THE LINK BETWEEN HYPERCHOLESTEROLEMIA AND TENDON PATHOLOGY

by

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Abstract

Tendinopathy is a major cause of morbidity in athletic populations and in the work force, traditionally thought to occur as a consequence of an imbalance between damage (resulting from mechanical loading) and repair. However, one third of the cases for midportion Achilles tendinopathy occur in sedentary individuals, and recent data suggests an association between hypercholesterolemia and the occurrence of tendon rupture or tendinopathy. The aim of this study was to examine the link between elevated lipids and tendon pathology. We used an apolipoprotein E-knockout model (ApoE-KO), in which apolipoprotein E deficiency leads to development of atherosclerosis. We hypothesized that tendons from ApoE-KO mice fed a high fat diet, in comparison to those fed a regular chow diet and the wild type (non-atherosclerotic mice), will demonstrate increased lipid deposition, increased cross-sectional area, and increased expression levels of collagen genes (*Col1a1* and *Col3a1*), a growth factor gene (*TGF-β*), and an indicator of mast cell presence (*Cpa3*).

To test these hypotheses, ApoE-KO and control mice were fed a regular or high fat diet and sacrificed at different time points: 0, 15, and 30 weeks. The morphological properties were examined on H&E stained Achilles tendon sections while the lipid content was analyzed with Oil Red O staining. Tendon thickening was measured by ultrasonography of patellar tendon cross-sectional area. qPCR analysis was carried out on tail tendons at 30 week time point to analyze gene expression of *Cpa3, TGF-β, Col1a1, and Col3a1*.

ApoE-KO mice developed xanthomatous lesions, and showed less weight gain than control mice. ApoE-KO mice showed no appreciable changes in tendon histomorphology. Compared to control mice, ApoE-KO mice had lower tendon lipid content but demonstrated an increase in

tendon cross-sectional area. *Col1a1* gene expression levels were decreased in ApoE-KO mice. *Cpa3, TGF-β, and Col3a1* showed no differences between strains; however, *Cpa3* expression was decreased in mice that were fed the high fat diet. ApoE-KO mice demonstrated significant tendon alterations, demonstrating thicker tendons with decreased collagen expression. Future work is required to determine the mechanism involved and the potential impact on tendon function.

Preface

All animal procedures were performed according to the guidelines for animal experimentation approved by Animal Care Committee of University of British Columbia. The Animal Care Certificate number was A11-0027.

Part of the methods and results in the thesis (Chapter 2 and 3) were presented at Physiotherapy Association of British Columbia Conference and International Scientific Tendinopathy Symposium:

> Grewal, N., Granville, D., Reid, D., Scott, A. Hypercholesterolemia and Tendon Pathology. Physiotherapy Association of British Columbia Conference. Vancouver, British Columbia, April 2, 2011 and April 27, 2013.
>
> Grewal, N., Granville, D., Reid, D., Scott, A. Hypercholesterolemia and Tendon Pathology. International Scientific Tendinopathy Symposium. Vancouver, British Columbia, September 27-29, 2012.

For gene expression analysis, cDNA synthesis and qPCR experiments were done in the Scott laboratory by Aishwariya Sharma. Statistical analysis was done by Dr. Rick White.

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List of Abbreviations and Acronyms

ApoEApolipoprotein E

ApoE-KOApolipoprotein E-knockout

bFGFBasic fibroblast growth factor

BMP-12Bone morphogenic protein 12

C57Bl/6C57 black 6

cDNAComplementary DNA (deoxyribonucleic acid)

Col1a1Collagen type I, alpha 1

Col3a1Collagen type III, alpha 1

COX2Cyclooxygenase 2

Cpa3Carboxypeptidase A3

CSACross-sectional area

ECMExtracellular matrix

ESEmbryonic stem

GAGGlycosaminoglycan

H&EHematoxylin and eosin

HDLHigh density lipoprotein

HDL-CHigh density lipoprotein-cholesterol

IGF-1Insulin-like growth factor 1

IL-1βInterleukin 1 beta

IL-6Interleukin 6

iNOSInducible nitric oxide synthase

JNKc-JUN N-terminal kinase

LDLLow density lipoprotein

LDL-CLow density lipoprotein-cholesterol

LLDLeg length discrepancy

MMPMatrix metalloproteinase

mRNAMessenger RNA

OCTOptimal cutting temperature

PCRPolymerase chain reaction

PDGFPlatelet-derived growth factor

PGE2Prostaglandin E2

qPCRQuantitative PCR

RNARibonucleic acid

SHGSecond harmonic generation

TGF-βTransforming growth factor beta

VEGFVascular endothelial growth factor

VLDLVery low density lipoprotein

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Last but not least, thank you to God for answering my prayers and giving me the strength to struggle and overcome the obstacles and difficulties.

Dedication

To my parents, for their endless love, support, and encouragement.

Chapter 1: Introduction

1.1 Overview

Overuse injuries account for 30-50% of all injuries related to sports (Kannus, 1997). Tendon disorders are a major problem for those participating in competitive and recreational sports, and cause morbidity in both elite and recreational athletes. Tendon disorders are seen in physically active workers as well, and the prevalence of musculoskeletal symptoms increases with duration of employment (Forde, 2005). Tendinopathy and tendon degeneration might result from various factors such as overuse, poor vascularity, lack of flexibility, genetic makeup, sex, and endocrine or metabolic factors. Much attention has been given to the overuse aspect of this injury, but not enough to its occurrence in sedentary individuals as they age.

Cardiovascular diseases are a major cause of death in the Western society, and they result from interactions among multiple genetic and environmental factors (Berg, 1983). Hypercholesterolemia (a condition characterized by high levels of cholesterol in the blood) has been linked to pathophysiology of cardiovascular diseases; however there is not enough data regarding its potential effects on the musculoskeletal system. The gene encoding Apolipoprotein E (ApoE) involved in lipid metabolism is considered to be one of those factors causing hypercholesterolemia, eventually leading to atherosclerosis. As a high affinity ligand for the Apolipoprotein B and ApoE (LDL) receptor and chylomicron remnant receptor, it allows the specific uptake of ApoE-containing particles by the liver (Curtiss, 2000).

The effect of hypercholesterolemia on tendon pathology has been shown in few studies. Serum total cholesterol has been shown to be a factor associated with Achilles tendon ruptures. Murano

et al. examined the potential relation between Achilles tendon rupture and total cholesterol level in patients with familial hypercholesterolemia, a genetic disorder characterized by high cholesterol levels, specifically low-density lipoprotein (LDL). Serum concentrations of total cholesterol, LDL-cholesterol (LDL-C), and triglycerides were significantly higher in the Achilles tendon rupture group than control group; while high-density lipoprotein cholesterol concentration was significantly lower (Murano, 1993; Zehntner, 1984). Lipid deposition is also known to occur in tendons (Adams, 1974; Jozsa, 1984). Patients with familial hypercholesterolemia develop tendon xanthomas, which are deposits of lipid and connective tissue consisting of cholesterol and cholesterol esters (Kruth, 1985). Xanthomas also consist of foam cells, which are tissue macrophages that have phagocytosed the lipid components of lipoproteins deposited in tissues (Parker, 1973). Xanthomas can develop in the Achilles tendon (Fahey, 1973), with lipid infiltrating the tendon and tendon sheath (Galloway, 1970), resulting in a diffuse and irregular thickening of the tendon. The increase in tendon size may interfere with function and produce pain (Fahey, 1973). With the Achilles tendon being one of the main sites for xanthomas, it can be used to study lipid deposition.

Hypercholesterolemia can affect tendons in several ways. The deposits of cholesterol byproducts have been implicated in the formation of tendon xanthomas (Bahr, 2002). The tendon xanthomas may change the mechanical properties of the tendon and result in an increased rate of tendon ruptures. Hypercholesterolemia may also alter the tendon's extracellular matrix (and in turn biomechanical properties) in a way that leads to impaired healing or homeostasis (Abboud, 2012). An example was shown in a research study in which embryonic fibroblasts were used to

study the effect of hyperlipidemic rat serum on connective tissue cell isolated from embryonic tendons. It was found that embryonic fibroblasts reacted differently to hyperlipidemic rat serum, which was less likely to stimulate the synthesis of non-collagenous proteins or the incorporation of glycosamine and cytidine compared to serum with normal cholesterol levels. This suggested that elevated cholesterol levels may impair healing or alter the biomechanical properties in a manner that could promote injury (Ronnemaa, 1975).

Hypercholesterolemia may also cause changes in collagen levels and growth factors that play a role in tendon function, injury, and repair. Type I is the main type of collagen in tendons. In tendinopathic and ruptured tendons, there is a decrease in the proportion of collagen I and a significant increase in type III collagen (Maffulli, 2000). Reduced number of cross-links in type III collagen, compared to type I collagen, is responsible for reduced tensile strength of the tendon (Jozsa, 1990). Growth factors, such as TGF-β, are involved in embroyonic differentiation and healing of tissues. Not only do they influence the process of healing, but they also stimulate cell proliferation and differentiation among many of its other functions (Kader, 2007). In order to detect if hypercholesterolemia affects changes in collagen levels and growth factors, gene expression studies can be done to measure mRNA levels of Col1a1 and Col3a1 for collagen I and III respectively, growth factor TGF-β, and mast cell indicator Cpa3.

High cholesterol levels can also cause a change in biomechanical properties. It has been shown in a previous research study that patellar tendons of hypercholesterolemic mice showed reduced baseline elastic modulus and strength compared with controls, when tested to failure (Beason, 2010). The tensile strength of tendon is dependent on its thickness and collagen content.

Hypercholesterolemia may also cause changes at the molecular level, which can be analyzed by quantitative PCR to measure mRNA levels of genes associated with normal tendon function or with diseased tendon.

