate the environment that the cells experience in native tendon tissue. This would include investigating the cell population within the model and compare it to the one in native tissue to assess whether certain cell types are being favored. A method that could be used to assess this is flow cytometry, which is a method be used for characterization of single cells in samples with mixed cell populations [103]. Moreover, it has been shown that intercellular communication between tendon cells and macrophages resident in tendon tissue influences the healing response [104]. Further development of the model to include this interaction could thereby be obtained by co-culturing macrophages together with the tendon cells extracted from the collected biopsies. Another factor that cells experience in the native environment of Achilles tendons is the loading that naturally occurs *in vivo* during physical movement, and this would also be beneficial to be able to include in the model. Although incorporating mechanical loading into the model would help with better replicating the native environment that cells experience *in vivo*, the loads produced by a young individual and an older one would likely differ significantly, given that physical movement generally decreases with age. This difference would be hard to replicate in the *in vitro* model but however, introducing loading

could provide information regarding whether cells from young and old individuals respond differently to mechanical loading in regards to forming new tendon tissue post-injury.

6
Conclusion

The aims of this study were to investigate age-related differences in mechanical and biological properties of tendon constructs formed from human primary tendon cells. In the results and discussion of this report, it is demonstrated that there is no major differences in younger and older cells capability to form new tendon tissue in a 3D *in vitro* model. There was no difference in either the structural nor the material properties of the newly formed tendon tissue between the younger and older samples. No differences could be seen in the majority of the tested biological properties either, including cell health, migration, cell morphology, collagen type I and type III content and gene expression, with the exception of two genes (DCN and S100A4) linked to the ECM and fibrotic healing. Both of these genes showed an increased expression in the younger group at different time-points after seeding in the model which is contradictory to what previous studies have shown. Therefore it would be interesting to further study these genes and to also extract RNA directly from human biopsy samples to achieve a more accurate representation of gene expression of cells in the native Achilles tendon tissue. It would also reduce the risk of involuntary selection for certain cell types and subpopulations of tenocytes, which may be the case when using an *in vitro* model. With that being said, further examining which cell types that are present in the model in comparison with cell types in native healing tendon tissue would also be beneficial. To conclude, these results are promising for older patients as it implies that aging does not affect the cells ability to create new and strong tendon tissue after injury. Further studies are needed to validate these results, with larger patient groups and more extensive testing, especially since it has been proven that older patients experience worse functional outcomes. However, the takeaway of this study should still be that increasing age does not impair the tendon cells' ability to form new tendon tissue.

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A

Appendix 1

A.1 Extraction and Culturing of Primary Tendon Cells

Collect sample

1. Collect samples from the operating theater in PBS containing 1% penicillin/streptomycin (approx. 20 mL in a 50 mL tube).
2. Remove all visible non-tendon tissue and cut the whole tendon tissue into pieces in the size of about 2x2x2 mm.

Digestion

1. Transfer the pieces to a new petri dish with PBS to wash them. Repeat once more.
2. Digest in DMEM/F12 + 20% FBS + 1 mg/mL collagenase (type II) overnight in the incubator (17-24 h).
3. Shake the tube and pipette up and down the following day, clumps should be dissolved. If not properly digested, place it back in the incubator for 2 h.
4. Centrifuge at 600 g for 6 min.
5. Retain the supernatant and transfer it to a new tube.
6. Wash the pellet in culture medium.
7. Centrifuge all tubes at 600 g for 6 min.
8. Aspirate the supernatant and add new culture medium.
9. Centrifuge at 600 g for 6 min.
10. Aspirate the supernatant and resuspend the pellet in culture medium.
11. Seed out in a cell culture flask.
12. Change medium the next day to remove debris.
13. Cultivate cells to confluency.

A.2 Preparation of the Wells Utilized in the Tendon Construct Formation

1. Mix 9 parts Dow SYLGARD 184 silicone base and 1 part silicone curing agent thoroughly and pour into each well in a six well plate to a thickness of a few millimeters.
2. Place the plate in an incubator at 55 °C overnight to cure.
3. Make two markings 1.5 cm in from the edges of the well to mark the placement of the suture ends.
4. Cut 8 mm long suture pieces and use insect needles to fasten the sutures in the silicone in the bottom of the well.

A.3 Cell Maintenance

Trypsinization

1. Take out the cells and aspirate the medium.
2. Wash the flask with approximately 12-13 mL PBS.
3. Remove the PBS.
4. Add trypsin and make sure that it covers the entire bottom.
   - 1 mL per 25 cm^2 flask.
   - 3 mL per 75 cm^2 flask.
   - 5 mL per 175 cm^2 flask.
5. Place in the incubator with the lid sealed for 5-7 min and continually check if the cells are loose to avoid over trypsinization.
6. Gently hit the flask against the table to loosen as many cells as possible before adding 15 mL medium into the flask to inactivate the trypsin.
7. Rinse a flask a few times with the media before transferring it into a tube.
8. Centrifuge the cells for 6 min at 600 g.
9. Aspirate the supernatant and resuspend the cell pellet in the appropriate medium.

Freezing of cells

1. Trypsinate the cells according to the trypsinization protocol above.
2. Resuspend in 1 mL cold freezing medium per 175 cm^2 flask.
3. Transfer to a cryo tube.
4. Put the tube immediately in a cell freezing container and place it in the -80°C freezer.
5. After 2-5 days, transfer the cells to the -140°C freezer and note the position in the database.

Thawing of cells

1. Prepare a 50 mL tube with 20 mL of warm culture medium.
2. Transfer 1 mL of warm culture medium to the cryo tube with frozen cells and carefully pipette up and down to thaw the cells.
3. Transfer the liquid to the 50 mL tube containing warm culture medium.
4. Repeat step 2 and 3 so that the entire volume in the cryo tube is transferred to the 50 mL tube.
5. Centrifuge the tubes for 6 min at 600 g.
6. Aspirate the supernatant and resuspend the cell pellet in 1 mL culture medium.
7. Add an additional 24 mL of culture medium to the tube before transferring the entire volume to a cell culture flask.
8. Gently move the culture flask against a flat surface to distribute the cells evenly before placing it in the incubator.

Passaging of cells

1. Trypsinate the cells according to the trypsinization protocol above.
2. Resuspend the pellet in 1 mL culture medium and add an additional amount of medium depending on the desired amount of flasks.

3. Divide in new flasks and move the flask to distribute the cells evenly before placing them in the incubator.

A.4 Formation of Artificial Tendon Constructs

Preparation of the 6-well plates

1. Put the plates in an ethanol bath for 45 min.
2. Take out 1.5 mL tubes, 1/well, put them in racks and label them. Add an extra tube for cell counting.
3. When the plates are ready, take them out and place them in the LAF-bench. Aspirate the ethanol on the plates thoroughly and let the remaining ethanol evaporate with the lid semi-covering the wells.
4. Wash the wells with PBS, approximately 4 mL/well, and repeat it once more.

Preparation of the cells

1. Trypsinate the cells according to the trypsinization protocol found in A.3.
2. Scratch the tube on the bench to loosen the pellet before adding 1 mL of construct medium and pipette up and down until the cells are in solution.
3. Add more medium to a final volume of approximately 4 mL and mix.

Cell counting

1. Add 10 μL of trypan blue to a 1.5 mL tube and add 10 μL of cells and mix.
2. Add 10 μL to a cell counting chip.
3. Count the cells in 3 separate squares and calculate the mean.
4. Dilute the cells to obtain a final concentration of 333333 cells/mL.

Construct formation

1. Add 600 μL cell/1.5 mL tube and add 10 μL aprotenin and mix.
2. Add 200 μL fibrinogen and 4 μL thrombin to one of the tubes, mix and transfer the entire volume to the well.
3. Swirl the plate against the bench to ensure the gel is evenly distributed in the well.
4. Repeat the previous step for the next well.
5. When all wells are finished, let the fibrin gel solidify in the incubator for approximately 1 h before adding 3 mL of medium to each well.

Loosening of edges

1. After 48 h, aspirate the medium from the wells.
2. Use a pipette tip to carefully loosen the edge of the fibrin gel from the wall of the well to allow for contraction.
3. Add 3 mL of fresh construct medium to each well and place the plate back in the incubator.