1.2 Scope/significance of problem in workers, athletes, and sedentary individuals

Tendon injuries affect a large number of individuals and account for enormous associated costs. They are increasing as the population continues to age and remain active. Tendinopathy, mainly associated with sports and physical activity, is a major medical problem of tendons resulting in many cases from overload and overuse (Woo, 2007). In workers and athletes, the associated pain results in reduced physical activity and productivity, missed work days, and a large amount of costs to Worksafe BC. Overuse is considered to be one of the causes of tendinosis and tendinopathy, which develop when tendons are exposed to forceful loads and/or high repetition (O'Brien, 1997). However, there may be other factors contributing to tendon injury. Hypercholesterolemia affects approximately 17% of the United States population 20 years and older (Schober, 2007). High levels of total cholesterol, triglycerides, LDL have all been associated with cardiovascular diseases such as atherosclerosis, in which the soft tissue vascular endothelium is damaged (Abboud, 2012). In the U.S. population, 66.5% of patients diagnosed with diabetes also have cholesterols levels higher than normal, alongside cardiovascular heart disease or other risk factors (Harris, 1991). Furthermore, studies have shown those patients with even a mild insulin resistance show changes in their dyslipidemic profile, which is an increase in triglycerides (TG) and a decrease in high density lipoproteins (HDL), but no change in LDL (Laws, 1992; McLaughlin, 2005).

1.3 Tendon structure and function

1.3.1 Gross anatomy and cellular features

Tendons are load bearing structures that transmit forces generated by muscles to bone, making joint movement possible. They are white in color and have a fibroelastic texture, showing great resistance to mechanical loads (Jozsa, 1997). They are made up of tightly packed, longitudinally oriented collagen fibrils, consisting mainly of type I collagen, with some type III collagen, cross-linked to provide stiffness. The dry mass of tendons is about 30% of the total tendon mass, while the rest of the 70% is made up of water. Type I collagen accounts for 65-80% of the dry mass of tendons, whereas elastin makes up approximately 2% of the dry mass (O'Brien, 1997; Hess, 1989). The main cell population in tendons responsible for producing collagen is elongated tenocytes, which are fibroblast-like cells that lie between collagen fibers (Kirkendall, 1997). Due to their proliferation ability, tenocytes are important for the maintenance of a healthy tendon. They consistently undergo metabolic and repair activity which involves collagen synthesis and breakdown. This ongoing process is regulated by how much loading the tendons are exposed to. Upon mechanical loading, calcium-dependant signalling is activated leading to stimulation of extracellular matrix gene expression. Therefore, a loading stimulus is required for tenocytes to maintain the normal tendon structure (Jozsa, 1997).

Tendons have a multi-unit hierarchical structure that consists of collagen molecules, fibrils, fiber bundles, fascicles, and tendon units running parallel to the tendon's long axis (Figure 1.1). The smallest structural unit of a tendon is the fibril (Wang, 2006) that consists of triple helix polypeptide chains of tropocollagen (Jozsa, 1997). The next level of tendon structure is formed

by fibers, which are made up of collagen fibrils and bound by endotenon (Kastelic, 1978). Endotenon is a thin layer of connective tissue that contains blood vessels, lymphatics, and nerves (Kastelic, 1978; Ochiai, 1979). A bunch of collagen fibers form a primary fiber bundle (subfascicle) and a group of primary fiber bundles forms a secondary primary bundle called a fascicle. Furthermore, a group of secondary fascicles form a tertiary bundle, which makes up the tendon surrounded by epitenon (Jozsa, 1997). Epitenon is a fine, loose connective-tissue sheath that contains vascular, lymphatic, and nerve supply to the tendon (Kastelic, 1978). Tendons are also surrounded by a third layer of connective tissue called the paratenon. It mainly consists of type I and III collagen fibrils, elastic fibrils, and synovial cells that line the inner surface of paratenon. Due to its elasticity function, it allows free movement of the tendon against surrounding tissues (Jozsa, 1997). The epitenon and the paratenon make up peritendon, which reduces friction with the adjacent tissue (Wang, 2006).

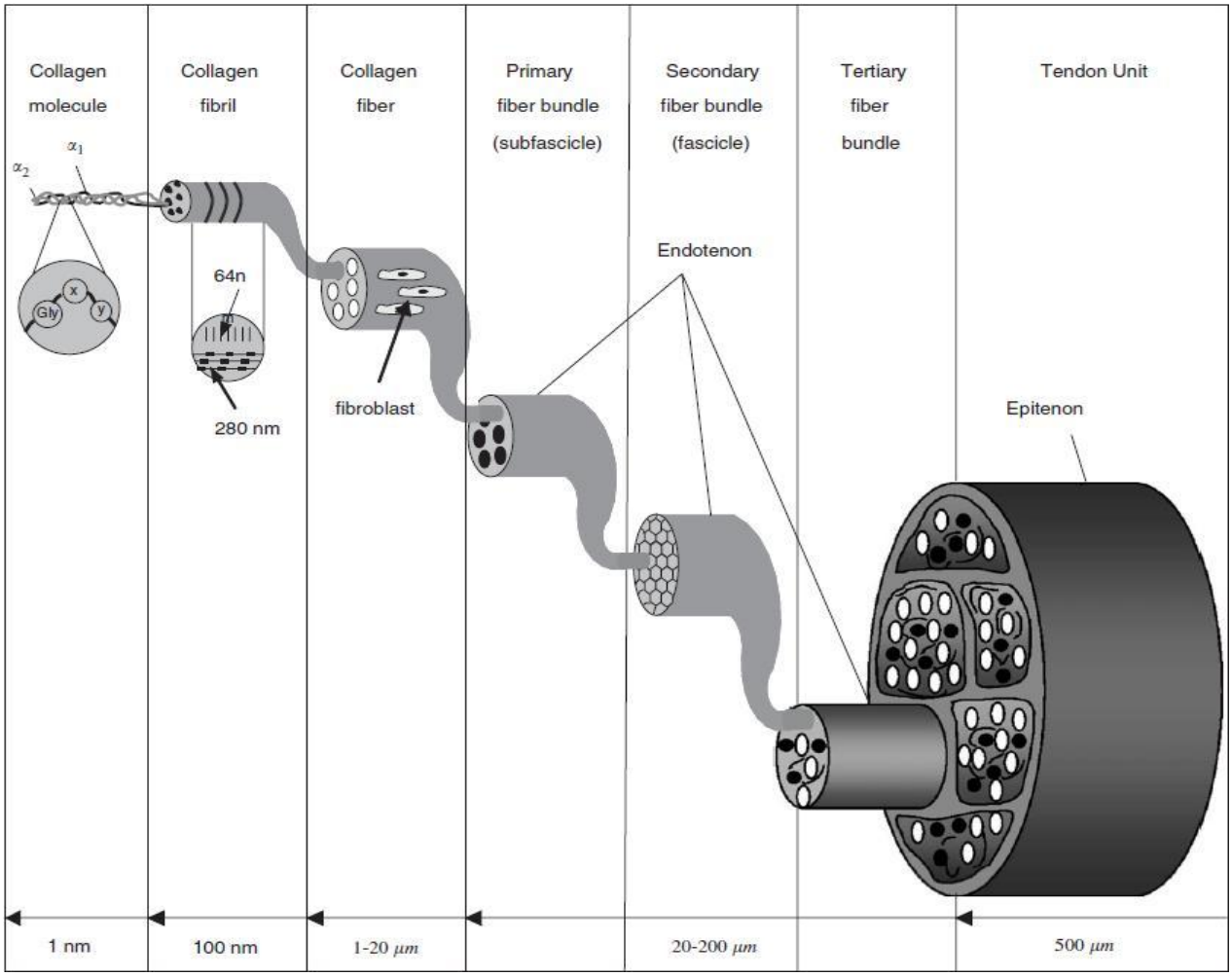

Figure 1.1 Schematic of a multi-unit hierarchical structure of the tendon (Wang, 2006, reproduced with permission).

1.3.2 Extracellular matrix components and organization

The extracellular matrix (ECM) of tendon consists of the structural proteins, collagen and elastin, and ground substance. The most abundant type I collagen makes up about 65-80% of the dry mass, and 90% of the total collagen (Jozsa, 1997). Collagen in the matrix is cross-linked, which

increases the Young's modulus and reduces its strain at failure (Wang, 2006). On the other hand, elastin makes up only about 2% of the tendon's dry mass (Jozsa, 1997).

The ground substance in the ECM surrounds the collagen and consists of proteoglycans, glycosaminoglycans (GAGs), structural glycoproteins, and other small molecules. Proteoglycans are negatively charged hydrophilic molecules made of a protein core with covalently attached GAG. They are entrapped within and between collagen fibrils and fibers, and allow rapid diffusion of water soluble molecules and migration of cells (Jozsa, 1997). In the ECM, they trap water and affect the viscoelastic properties of the tissue, therefore helping the tissue resist compressive forces (Xu, 2008). Proteoglycans and GAGs play an important role in physiological processes in tendon such as ion transport, water retention, diffusion of nutrients, mediating cell-matrix interactions, resistance of compression, and sequestration of growth factors and enzymes in the matrix (Hardingham, 1992; Yoon, 2005). Examples of proteoglycans are aggrecan and decorin. Aggrecan holds water within the fibrocartilage and resists compression (Vogel, 1989). Decorin, a leucine-rich proteoglycan located on the surface of the middle portion of collagen fibrils (Graham, 2000), facilitates fibrillar slippage during mechanical deformation (Pins, 1997).

Glycoproteins are macromolecules that consist of a large protein fraction and a small glyceridic component. The adhesive glycoproteins are a subgroup of the matrix glycoproteins that can bind other macromolecules or cell surfaces together (Jozsa, 1997). Examples of such glycoproteins are fibronectin (Jozsa, 1991) and thrombospondin (Miller, 1988). Fibronectin is involved in degeneration and repair processes of tendons, while thrombospondin mediates cell matrix interactions that occur in normal homeostasis and in repair and regeneration processes.