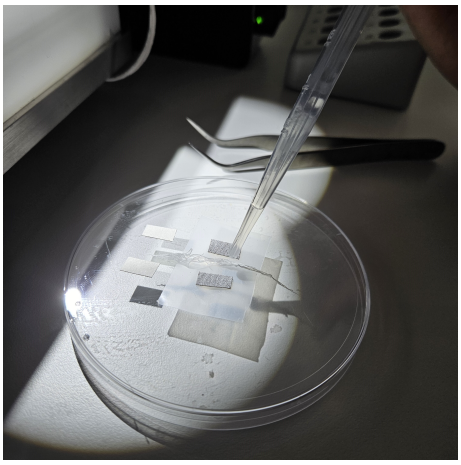
A.5 Mechanical Testing

Preparation

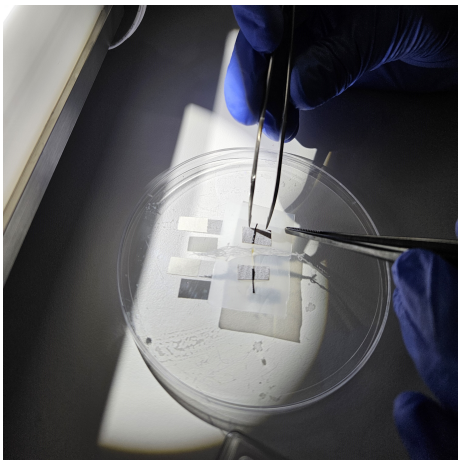
1. Fill the water bath of the mechanical testing machine and allow it to heat up to 37 °C.
2. Cut fine sandpaper into small rectangles and place two rectangles with the distance about the length of a construct in between them on top of a piece of parafilm.
3. Place a small piece of gauze wetted with PBS in between the sandpaper rectangles, as seen in figure 26a, to avoid the construct drying out during the mounting process.

Mount the constructs

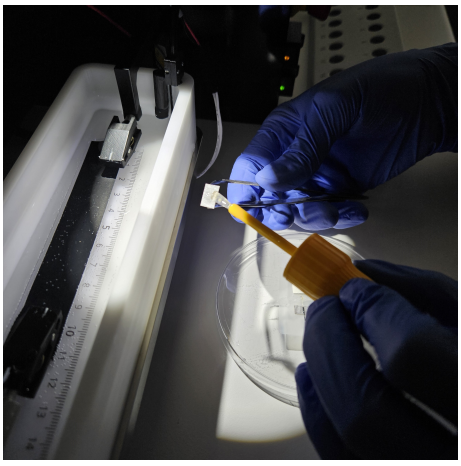
1. Fully aspirate the media from the well.
2. Remove the needles keeping the suture thread in place.
3. Lift the construct carefully and place it on the two sandpaper rectangles so that the ends of the suture and a small part of the construct is on top of each rectangle, as seen in figure 26b.
4. Add some superglue on a third and fourth sandpaper rectangle and place them on top of the other rectangles and constructs, as seen in figure 26c and figure 26d.
5. Transfer the entire package and mount it in the mechanical testing machine.
6. Start the testing program (displacement of 4 mm/min until failure).
7. Repeat for all the constructs.



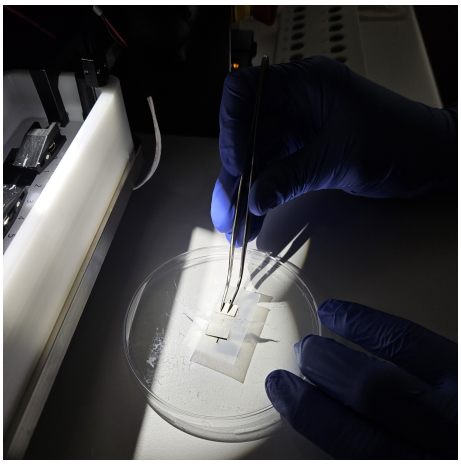
(a) *Wetting the gauze in between the two sandpapers with PBS*



(b) *Transfer the construct from the well onto the mounting stage*



(c) *Add glue to another piece of sandpaper*



(d) *Glue the two sandpapers and the construct together*

Figure 26: *Mounting procedure of tendon constructs prior to the mechanical testing.*

A.6 Cell Health Assays

Prior to CCK-8 assay

1. Take morphological pictures with a microscope and 10X zoom for DNA analysis.
2. Transfer 2 x 1 mL of the conditioned media into two tubes with screw lids for LDH analysis and put them in liquid nitrogen.

CCK-8 assay

1. Make CCK-8 medium by mixing 10 parts construct media and 1 part CCK-8 solution.
2. Aspirate the remaining medium from the well and add 3 mL CCK-8 media into each well.
3. Add 1 mL of the CCK-8 medium to an empty plate for control.
4. Incubate the plate for 1 h before transferring 350 μL from each well (including control) to a new well on a 24 well plate and place it in the fridge.
5. Repeat the previous step after 2 h and make sure that all samples are at room temperature.
6. Transfer 100 μL for each patient and time point to three separate wells in a 96 well plate and do the same for the controls.
7. Place the plate in the plate reader that is set at 450 nm and measure the absorbance.

LDH assay

1. Thaw the samples of conditioned medium by placing them in room temperature for approximately 30 min.
2. Prepare the standard curve by adding a gradient consisting of 6 wells with different known concentrations of NADH standard solution, ranging from 0 nmol to 12.5 nmol. Fill each well with the assay buffer to a final volume of 50 μL.
3. Add 50 μL of each sample of conditioned medium to separate wells.
4. Mix 48 μL assay buffer and 2 μL substrate mix per well and add 50 μL to all wells.
5. Place the plate in the plate reader and record the absorbance at 450 nm every two minutes for 30 minutes at 37 °C.
6. Calculate the enzymatic activity of LDH using the manufacturer's instructions.

A.7 Gene Expression Analysis

1. Prepare one tube with a screw lid for each well with 5 stainless steel beads, 5 pieces of silicon carbide beads and 1 mL of Trizol, label them and place on ice.
2. Aspirate the medium and wash each well with 3 mL cold PBS.
3. Aspirate the PBS, remove the needles, scrape up half of the gel and suture with a cell lifter and transfer it into the prepared tubes.
4. Add some PBS on top of the remaining gel to prevent it from drying out in preparation for histological analysis.
5. Shake the tubes in a bead beater (MP, FastPrep-24 Classic) on the 5.5 setting for 20 s, cool them on ice for 2 min and then shake them again before freezing them in liquid nitrogen.

After this step the tubes can either be placed in a -80 °C freezer or further analyzed directly.

RNA extraction

1. Spin shortly to remove Trizol from the lid.
2. Add 100 μL BCP per 1000 μL Trizol.
3. Vortex for 15 seconds, max speed, do this step as fast as possible.
4. Leave for 2-15 minutes at R.T.
5. Spin at 12000 g for 15 minutes at 4 degrees. Mark the lids on which side is up in the centrifuge. The mixture will now separate into 3 phases. Top = aqueus phase that contain the RNA, the mid phase contains DNA and the lower red phase contains proteins, including the RNAses.
6. Label 1.5 mL tubes and add 4 μL of glycogen.
7. Carefully transfer the top phase (set the pipette on 450 μL). Make sure not to touch any other phase. The remaining phases can be saved for later. Transfer this phase to a 1.5 mL tube containing 4 μL of glycogen.
8. Add an exactly equal volume (important) of isopropanol to the 1.5 mL tube to precipitate the RNA. Mix by inversion.
9. Leave for 5-10 min in room temperature.
10. Spin at 12000 g at 4-25 degrees for 8 minutes.
11. Carefully remove the supernatant with a pipette.
12. Add 1 mL 75% ethanol (RNAse free). Wash sides by inversions.
13. Spin at 7500 g 4-25 degrees for 5 minutes.
14. Carefully remove the supernatant with a pipette.
15. Spin at 7500 g 4-25 degrees for 1 minutes.
16. Remove the remaining supernatant.
17. Add 100 μL RNAse free water on top of the pellet.
18. Leave for 5-10 minutes at room temperature.
19. Vortex thoroughly to dissolve pellet.
20. Add 10 μL of 3M NaAc pH5.5 (RNAse free) and mix (no vortex but vigorously shaking).
21. Add 200 μL 96% ethanol (RNAse free). Wash sides by inversions.
22. Leave for 2-15 min in room temperature.