1.3.3 Biomechanical function

The physical characteristics of tendons are great tensile strength, good flexibility, and considerable inextensibility. This is due to the molecular and supermolecular structure of collagen and its elastic fibers. Tendons have a good ability to withstand tensile or stretching forces but are less able to withstand the shear and compressive forces transmitted by the muscles. They also have the ability to change the direction of the pull because of their flexibility and resistance to extension (Jozsa, 1997). The parallel arrangement of fiber bundles enables the tendons to transmit high muscle forces to the bone for movement with minimal energy loss and deformation (Kirkendall, 1997).

Tendons are viscoelastic materials and sensitive to different strain rates. They undergo stress-relaxation, creep, and hysteresis, with the first two being the main features of biomechanical properties. As seen in figure 1.2A, stress-relaxation means that with the same degree of extension (deformation), the load required to maintain that extension decreases over time (Best, 1994). On the other hand, creep means that with a constant load, length (deformation) increases over time (Jozsa, 1997) (Figure 1.2B).

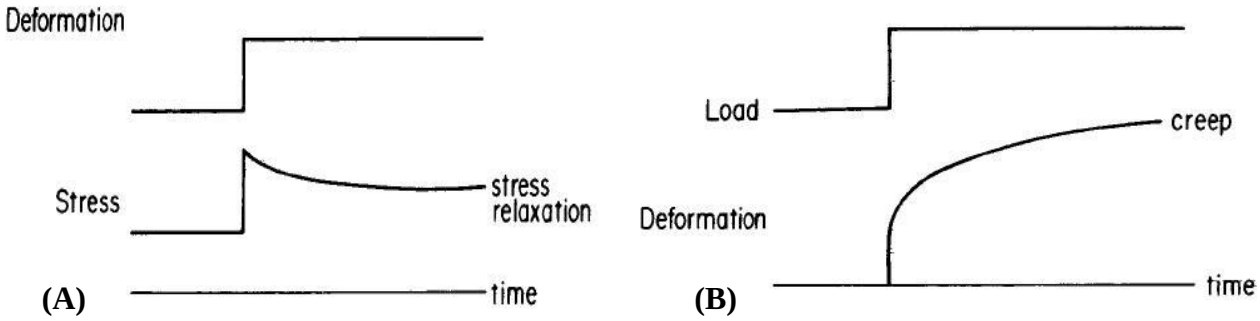

Figure 1.2 Graphic representation of the biomechanical properties of tendon. (A) Stress-relaxation (B) Creep (Kirkendall, 1997, reproduced with permission).

The characteristic mechanical behavior of tendon's unique structure and composition is illustrated by a typical stress-strain curve (Figure 1.3) (Wang, 2006). In the resting state, the collagen fibers and fibrils of the tendons have a wavy configuration. In the toe region, the wavy configuration of the tendon disappears when it is stretched by about 2%, corresponding to straightening of the fibers. The tendon resumes its normal wavy appearance when the tensile force is released (Jozsa, 1997). In the linear region of the curve, the tendon is stretched less than 4% and the collagen fibers lose their wavy appearance becoming more parallel. The slope of the curve in this region is referred to as the Young's modulus and is the elastic stiffness of the tendon (Wang, 2006). When the tendon is stretched more than 4%, microscopic tearing of the tendon occurs. The collagen fibers slide past one another as cross-links fail. Any further stretch leads to rupture and recoil of the tendon at the ruptured end. When the muscle-tendon-bone unit fails, the locations are bone, bone-tendon junction (osteotendinous junction), or muscle-tendon junction (myotendinous junction) (Kirkendall, 1997). These are the changes that occur in tendon rupture when the forces on the tendon exceeds its tensile strength. On the other hand, tendinosis

is described as intratendinous degeneration characterized histologically by intratendinous collagen degeneration, fiber disorientation, and vascular growth. There is no inflammatory cell infiltrate or swelling of the tendon sheath in tendinosis, but there can be a palpable tendon nodule (Khan, 1999). Tendinosis results in a decreased stiffness and Young's modulus, and tendinopathy alters mechanical and material properties of the Achilles tendon (Arya, 2010).

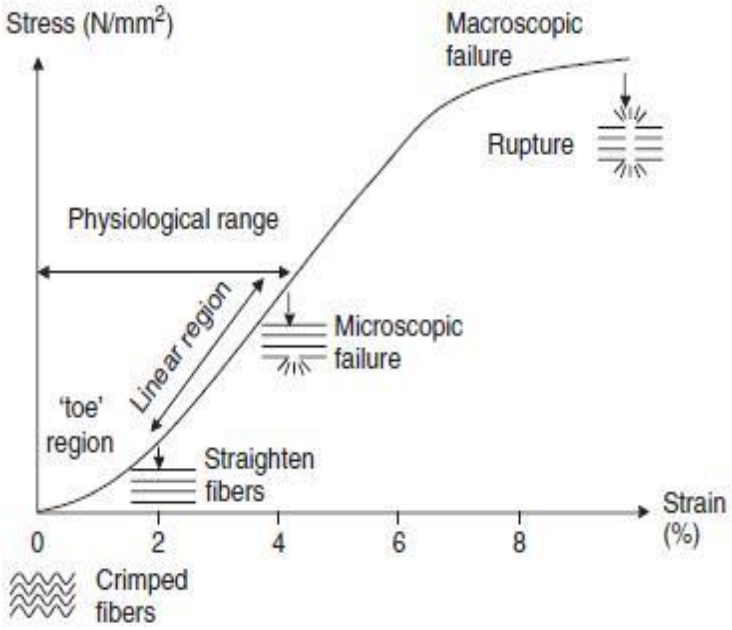

Figure 1.3 Stress-strain curve of a tendon (Wang, 2006, reproduced with permission)**.**

1.3.4 Biomechanical testing: pilot study

I conducted a pilot study to biomechanically test mice patellar tendons. Using the blunt end of the scalpel handle, the quadriceps muscle was removed by scraping. The tibia of the tibia-patellar tendon-patella complex was molded in glue and fixed with flat metal clamps provided by Bose. Custom-made grips were designed to hold the patella in place. The patella was clamped in

between two plastic screws with concave tips on the custom-made grips and tightened to hold it in place. Biomechanical testing was performed on a Bose ElectroForce 5100 Biodynamic test instrument (Bose Corporation, Eden Prairie, Minnesota) with a 22N load cell on tendons from C57Bl/6 mice.

The instrument was tuned in load and displacement control using Manual TuneIQ with a patellar tendon. Load control TuneIQ settings were: level 1, 0.5N; level 2, 1.5N; frequency, 2 Hz; 1000 cycles; and absolute position. Displacement control TuneIQ settings were: level 1, 0mm; level 2, 0.5 mm; frequency of 0.5 Hz; 1000 cycles; absolute position. Once the TuneIQ was successful, the sample to be tested was clamped on to the instrument.

Each tendon was preloaded to 0.5N; at this point the length of the tendon can be measured (Lo) with a digital caliper. Preconditioning was established with loads of 0.5N to 1.5N at a frequency of 2 Hz in 10 cycles in load control (Sereysky, 2012). A compensation rate of 0.5% was applied to adjust for the decrease in load overtime due to stress-relaxation behavior of a tendon. Constant ramp to failure test was performed at a rate of 0.167 mm/s in displacement control (Rigozzi, 2009). Preconditioning and preload (as seen in figure 1.4A) were done to obtain a uniform zero point and to allow the sample to settle into the testing apparatus (Provenzano, 2002).

Displacement and load data computed from the software can be used to calculate stress-strain values. Strain is calculated by dividing the change in length by the original length of tendon. Stress is calculated by dividing the load by initial cross-sectional area. The following parameters can be calculated for each group: maximum load to failure, maximum normalized load, strain at

maximum normalized load, and stress at maximum normalized load. The load-displacement curve (Figure 1.4B) and stress-strain curve can be produced from this data.