23. Spin at 12000 g at 4-25 degrees for 8 minutes.
24. Carefully remove the supernatant with a pipette.
25. Add 1 mL 75% ethanol (RNAse free). Wash sides by inversions.
26. Spin at 7500 g 4-25 degrees for 5 minutes.
27. Carefully remove the supernatant with a pipette.
28. Spin at 7500 g 4-25 degrees for 1 minute.
29. Remove the remaining supernatant.
30. Air-dry the pellet for 5 min, exactly! Place the tube lying with the lids open on the bench. Be careful to not completely dry the pellet as it will be difficult to resuspend.
31. Add 10 μL RNAse free water on top of the pellet.
32. Leave for 5 minutes at room temperature.
33. Vortex.
34. Leave for 1 minute at room temperature.
35. Vortex and spin.
36. Take out 3 μL to a new tube for quantification and quality measurement.

The extracted RNA can then be stored in a -20 °C freezer until it is transcribed into cDNA. The protocol described below is from the supplier's user manual and can be found here [105].

Reverse transcription

1. Thaw components of the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems on ice.
2. To a tube on ice add 2.0 μL 10X RT Buffer, 0.8 μL 25X dNTP Mix, 2.0 μL 10X Random Primers, 1.0 μL Multiscribe Reverse Transcriptase and 4.2 nuclease-free water for each reaction to create a 2X RT master mix.
3. Mix 10 μL of the 2X master mix and 10 μL of the RNA sample, also include samples with universal human reference RNA that later can act as a standard curve during gene expression.
4. Load the thermal cycler and run the program displayed in table 4.

Table 4: *Program for the thermal cycler.*

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	25 ° C	37 ° C	85 ° C	4 ° C
Time	10 minutes	120 minutes	5 minutes	Hold

Samples containing cDNA can then placed in the fridge for short term storage.

Gene expression analysis

1. Design the plate layout so that no replicates of the samples are included but a standard curve with duplicate samples and a 1:4 serial dilution in 5 steps is included along with a negative control.
2. In a tube mix 7.5 μL TaqMan mastermix, 0.75 μL TaqMan primer probe, 5.25 μL nucelase free water for each reaction.
3. Transfer 13.5 μL of the mixture to each well and add the samples according to the previously designed plate layout.

4. Load the Real-Time PCR system and run the program displayed in table

Table 5: *Program for the Real-Time PCR system.*

Settings	Step 1	Step 2	Step 3	Step 4
Temp.	95 ° C	95 ° C	60 ° C	Go to step 2
Time	10 minutes	15 seconds	1 minute	40X

A.8 Histological Analysis

After harvesting the first half of the gel for qPCR, 1 mL of PBS is added to the wells so that the remaining gel does not dry up.

1. Prepare “Peel-A-Way”-boxes, one for each sample, by labeling them and adding OCT so that it covers approximately one third of the box.
2. Aspirate the PBS from the wells and carefully remove the anchor needles and the suture thread with sterile tweezers and a scalpel.
3. Use a cell lifter to gather the remaining gel into a clump and transfer it to the middle of the prepared box and make sure that the gel is properly submerged in the OCT.
4. Add liquid nitrogen to an ice box inside a fume-hood.
5. Pour isopentane in a metal bowl and place the bowl in the liquid nitrogen until it freezes at the edges of the bowl.
6. Place the "Peel-A-Way"-box into the metal bowl with the help of large metal tweezers and gently stir the metal bowl so that the box does not stick to the bottom of the bowl.
7. Move the box to the -18 °C freezer once the content is completely frozen to let any isopentane residue evaporate.
8. The frozen cube of sample embedded in OCT can then immediately be sliced into 8 μm cryosections in a cryotome or transferred to -80 °C for long term storage.

Hematoxylin and Eosin staining

1.	Air dry the glass slide samples at room temperature	15 min
2.	Fix in 4% formaldehyde	3 min
3.	Wash with running tap water	5 min
4.	Stain with Harris hematoxylin	5 min
5.	Wash with running tap water	5 min
6.	Differentiate in acid alcohol	5 dips
7.	Wash with running tap water	10 min
8.	Stain with Eosin	5 min
9.	Rinse quickly 3 times in MQ water	
10.	Dehydrate rapidly to xylene:	
	• 70% ethanol	30 sec
	• 95% ethanol	60 sec
	• Abs ethanol	90 sec
	• Xylene	2 min
	• Xylene	2 min
11.	Mount in Pertex and leave to cure overnight.	

Alcian Blue staining

1.	Air dry the glass slide samples at room temperature	15 min
2.	Fix in 4% formaldehyde	10 min
3.	Wash with running tap water	5 min

4. Rinse in MQ water
5. Incubate in 3% acetic acid — 3 min
6. Stain with Alcian blue solution — 15 min
7. Wash with running tap water — 5-10 min
8. Rinse in MQ water
9. Counterstain in Nuclear Fast Red solution — 2 min
10. Wash with running tap water — 5 min
11. Dehydrate to xylene:
    - 70% ethanol — 2 min
    - 95% ethanol — 2 min
    - Abs ethanol — 2 min
    - Xylene — 2 min
    - Xylene — 2 min
12. Mount in Pertex and leave to cure over night

Immunofluorescence

1. Air dry the glass section samples at room temperature — 15 min
2. Fix in pre-chilled methanol and place it in -20°C — 10 min
3. Remove sections and let them air dry at room temperature — 10 min
4. Apply a barrier around the sample using ImmEdge pen
5. Hydrate in PBTD — 5 min
6. Place sections on a slide and block them by adding 200 μL of 5% v/v normal goat serum in PBTD — 1 h
7. Incubate with primary antibodies Anti-Collagen I, rabbit mAb [EPR7785], abcam ab138492 (100X dilution) and Anti-Collagen III, mouse mAb [FH-7A], abcam ab6310 (100X dilution) mixed in PBTD with 2.5% v/v normal goat serum at 4°C — min. 18 h
8. Equilibrate at room temperature — 30 min
9. Wash in PBTD — 3 x 5 min
10. From this step, shield sections from light. Incubate with secondary antibody, invitrogen Goat anti-Rabbit [A32731] and invitrogen Goat anti-Mouse [A32742] (400X dilution), mixed in PBTD with 2.5% v/v normal goat serum — 1 h
11. Wash in PBTD — 3 x 5 min
12. Counterstain with DAPI — 5 min
13. Wash in PBTD — 2 x 5 min
14. Mount slides in ProLong Gold Antifade Mountant and leave to cure

A.9 Wound Healing Assay

Preparation of the cells

1. Trypsinate the cells according to the trypsinization protocol found in A.3.
2. Scratch the tube on the bench to loosen the pellet before adding 1 mL of culture medium and pipette up and down until the cells are in solution.
3. Add more medium to a final volume of approximately 4 mL and mix.

Cell counting

1. Transfer 10 μL cells into an 1.5 mL tube and mix with 10 μL trypan blue before transferring 10 μL to a cell counting chip.
2. Count the cells in 3 separate squares and calculate the mean.
3. Calculate how to dilute the cells to obtain a final concentration of 100000 cells/mL.

Seeding

1. Mark the wells with three horizontal lines that will work as a guide when taking pictures.
2. Paint a dot at the beginning and end for each scratch to help keep track of them.
3. Add 3 mL of the cell-solution to each well on a 6-well plate.
4. Incubate for 40 h.

Scratching

1. After 40 h make two scratches in each well, one with a 200 μL pipette tip and one with a 1000 μL tip.
2. Gently agitate the media and aspirate it.
3. Add 3 mL fresh media into each well.
4. Take pictures directly after making the scratches (note which spot based on the horizontal lines that is supervised to be able to take pictures of the same area at each time point) with a microscope with 4X zoom.
5. Then take pictures every 2 h at the same spot at the scratch made with the 200 μL pipette tip for 8 hours.
6. After 24 hours take pictures of the scratch made with the 1000 μL pipette tip.
7. Analyze the area of the gap at different time points using ImageJ to determine how fast the cells migrate.