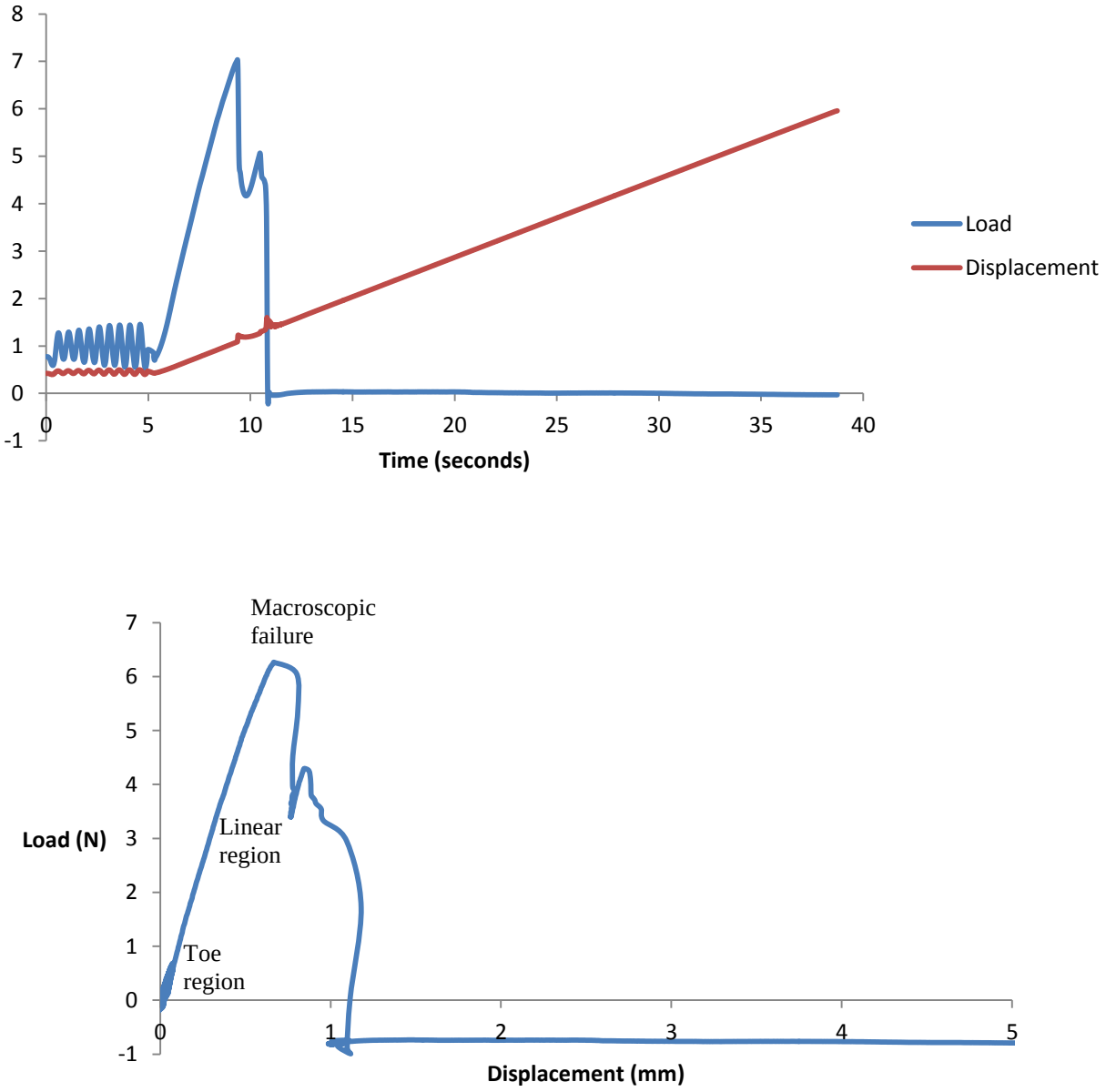

Figure 1.4 Load-displacement curve. (A) Change in load and displacement with time. Preconditioning is done in the first 5 seconds as illustrated by the sine wave. In this region, there is minimal change in displacement, but it increases when ramp to failure starts after 5 seconds at

a rate of 0.167 mm/s. (B) Patellar tendon of a C57Bl/6 mouse (approximate age 24-28 week old) ruptured at the tibial insertion with a maximum load of 6.26 N. The length was not measured for this tendon; therefore, stress-strain data could not be produced. The shape of the graph is similar to the stress-strain curve, showing a toe region, linear region, and macroscopic failure at 6.26 N.

1.4 Tendon physiology

1.4.1 Response to exercise

Tendons have been shown to undergo remodelling in response to exercise, and this response can be measured at the biomechanical, biochemical, and structural levels. Tendon has a capacity to adapt its structural and mechanical properties to the functional demands of the whole muscle-tendon unit (Buchanan, 2002).

With respect to biomechanical properties, endurance training results in increased tensile strength and stiffness of the tendon. In a study done by Viidik et al., the rabbit tibialis posterior and Achilles tendon had increased stiffness of approximately 10% and tensile strength increase by approximately 5%, when these rabbits were trained for 40 weeks on a running machine (Viidik, 1967, 1969). Another study by Woo et al. of swine digital extensor tendons, a 12-month training program of running at maximum speed increased ultimate strength by 62%, along with an increase in stiffness (Woo, 1980). Therefore, there seems to be a correlation between increased tendon strength and stiffness associated with long-term training; however, this response seems to be better in growing animals than in adult animals (Jozsa, 1997).

Since collagen is the most abundant protein found in tendon, theoretically, it can be expected that there may be changes in collagen concentration. However, research studies done on Achilles

tendons of trained rats (Vailas, 1985), and chickens (Curwin, 1988), or peroneous brevis tendons of rabbits (Viidik, 1967) did not show any difference in collagen concentration when compared to non-trained animals or birds. It has been reported that mechanical failure in collagen is not due to breakage of tropocollagen molecules but pulling apart of adjacent molecules (Wainwright, 1967). An important factor in determining the stiffness and tensile strength of collagen is an inter-molecular cross-link molecule called pyridinoline (Eyre, 1984). It was found that tensile strength of rat patellar tendon during healing was directly related to pyridinoline concentration. Therefore, changes in the mechanical properties of tendon due to exercise may be associated with a change in concentration of pyridinoline cross-links (Chan, 1998). On the contrary, physical training in growing chicks decreased the number of cross-links. Curwin et al. suggested that exercise leading to increased collagen turnover results in an accumulation of immature collagen with decreased cross-links (Curwin, 1988). Overall, the data suggests that training does not increase collagen concentration.

At a structural level, tendons of exercised rabbits, when compared to control animals, showed no difference in dry or fresh weights (Viidik, 1967). This finding was supported by few other studies that showed that training did not alter the dry weight of patellar tendon of trained rats (Vailas, 1985), or the Achilles tendon of trained chicks (Curwin, 1988). Studies of cross-sectional area (CSA) of tendons reported that there was in increase following long-term training (Woo, 1980; Birch, 1999). Microscopically, the collagen fibers and fibrils are thickened (Enwemeka, 1992), and these fibrils were more intensely packed in the Achilles tendon of rats after 30 days of exercise (Vilarta, 1989).

In summary, exercise in normal tendons immediately increases the uptake of blood flow, glucose, and oxygen. It improves protein production by tenocytes and increase in type I collagen production is observed even with a single exercise session (Langberg, 1999, 2007). An increase in cross-sectional area was also seen in tendons along with collagen fibril thickness and increase in concentration of nuclei. Long term training resulted in increased tensile strength and stiffness (Buchanan, 2002). The increased tenocyte activity leads to accelerated collagen synthesis and proteoglycan matrix (Jozsa, 1997).

1.4.2 Response to immobilization

Immobilization has a negative effect on tendons, even though it is a slow process due to low metabolic rate and vascularity of tendons (Kannus, 1992). The tensile strength of the tendon decreases along with a reduction in elastic stiffness and total weight of the tendon (Woo, 1982). At a microscopic level, collagen fibers become thinner and disorientated, with longitudinal splitting of the fibers (Jozsa, 1984). Cross-links decrease in number. Immobilization of hind limbs of young rats for five weeks showed a decrease in the number of the thick collagen fibrils, as well as the average diameter of the fibrils (Nakagawa, 1989). Proteoglycan content is also shown to be decreased, with tenocytes showing alteration in size and shape of mitochondria and nuclei (Jozsa, 1989). The activity of enzymes catalyzing collagen biosynthesis was decreased in immobilized Achilles tendons (Karpakka, 1991). The biosynthesis of new collagen is likely to be decreased during immobilization; however some studies show that hydroxyproline, an indicator of collagen concentration, remains unchanged during moderate immobilization. Overall, total hydroxyproline maybe decreased during immobilization due to reduction in collagen synthesis

(Akenson, 1973; Sayolainen, 1988). The effects of exercise and immobilization on tendon tissue are summarized in Figure 1.5.

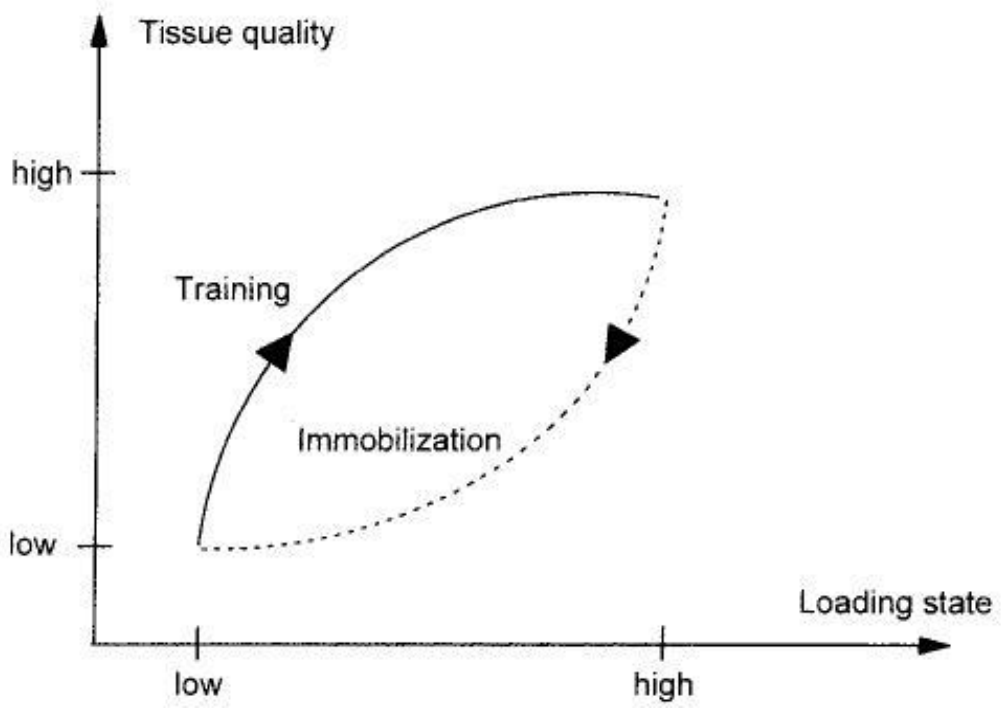

Figure 1.5 Schematic presentation of the effects of training and immobilization on the quality of tendon tissue (Kannus, 1997, reproduced with permission).

1.4.3 Effects of aging

As a person ages, changes are seen in both the collagenous and noncollagenous component of tendons (Jozsa, 1997). The collagen content does not change much in old age; however, there is a decrease in the proportion of proteoglycans and glycoproteins present. The elastic component and water content increase into early adulthood and decrease with old age. Cross-linking of tropocollagen molecules in collagen increases with aging, and this causes a decrease in collagen solubility (Best, 1994). The number of thick fibrils, as well as the diameter of these fibrils, is also decreased (Nakagawa, 1994). There is a decline in collagen turnover as the tenocyte number decreases with aging as well. With a reduction in tensile strength, the collagen becomes tougher,

its fibers shrink, and the tendon becomes stiff. With an overall decrease in elastin, proteoglycan, and water content, there is reduction in tendon elasticity as well. A decrease in tendon blood flow and tenocyte activity affects its repair ability. Due to all these changes, an aged tendon is weaker and more likely to tear from overuse injury (Jozsa, 1997).