B

Appendix 2

B.1 Correlation Between Age and Mechanical Properties

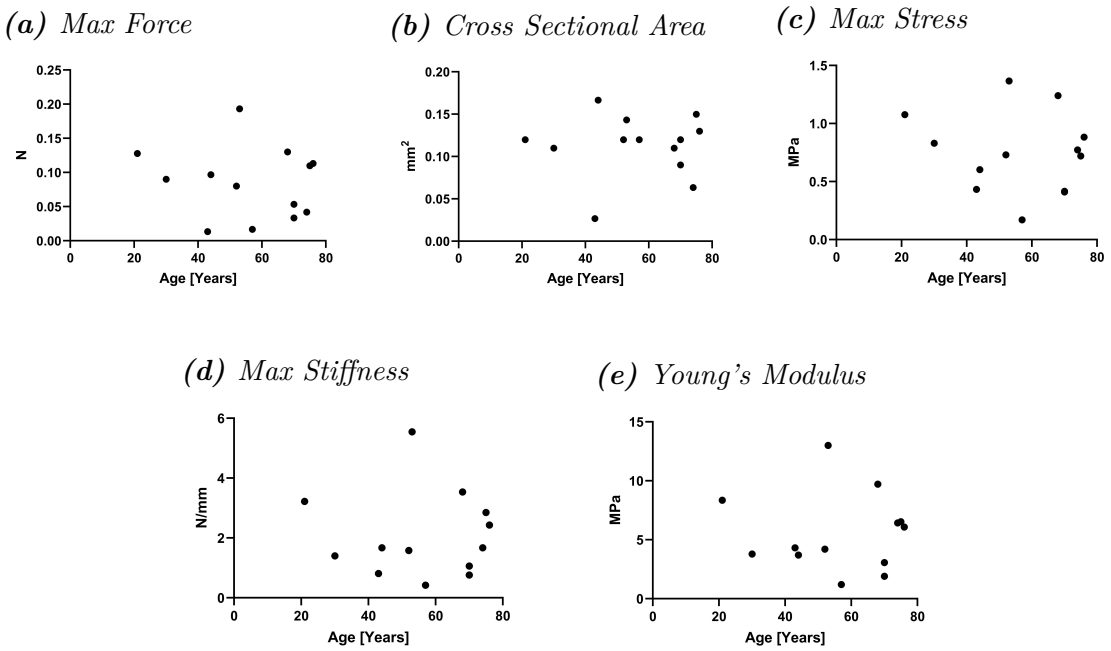


Figure 27: *Correlation analysis of the mechanical properties against age. (a) Maximum force with a p-value of 0.989. (b) Cross sectional area with a p-value of 0.683. (c) Maximum stress with a p-value of 0.686. (d) Maximum stiffness with a p-value of 0.803. (e) Young's modulus with a p-value of 0.968.*

B.2 Correlation Between Migration Rate and Original Wound Area

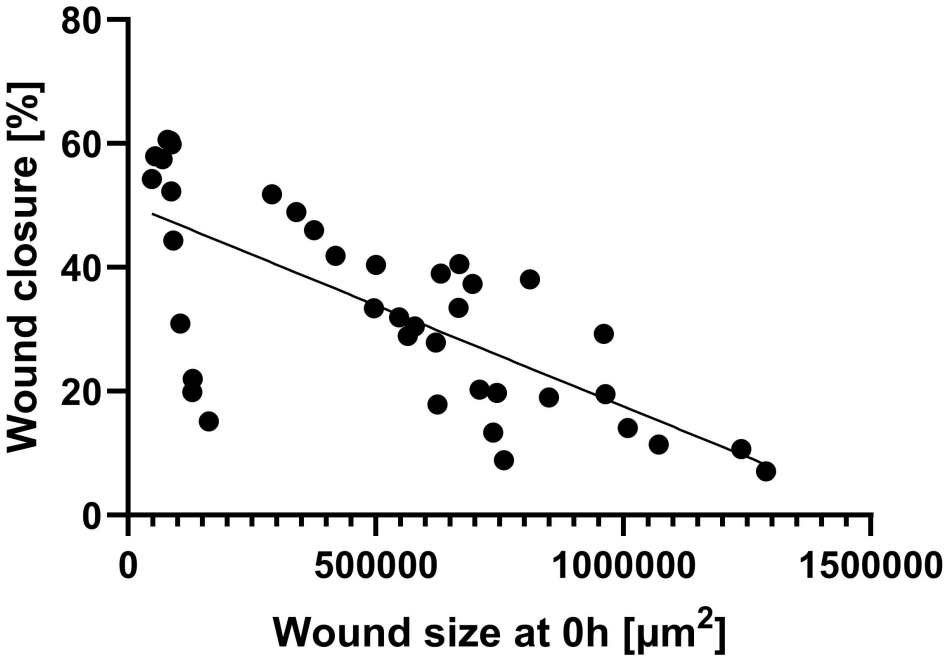


Figure 28: *Correlation between the migration rate and the original wound area, with a p-value of <0.0001.*