1.4.4 Gender influences

With respect to gender, most tendon injuries occur in males with a ratio of 7 to 1 and 4 to 1 in Achilles tendon ruptures (Wong, 2002). Sixty percent of the overuse injuries occur in males (Segesser, 1980). Majority of the Achilles tendon injuries from sports occur in males because of their higher rates of participation in sport (Kvist, 1994). Larger cohort studies have also found that females have a decreased risk for developing patellar tendinopathy (Cook, 2000). Women under the age of 30 are also at the greatest risk for overuse injuries (Maffulli, 2003), as the proportion of female participants has increased in the past few decades (Maffulli, 2002). Tendinopathy induced through work, which involves repetitive overloading of the muscle-tendon unit, is present in over 60% of women, with a mean age of 41 years. The upper limb is involved in 93% of the cases of which 63% occur in the forearm, and 24% is elbow epicondylitis (Kivi, 1984).

1.5 Tendon injury and repair

1.5.1 Phases of healing and cells

There are three phases of healing that are initiated by the body when tendons are injured. The homeostasis/inflammation is the initial phase that occurs immediately after tendon injury.

Erythrocytes, and inflammatory cells as neutrophils, enter the site of injury. Monocytes and macrophages predominate in the first twenty-four hours to phagocytose the necrotic material. As vasoactive and chemotactic factors are released, there is an increase in vascular permeability, initiation of angiogenesis, tenocyte proliferation, and recruitment of more inflammatory cells (Murphy, 1994). The migration of tenocytes to the wound initiates synthesis of type III collagen (Oakes, 2003). The second phase of proliferation/fibroplasias phase begins after a few days. The synthesis of type III collagen is increased and lasts for few weeks, along with an increase in water content and glycosaminoglycan concentrations, and fibroblasts are the predominant cells (Lin, 2004). The remodelling/maturation phase begins after about six weeks, with a decrease in cellularity, and collagen and glycosaminoglycan synthesis. It is further divided into consolidation and maturation stage (Tillman, 1996). In the consolidation stage (from six to ten weeks), (i) the repair tissue changes from cellular to fibrous, (ii) tenocytes and collagen fibers become aligned in the direction of stress (Hooley, 1979), and (iii) type I collagen is synthesized in increasing amounts (Abrahamsson, 1991). The maturation stage begins after ten weeks, in which fibrous tissue changes to scar-like tendon tissue over the ensuing year (Hooley, 1979; Parkas, 1973). There is more type I collagen compared to type III in this phase, with accumulation of collagen cross-links (Sharma, 2005).

Extrinsic and intrinsic healing are two models of tendon healing. In extrinsic healing, the tendons are thought to have no internal ability to heal on its own (Potenza, 1962, 1969), and require invasion of cells from the surrounding sheath and synovium (Sharma, 2005). Therefore, this type of healing results in the formation of adhesions due to inflammatory cells infiltration with

fibroblasts and new blood supply derived from surrounding tissues (Potenza, 1962, 1969). Cells from the tendon sheath produce less collagen and GAG compared to epitenon and endotenon cells (Sharma 2005). On the contrary, in intrinsic healing the tendon is believed to heal by proliferation of epitenon and endotenon tenocytes with intratendinous blood supply (Mass, 1991). Epitenon tenoblasts initiate the repair process through proliferation and migration (Manske, 1985). Internal tenocytes secrete larger and more abundant collagen fibers than epitenonon cells, thereby contributing to the intrinsic repair process (Fujita, 1992). Fibroblasts in the epitenon and tenocytes synthesize collagen during repair. Collagen is initially synthesized by epitenon cells, and later by endotenon cells (Ingraham, 2003).

There are fewer complications and improved biomechanics with intrinsic healing. With this type of healing, a normal gliding mechanism is preserved within the tendon sheath (Koob, 2002). On the other hand, extrinsic healing leads to scar tissue which results in adhesion formation, and this disrupts gliding. The biochemical and mechanical properties of healed tendon never match those of an intact tendon. For instance, the rupture force of a spontaneously healed transected sheep Achilles tendon was only 56.7% of normal at 12 months (Bruns, 2000), which could be due in part to the absence of mechanical loading, and thus de-adaptation of the tendon as a whole, during the immobilization period (Sharma, 2005).

1.5.2 Growth factors

Cellular growth factors and cytokines are important for their roles in embryonic tissue differentiation and during wound healing (Gabra, 1994; Goomer 2000). Growth factors stimulate cell differentiation, chemotaxis, angiogenesis, and ECM synthesis (Mason, 1941; Nakamura,

1998). The influence of growth factors on tendon healing was studied by comparing the cytokine profiles of injured and normal tendons. It was shown that wounding and inflammation cause release of growth factors (Nakamura, 1996; Party, 1978).

Growth factors such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) are upregulated throughout tendon repair. They are produced by intrinsic and extrinsic cells and have dose dependant effects. Not only do they require specific receptors to be active, but they also work together with other signalling molecules (Molloy, 2003).

(i) Platelet-derived growth factor: PDGF is a group of dimeric polypeptide isoforms consisting of three types of structurally similar units (Molloy, 2003). Healing tendons have an increased concentration of PDGF. PDGF influences the rate of fibroblast proliferation; also, chemotaxis and collagen synthesis are somewhat elevated (O'Brien, 1992). PDGF is thought to play a significant role in the early stages of healing, by inducing the synthesis of other growth factors, such as IGF-1 (Lynch, 1989). Yoshikawa et al. show the role of PDGF during tissue remodeling. It was observed to stimulate both collagen and non-collagen protein production and DNA synthesis in a dose-dependent manner (Yoshikawa, 2001).

(ii) Insulin-like growth factor: The upregulation of IGF-1 during the initial repair process and the inflammatory phase stimulates the migration and proliferation of fibroblasts and inflammatory cells to the wound site (James, 2008; Kurtz, 1999). James et al. also

mentioned that IGF-1 may be stored as an inactive precursor protein in normal tendon, and released as a growth factor by enzymes upon injury. The local increase of IGF-1 after soft tissue injury, at both the mRNA and protein levels, is associated with corresponding up-regulation of its receptors (Bos, 2001; Edwall, 1989). In the later phases of remodeling, IGF-1 functions in stimulating synthesis of collagen and other ECM components. This is shown by in vitro studies and it has been reported that these effects are dose-dependent (James, 2008). Overall, IGF-1 stimulates the proliferation and migration of fibroblasts and other cells at the site of injury, followed by increase in collagen production and other ECM components during remodeling stages.

(iii) Transforming growth factor-β: In the healing process of an injured tendon, TGF-β plays an important role in directing the fibroblast migration (Roberts, 1988), neovascularization, and secretion of ECM proteins like procollagen type I and III (Kashiwagi, 2004). It is normally expressed in the process of inflammation and reorganization at 4 and 7 days, but its expression decreases at 2 and 4 weeks (Chan, 2003), which may be due to loading and mobilization of the tendon (Eliasson, 2009). It is also shown that this TGF-β expression increases again at 8 weeks. The changes in expression reflect the initial inflammation and activation of cells, followed by intermediary period, and then the remodeling of the tendon with increased apoptosis of cells (such as fibrocytes) that are no longer necessary (Jorgensen, 2005; Wurgler-Hauri, 2007). In a healing tendon, TGF-β induces the expression of collagen I and III,

and accelerates the healing process by improving the mechanical properties of the tendon (Hou, 2009); Kashiwagi, 2004).

(iv) Vascular endothelial growth factor: During early granulation tissue development, VEGF is required for the formation of an initial vascular plexus. In neovascularization, which occurs during the repair stage of tendon healing, VEGF contributes to vascular bud formation and endotheliocyte migration (Muller, 2012). VEGF is expressed in the healing tendon, along with increased expression of receptors 1 and 2 (Petersen, 2003). In a healing tendon, VEGF increases vascularization from 1 to 8 weeks after rupture (Hou, 2009). It is essential for establishing and maintaining the vasculature present in the endotenon and epitenon (James, 2008).

(v) Basic fibroblast growth factor: bFGF increases cellularity during the early healing process in a healing tendon (Chan, 2008). In the ECM, it increases lubricants and matrix metalloproteases (MMPs) during the first week, therefore, creating the space needed for additional tenoblasts and tenocytes (Thomopoulos, 2010). The initial increase in bFGF stimulates the proliferation of tenocytes and fibroblasts needed for ECM repair (Muller, 2012).

(vi) Bone morphogenic protein -12: BMP-12 plays an important role in tendon healing as it increases tendon callus and improves mechanical properties such as, strength and stiffness (Forslund, 2003; Majewski, 2008). In the first week of healing, BMP-12 leads to a more organized tendon tissue (Murray, 2007), higher volumes of collagen I, and an earlier of fibroblasts to fibrocytes (Majewski, 2008).

1.6 Epidemiology of tendinopathy

1.6.1 Prevalence of different tendinopathies

Overuse injuries of tendons are usually induced through work or sports due to overloading of muscle-tendon work unit, and tendinopathy is the most commonly diagnosed musculoskeletal disorder (Forde, 2005). Work induced overuse injuries are of upper extremities and a risk for those manual workers who engage in repetitive or forceful movements (Jozsa, 1997). Common upper extremity tendinopathies include rotator cuff injury, lateral and medial epicondylitis, and De Quervain's tenosynovitis. Shoulder tendinopathies in physical workers have an incidence of 4% to 56% for hand and wrist tendinopathies, and this risk is increased with a combination of high force, repetition, or exposure to vibration during repetitive work (Werner, 2005).

Lateral epicondylitis or tennis elbow is common in athletes of all ages, with an incidence of 9% to 40% in tennis players (Maffuli, 2003; Scott, 2006). It is 5 to 10 times more common than medial epicondylitis (Hume, 2006), and also 2 to 3.5 times more frequent in people over the age of 40 (Scott, 2006). Rotator cuff tendinopathy represents 18% of overuse injuries in adult athletes (Maffuli, 2003), and anterior shoulder pain due to this condition is often present in swimmers (Scott, 2006). Bicipital tendinopathy is the most common occurring pathology in the wheelchair population (Finley, 2004), as the shoulder is reported as the most common site of pain (Kaplan, 2005). De Quervain's disease seems to be the best known form of paratendinopathy of the wrist and hand, which is caused by stenosing tenosynovitis of the first dorsal compartment of the wrist. It is approximately six times more common in women than in men (Maffuli, 2003).

Achilles tendinopathy is the most prevalent tendinopathy of lower extremities with a frequency of 50% in elite endurance athletes and 5.9% in sedentary individuals (Scott, 2006; Fredberg, 2008). It is highest in individuals participating in sports such as long-distance running, tennis, badminton, and soccer (Kvist, 1994). Mid-portion tendinopathies and insertional problems are the most common clinical Achilles disorders (Jarvinen, 2005).Achilles tendinopathy is more prevalent in males because of their higher rate of participation in sports (Kvist, 1994). One quarter of the athletes that are treated for a knee injury are diagnosed with tendinopathy. The most common knee disorder is the jumper's knee, which has an incidence of 7 to 40%. Patellar tendinopathy represents two-thirds of knee conditions induced by volleyball or basketball practice (Scott, 2006). Males are affected twice as often as females due primarily to their activity levels (Mafulli, 2003). Hamstring tenosynovitis accounts for 3% of knee problems and is present in patients active in sprinting, jumping, and soccer (Maffuli, 2003). Greater tronchanteric pain syndrome is mainly caused by tendinopathy of the gluteus medius tendon (Bard, 2009). It has an incidence of 1.8 patients per 1000 per year, and a higher prevalence in women and in patients with coexisting back pain, osteoarthritis, iliotibial band tenderness, and obesity (Williams, 2009).

1.6.2 Risk factors

There are intrinsic and extrinsic risk factors that interact, making an individual more susceptible to tendon injury. The most common intrinsic risk factors are alignment abnormalities, leg length discrepancy, muscle weakness and imbalance, decreased flexibility, joint laxity, female sex, aging, and overweight obesity (Josza, 1997).

The most common misalignment in the foot is hyperpronation. Excessive and prolonged pronation during walking or running creates increased stress on the soft tissue of the foot, resulting in an angular traction (whipping or bowstring action) on the Achilles tendon. It can cause secondary effects to the lower extremities resulting in lower leg and knee problems, and can also occur because of anatomic reasons such as leg length discrepancy (LLD) (Renstrom, 1985). LLD of 5-6mm may be symptomatic; however, proper shoes with orthotics are required for 10 mm discrepancy or more to prevent overuse symptoms. Biomechanical alterations may occur due to LLD such as the pelvic tilting to the shorter side resulting in lumbar scoliosis and compression of the inner intervertebral disc, along with excess foot pronation of the foot on the longer or shorter side. Therefore, LLD is may be a factor in development of lower back pain, hip osteoarthritis, patellar tendinitis, and stress fractures (Renstrom, 1991). Muscle imbalance refers to asymmetry between the extremities, or the agonist and antagonist muscles in one extremity. No direct relationship has been found between muscle weakness or imbalance, and injury. It is also not clear if muscle imbalance is a causative factor for reinjury as persistent joint instability, pain, swelling, and impairment of neuromuscular coordination are also involved (Jozsa, 1997).

Musculotendinous flexibility is also important for physical performance and for prevention of sports injuries such as muscle spasm, muscle rupture, tendinitis, and patellofemoral pain syndrome. As with muscle imbalance, there is not enough evidence that musculotendinous tightness is the cause of injury (Jozsa, 1997). Joint laxity or instability can be caused by a ligamentous injury, and lead to problems in knee, ankle, and shoulder joints, and their tendons. The participation of women in sports has increased over the years, but studies also show that

there is a higher incidence of tendon injuries among women than men during athletic endeavours (Drinkwater, 1988; Kannus, 1987). This could be due to their weaker musculoskeletal system not being able to absorb the repetitive impact loads on the body, compared to the musculoskeletal system of men of equal body weight. Women have less muscle mass per body weight (20 to 25%) than equally trained men (40%), and their overall muscle strength averages about two-thirds to that of men (Drinkwater, 1988). Along with other functions of the body, the musculoskeletal system is also affected with age. Sports injuries in elderly athletes are more frequently overuse-related rather than acute, and more commonly degenerative (Jozsa, 1997). Being overweight has been associated with many diseases such as diabetes, hypertension, stroke, osteoarthritis, gall stones, and lung emphysema. In full weight bearing recreational physical activities, being overweight may be a problem due to development of knee and hip osteoarthritis (Hartz, 1986). Tendon overuse symptoms may also be associated with overweight (Jozsa, 1997).

The extrinsic risk factors are those that act externally on the human body. The most common extrinsic risk factors are training errors, poor environmental conditions, and equipment. Training errors account for 60-80% of tendon and other overuse injuries (James, 1978). These result from a distance that is too long, a training intensity that is too high or progressed too quickly, and excessive hill work (whether uphill or downhill). Poor technique and fatigue also play a role in injury development, and if repeated continually, can cause tendon overuse injuries. Fatigued muscles have a decreased ability to absorb repetitive shock or stress, and can result in overuse injuries such as tennis elbow, Achilles tendinopathy, and medial tibial stress syndrome (Jozsa, 1997). Environmental conditions such as extreme temperatures, humidity, and high altitude can

all play a role in overuse injury pathogenesis. In sports like downhill or cross-country skiing, ski jumping, and mountain climbing, low temperature can inhibit contractility and decrease the shock absorption capacity of the tendon muscle units. Improper equipment may promote overuse injuries. For instance, a tennis racket that is too stiff in composition or strung too tightly can transmit increased forced and initiate symptoms of tennis elbow. Use of proper equipment is a way to prevent many sports injuries (Jozsa, 1997).

1.7 Summary: literature on link between tendinopathy and cardiovascular disease/lipids

Hypercholesterolemia is defined as the presence of high levels of cholesterol in the blood. It is a risk factor for many conditions such as cardiovascular disease (Romero-Corral 2006), kidney disease (Chalmers, 2006), and type 2 diabetes mellitus (Smith, 2001). Hypercholesterolemia has been reported to affect approximately 17% of the US population 20 years and older (Schober, 2007). Recent research suggests that some musculoskeletal problems may be linked to hypercholesterolemia. Tendinopathy, a major cause of morbidity in the work force (Pottie, 2006), has traditionally been thought to occur as a consequence of increased mechanical loading (i.e. overuse) (Gaida, 2008). However, one third of the cases for midportion Achilles tendinopathy occur in sedentary individuals, and it is often observed in overweight individuals (Rolf, 1997). In the study by Rolf et al (Rolf, 1997), surgeries were performed on patients suffering from achillodynia. Most of the cases were associated with sports activity, but 31% of the patients had no association with sports or excessive physical activity.

Cross sectional data from research studies suggested that patients with chronic Achilles tendinopathy demonstrated dyslipidemia, characterized by lower HDL-C and higher TG levels (Gaida, 2009). Similarly, Ozgurtas et al. reported increased total cholesterol, LDL-C and TG levels, and lower HDL-C levels in patients with Achilles tendon ruptures compared to patients without rupture. The mean age was 25.7 years in the study group of 47 patients and 32.6 years in the control group of 263 subjects. Blood samples were obtained from the patient group within 6-8 hours following Achilles tendon rupture (Ozgurtas, 2003). Dyslipidemia is also associated with rotator cuff tears. Compared to controls, patients with torn rotator cuff tendons had increased total cholesterol, TG, LDL-C and decreased HDL-C (Abboud, 2010). Serum levels and lipid profiles were collected from 74 patients with rotator cuff tears, with mean age of 66.3 years. The control group consisted of 73 patients with shoulder complaints unrelated to tendons (mean age 67.4 years).

Another research study further supports the deposition of lipid in tendons. Tissue samples prepared from tendons from necropsy from subjects ages 5-88 were stained in Oil Red O, Sudan black, and bromine Sudan black. Lipids accumulated in tendons in ages 40 and on. Lipid deposits in tendons were extracellular and arranged as finely dispersed droplets along the axis of collagen fibers. Histochemical results showed that the tendon deposits contained mainly cholesterol in its esterified form (Adams, 1974).

The link between hypercholesterolemia and tendon pathology is highlighted by research on tendon xanthomas, a dramatic but common occurrence in patients with severe, familial hypercholesterolemia (Tsouli, 2005). Tendon xanthomas are characterized as a painful

thickening of the tendon which can lead to spontaneous tendon rupture (Fahey, 1973; Lehtonen, 1981); the pathology is driven by an accumulation of cholesterol in tendon both extracellularly and intracellularly (Kruth, 1985). An increase in tendon size is observed in Achilles tendon xanthoma due to lipid infiltrating the tendon and the surrounding tendon sheath, as well as a local inflammatory reaction with peritendinous edema, the combination of which results in a painful thickening (Galloway, 1940).

1.8 Animal models for studying tendon

Animal models are used to study human diseases to allow in-depth studies on etiology, molecular mechanisms, and potential treatment. Variation among animals is easier to control, making it possible to isolate the effect of a single factor. Unlike humans, it is easier to obtain tissue samples in animals, even at early time points in the pathological process.

Using a single animal model would not be realistic to study all aspects of tendinopathy, as every animal model has its advantages and disadvantages. To be considered a valid model for tendinopathy, the animal needs to consistently replicate clinical, histopathological and functional characteristics of tendinopathy in humans (Lui, 2010). Horses and dogs have been used in the past and developed tendinopathy when trained and raced (Dowling, 2000; Fransson, 2005). Due to the anatomical differences between quadriceps and bipeds, along with their large size and high costs, they are not considered practical laboratory animal models for tendinopathy (Lui, 2010). Current animal models frequently being used are rats and rabbits as they are less costly and

easily available compared with large animals. The larger tendons in rabbits provide larger samples for analysis, and make it easier for manipulation during surgical procedures, whereas rats are more resilient and have frequently been used in studies of running-induced tendinopathy. Despite this, both animal models are popular but having a better understanding about their behaviour and physiology is essential (Lui, 2010).