B.3 Correlation Between Migration Rate and Age

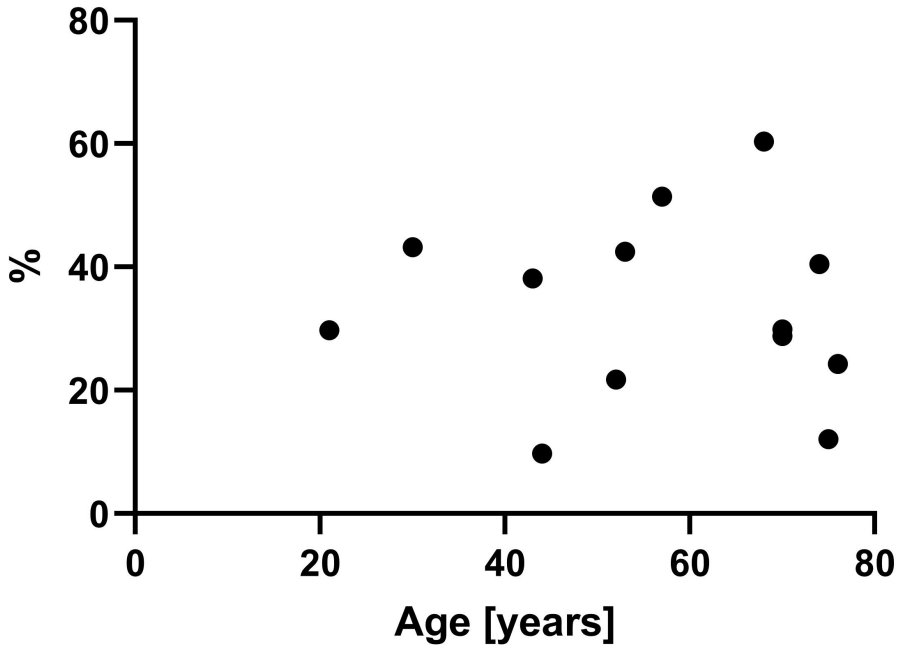


Figure 29: *Correlation between the migration rate and age, with a p-value of 0.600.*

B.4 Correlation Between Contraction Rate and Age

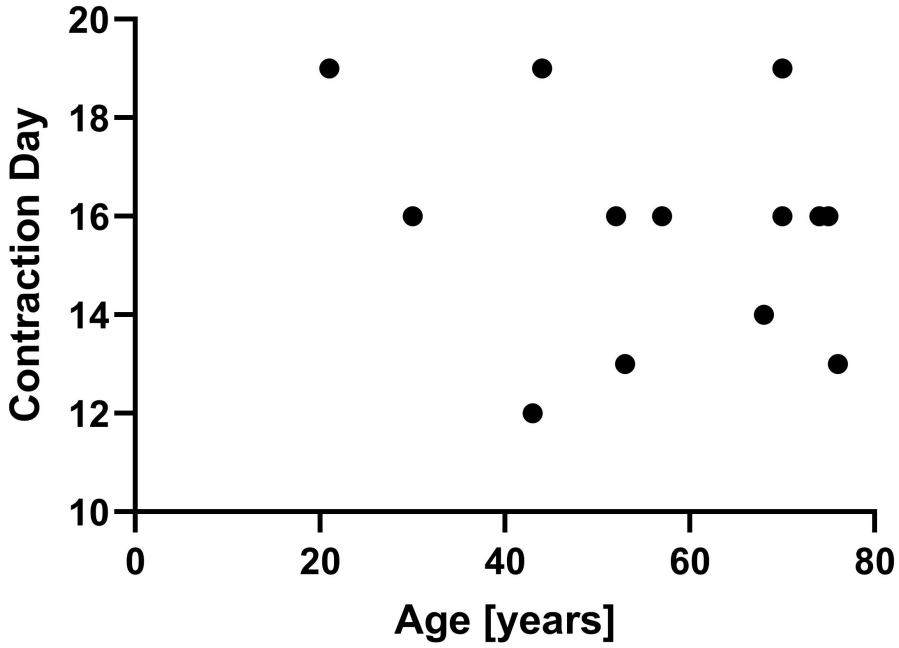


Figure 30: *Correlation between the contraction rate and age, with a p-value of 0.542.*

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