One of the challenges in using animal models is that tendinopathy is defined as an activity related pain with tendon thickening. This causes some limitations as it is not possible to directly ask the animal how much pain it experiences in response to its athletic performance. Rather, the animal is monitored, and the pain is related to gait pattern or withdrawal reflex. However, these patterns are easier to observe in large animals like horses than small animals like mice and rats. As mentioned earlier, there is no validated animal model for all tendinopathies observed in humans. Mice are considered an appropriate species for human diseases mainly due to their lower cost, short life cycle, and availability. Transgenic and knockout mice provide tools for understanding the pathophysiology of tendinopathy. Even then, the use of mice is limited as its small size causes difficulty in manipulation such as intratendinous injection, histology, biomechanical testing, functional testing, and sample amount. Overall, it is necessary to study the behavioural changes, such as stress, pain, and discomfort, during the establishment and use of the animal models (Liu, 2010).

1.9 Summary of findings from ApoE-deficient mice

1.9.1 Importance of ApoE

ApoE is one of the lipoprotein transfer genes that functions in a receptor-mediated lipoprotein removal from the blood. It is a glycoprotein synthesized in the liver and brain, and a constituent for all lipoproteins except LDL. Furthermore, it is a ligand for receptors that clear chylomicrons and very low density lipoproteins (VLDL) remnants (Meir, 2004). Synthesized by monocytes and macrophages in vessels, it has effects on cholesterol homeostasis and inflammatory reactions in atherosclerotic vessels (Curtiss, 2000).

1.9.2 ApoE-deficient mouse

Genetic variation at the ApoE locus is suggested to be associated with increased risk of developing atherosclerosis and hypercholesterolemia. Knockout mice are used to study gene function by creating mice without the desired gene. Mice deficient in ApoE is the first animal model used for atherosclerosis, and to show the importance of high density lipoproteins in preventing atherosclerosis (Plump, 1995).

Mice are considered the mammalian system of choice to study the genetic contributions to disease because of their advantages like easy breeding, short generation time, and availability of inbred strains. They are normally resistant to atherosclerosis but ApoE deficiency leads to severe hypercholesterolemia that makes them highly susceptible to this disease (Jawien, 2004). This condition is known to be similar in humans (Plump, 1995). However, there are differences as well, the most obvious being the life span of a mouse which is 2 years, compared to about 75 years in humans. Mice also weigh much less, and their lipid profile is very different that in

humans. Mice carry most of their cholesterol on HDL lipoproteins whereas human carry about 75% of their plasma cholesterol on LDL (Jawien, 2004).

ApoE deficiency results in an inability to clear plasma lipoproteins. When fed a low-fat diet of mouse chow, ApoE-deficient mice develop significant hypercholesterolemia and have increased sensitivity to dietary fat and cholesterol (Plump, 1995). They have high levels of plasma cholesterol (500 mg/dl) compared with wild type mice (60-90mg/dl). On high fat diet, their plasma cholesterol levels increase to greater than 2000 mg/dl (Curtiss, 2000), and they also develop complex lesions. Macrophages are present in these lesions as their function is to phagocytose modified lipids, lipoproteins, and cell debris, which leads to formation of foam cells (Jawien, 2004).

1.10 Objectives

The overall objective is to study the link between hypercholesterolemia and tendon pathology by using the Apolipoprotein E-knockout (ApoE-KO) mice model. The ApoE-KO mice will be fed a high fat or regular diet starting at 7 weeks of age. This diet is known to cause rapid development of hypercholesterolemia and atherosclerosis, and is widely used by academic and industry research as a valid model of cardiovascular disease (Plump, 1995). The animals will be sacrificed at 0, 15, and 30 weeks. Tendon structure and function will be examined using validated measures. C57Bl/6 mice will be used as controls for both on and off high fat diet.

1.11 Aims

To determine if, after 15 or 30 weeks on either a normal or high fat diet, ApoE-KO mice demonstrate the following changes, in comparison to wild type controls:

1) An increase in lipid deposition in the Achilles tendons.
2) An increase in the cross-sectional area of patellar tendons.
3) Changes in gene expression of *Cpa3, TGF-β, Col1a1,* or *Col3a1* levels in the tail tendons.

1.12 Hypotheses

We hypothesize that ApoE-KO mice will demonstrate, in comparison to wild type mice (WT):

1) Increased lipid deposition in the Achilles tendon, as measured by the positivity ratio of Oil Red O staining
2) Increased patellar tendon cross-sectional area, as measured by high resolution ultrasound
3) Increased gene expression levels of *Cpa3, TGF-β, Col1a1,* and *Col3a1*, as measured with quantitative PCR

We further hypothesize that mice fed a high fat diet will demonstrate the same changes outlined above, in comparison to mice receiving a normal diet.

1.13 Significance of research

Tendon problems and cardiovascular disease are both very prevalent in our society, and the paradox has often been noted that tendon problems are associated with increased mechanical demand on the one hand (e.g. athletes, workers), and with sedentary behavior and hypercholesterolemia on the other hand. The latter finding has received very little scientific or clinical attention. Studies on high cholesterol and tendon pathology can allow us to determine the link between the two, and if mechanism found, potentially therapeutic interventions can be used. In the future, the presence of tendinopathy could be seen as a prompt to measure serum cholesterol.

Chapter 2: Materials and Methods

2.1 Mice

All animal procedures were performed according to the guidelines for animal experimentation approved by Animal Care Committee of University of British Columbia. Male wild type C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and ApoE-KO mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). The knockout mice were derived from a C57Bl/6 background. ApoE-KO mice were bred on site and housed at the animal facility at Jack Bell Research Centre (JBRC).

2.2 Diet

All mice were fed either a high fat (21.2% fat, TD.88137, Harlan Tekland, Madison, WI) or regular chow (PicoLab Mouse Diet 20: 5053, LabDiet; Richmond, IN) diet beginning at 7 weeks of age for either 15 or 30 weeks. At 7 weeks of age, the animals were weighed and their initial age and weights were recorded (see Appendix A). While on diet, mice were checked twice per week and weighed once per week, and their weights were recorded on the Animal Monitoring Sheet (see Appendix B), along with any abnormalities observed. Their daily food intake was estimated by weighing food given and food remaining at each feeding, which was also done once a week. If mice developed cutaneous xanthomas or skin lesions, they were monitored closely every day. At the early signs of xanthoma development, the mice had their nails trimmed and hair shaved under anesthesia, and the corticosteroid ointment Theraderm was applied directly to the skin lesions. The degree of severity of skin lesions was determined by the animal care staff at

JBRC. Animals with severe skin lesions were considered for euthanasia if they exhibited ongoing distress and pain medication did not improve the condition within 6-7 days.

2.3 Tissue collection and processing

At their respective time points, the mice were weighed again (see Appendix A) and anesthetized. Isoflurane was used as an anesthetic agent with a calibrated vaporizer. The animal to be anesthetized was placed inside an induction chamber. The chamber was loaded with 5% isoflurane and 2 liters/minute oxygen for one to two minutes. The mouse was monitored closely by observing respiration changes. Once the breathing became slow but frequent, the pain reflex was checked (toe pinch of hind limb). If there was no response, the mouse was transferred onto a heating pad and isoflurane delivery continued through a mask it. The pain reflex was assessed again, i.e. loss of toe pinch reflex. The vaporizer was adjusted to 2-3% isoflurane to ensure proper depth of anesthesia.

Following anesthesia, the mouse was placed on the heating pad in dorsal recumbency (on its back). Blood was drawn using a cardiac puncture procedure under general anesthesia, isoflurane. By placing fingers around the chest to secure the mouse and using a 1-3 ml syringe with a 21-22g needle, the needle was inserted into the chest caudal to the xyphoid process at a 30 degree angle aiming at the left shoulder. Approximately 1 ml of blood was collected, following which it was ensured that the mouse was dead by performing cervical dislocation. These mice were then used to harvest tendon samples: tail, patellar, and Achilles tendons.

Two tail tendons were harvested from each mouse and stored in an RNA preservative (RNAlater) at -20°C for qPCR gene expression experiments. Patellar tendons were dissected

from both limbs of each mouse with patella-patellar tendon-tibia as one unit. They were wrapped in phosphate buffered saline-soaked gauze and frozen at -20°C until testing. Achilles tendons were removed with the gastrocnemius muscle attached, frozen with Tissue Tek Optimal Cutting Temperature (OCT) Compound, and stored at -20°C until further processing.

Mice that developed cutaneous xanthomas were treated accordingly and monitored closely. If there was no improvement, and the xanthomas worsened (greater than 1 cm^2) and caused obvious discomfort, the mouse was sacrificed before its time point. Tendons were collected from three of these mice and only included in the analysis of qPCR experiments, but not for morphological measurements (histology or cross-sectional area).

2.4 Histology

Frozen samples of Achilles tendon embedded in OCT were sent to a certified histology laboratory for hematoxylin & eosin (H&E) and Oil Red O staining (Centre for Translational and Applied Genomics, BC Cancer Agency, Vancouver, B.C.). For H&E staining, the frozen section slides of Achilles tendons were fixed in formalin, washed in water, and stained in Selectech Hematoxylin 560 (Leica Biosystems, Concord, ON). After staining, the slides were washed in water, differentiated in Selectech Define with base Selectech Bluing (Leica Biosystems, Concord, ON). Once again, these slides were washed in water, followed by staining in Selectech alcoholic Eosin Y 515 (Leica Biosystems, Concord, ON). The changes were done in 95% alcohol, 100% alcohol, and xylene, and then mounted. The H&E stained slides were viewed under a Zeiss Axiophot Upright Light Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

For Oil Red O staining, sections of frozen tissue were cut in the cryostat onto charged slides. These slides were rinsed in 70% isopropanol and stained with a solution of Oil Red O. Working Oil Red O solution was made with 70 ml stock Oil Red O (approximately 0.5g per 100 ml of 100% isopropanol) and 30 ml dH_2O. Before mounting with ImmunoHistoMount™ (ImmunoBioScience Corp, Mukilteo, WA, USA), the slides were counterstained with hematoxylin. Oil Red O stained slides were also viewed using the same microscope as for H&E stained slides, and were blinded using a random number generator. These slides were sent to St. Paul's Hospital (Vancouver, B.C., Canada) for digital slide scanning using an Aperio Scancope XT (Aperio Technologies, Vista, CA, USA). This is a brightfield system that digitizes entire microscope slides at 20X and 40X magnification and provides high resolution images – approximately 0.5 microns/pixel for 20X and 0.25 microns/pixel for 40X scans (Model: Aperio Scanscope XT, 120 slide capacity; Objective lenses: Olympus 20X/0.75NA Plan Fluor, Olympus 40X/0.75NA UPlanFL N). The digital scanned images were viewed and the Oil Red O staining was quantitated using ImageScope™ viewer software ((Aperio Technologies, Vista, CA, USA). The digital magnification was set at 2X, and the largest tendon region visible on the screen was chosen as the area of interest. Any folded regions of the tendon and aponeurosis were excluded. The largest possible tendon region was selected using a rectangle tool, followed by selecting the Positive Pixel Count algorithm (Version 9.1) for analysis. The hue value was changed to zero (red hue). A trial analysis confirmed that the algorithm successfully captured only red hued pixels.

2.5 Cross-sectional area

Prior to measuring cross-sectional area (CSA), the patellar tendons were trimmed to remove as much of the retinaculum and peritendinous tissue as possible, using the EMZ Zoom Stereo Microscope (Meiji Techno Co. Ltd, Santa Clara, CA, USA). Ultrasonography was used to determine the cross-sectional area of the tendon. The patella was clamped on one side into a metal clamp, and tibia on the other side, leaving the patellar tendon in the middle. The whole specimen with the metal clamps was submersed in phosphate buffered saline (PBS). CLI 1500 Water-Path Diagnostic Ultrasound Probe 35 MHz transducer (E-Technologies, Inc., Davenport, IA, USA) was used to scan the tendon in a transverse direction with a sweep angle of ten degrees. The image captured in the Reflex Ultrasound Bio-Microscope software (E-Technologies, Inc., Davenport, IA, USA) was outlined to calculate the tendon's CSA. CSA was measured twice for each tendon, and the correlation coefficient was calculated for reliability. Test-retest reliability of patellar tendon CSA measurements had a correlation coefficient of 0.81.

2.6 RNA isolation and quantitative PCR

2.6.1 RNA isolation

Total RNA was extracted from tail tendons of both ApoE-KO and C57Bl/6 mice on high fat and normal diet for 30 weeks, following the methods used in mouse patellar tendon injury studies (Scott, 2011; Sharma, 2011). Tail tendons stored in RNAlater at -20°C were transferred to a new eppendorf tube, and washed three times with nuclease-free water. They were flash-frozen immediately in liquid nitrogen, followed by placing these frozen tendons in a liquid nitrogen-cooled vial together with a liquid nitrogen-cooled stainless steel ball. The tendons were

powdered in a tissue mill (Mikro-Dismembrator S, Sartorius, Goettingen, Germany) for 30 seconds at 3000 RPM in cryogenic tubes (Nalgene, Rochester, NY). 1ml of Trizol reagent (Ambion by Life Technologies, Burlington, Ontario, Canada) was added to the powdered tissue, followed by mixing until all the powder was dissolved. These samples were stored at -80°C for at least 30 minutes before continuing onto the extraction step.

2.6.2 RNA extraction

For extraction, the samples were thawed at room temperature. Equal amounts of chloroform (Fisher Scientific, Fair Lawn, New Jersey, USA) was added, mixed by shaking vigorously for 30 seconds, and incubated for 1-2 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes at 4ºC (Sorvall Legend Micro 21R Centrifuge, Thermo Scientific). The top aqueous layer containing RNA was transferred to a new tube and used for purification.

2.6.3 RNA purification

The RNA extracted was purified using the QIAGEN RNeasy® Mini Kit (Qiagen Inc., Toronto, Ontario, Canada) for the first four samples (49, 22, 23, 24) randomly chosen for the experiment. Only four samples were purified using this kit due to the availability of the materials. RNA yields were checked for these samples and compared with the rest of the samples. The housekeeping gene values were also checked for consistency, as their threshold cycle is expected to be between 14 and 16. The purification procedure was followed according to instructions provided by the manufacturer (Qiagen Quick-Start Protocol, steps 2-7). Ethanol was added to the samples, and transferred to the RNeasy spin columns. Several wash steps were carried out with the buffers provided in the kit. After the final wash, RNA was eluted with 30µl of RNase-free

water. The rest of the 50 samples were purified using Thermo Scientific GeneJET RNA Purification Kit. The procedure was followed as outlined in Thermo Scientific GeneJET RNA Purification Kit Handbook (page 6-7, steps 4-10). 100µl of RNA was eluted for all the samples purified using this kit. RNA concentrations were measured using the NanoDrop 2000 Spectrophotometer and software. All RNA samples were stored in -80°C until further use.

2.6.4 cDNA synthesis and qPCR

Each tail tendon sample was analyzed in triplicates for qPCR analysis, and standard errors of all the samples are listed in Appendix E. Sample with the lowest RNA concentration was used to calculate the amount of RNA needed for cDNA synthesis and quantitative PCR (qPCR). RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), as described in previous studies (Scott, 2011; Sharma, 2011). Gene expressions of *Cpa3, TGF-β, Col1a1,* and *Col3a1* were quantitated using qPCR on an ABI (Applied Biosystems) 7500 Fast system with SYBR green probe and primers sets for *Cpa3*, *TGF-β*, *Col1a1*, and *Col3a1* (Table 2.1). Each replicated was performed in triplicates. The quantity of mRNA for each gene was computed using $2^{-\Delta Ct}$ method.

Gene	**Forward Primer**	**Reverse Primer**
Cpa3	TTGGTCATGGACACAGGATCG	GTTGAGGTCAGTGCCAATGC
TGF-β	GCTGAACCAAGGAGACGGAA	ATGTCATGGATGGTGCCCAG
Col1a1	CGATGGATTCCCGTTCGAGT	GAGGCCTCGGTGGACATTAG
Col3a1	CAGGAGAAAAGGGTCCTCCC	ATACCCCGTATCCCTGGACC

Table 2.1 Primer sequences for qPCR

2.7 Statistical analysis

After consulting with a statistician, a linear model was used to analyze differences in weight among ApoE-KO and C57Bl/6, normal and high fat diets, at the 15 week and 30 week time points. For cross-sectional area, Oil Red O staining, and mRNA levels, a linear mixed model was used to determine if there were any significant differences according to strain (ApoE vs. C57Bl/6) or diet (regular chow vs. high fat) at 15 and 30 weeks. Normal quantile plots and residuals plots were graphed to ensure the assumptions of the tests were met (see Appendix C).

Chapter 3: Results

3.1 Morbidity and skin pathology

A total of 77 mice were used for this study of which 36 were C57Bl\6 and 41 were ApoE-KO mice. Ten of the ApoE-KO mice were euthanized prior to their designated time points due to cutaneous xanthoma or skin lesion development. Of all the 36 C57Bl\6 mice, 11 were assigned to no diet (i.e. baseline/0 week time point), 12 to regular chow, and 13 to high fat diet. The C57Bl/6 mice did not develop any skin lesions and all survived the 30 week period, even when fed a high fat diet. Specifically, the ApoE-KO mice developed xanthomatotic skin lesions. They also showed a decline in health compared to the wild type C57Bl/6 mice, resulting in increased morbidity including hair loss, signs of frailty, hair graying, skin lesions on back, neck, and irritation of ears, but the condition was more severe for mice on high fat diet. Of all the 41 ApoE-KO used in the study, 10 were assigned to no diet (i.e. baseline/ 0 week time point), 13 to regular chow diet, and 18 to high fat diet. For ApoE-KO mice fed regular chow diet, 4/13 (31%) showed signs of xanthomas or skin lesions, with the earliest case occurring at 10 weeks. 1/4 (25%) of these mice had to be sacrificed earlier than its experimental endpoint. When fed a high fat diet, 16/18 (89%) of the ApoE-KO mice developed skin lesions or ear irritations, with the earliest case occurring at 9 weeks. 9/16 (56%) had to be sacrificed earlier than their experimental endpoint. Two other mice, one on high fat and one on regular chow diet, were sacrificed due to conditions/diseases other than those resulting from the experiment.

3.2 Weight

Strain, week, and diet were all factors that had a significant effect on weight. The starting weights of mice of both strains are listed in Appendix A. Wild type C57Bl/6 mice were significantly heavier than ApoE-KO mice ($p<0.01$) (Figure 3.1). Mice that were fed a high fat diet were heavier than those on regular chow ($p<0.001$), and those who were sacrificed at 30 week time point were heavier than those sacrificed at 15 weeks ($p<0.001$). High fat diet resulted in a significant weight gain in mice overall at the 30 week time point but not at the 15 week time point. As illustrated in Figure 3.1, at the 15 week time point all mice had similar weights.

The interaction between diet and week demonstrates that mice on high fat diet at 30 week time point were significantly heavier than mice on high fat diet at 15 week time point. Within each time point, mice fed on high fat diet were significantly heavier than those on regular chow diet, as seen in Figure 3.1 ($p<0.001$). Furthermore, the interaction between diet and strain also demonstrates that high fat fed C57Bl/6 mice were significantly heavier than high fat fed ApoE-KO mice. Within each strain, high fat diet led to a significant increase in weight compared to regular chow diet ($p<0.05$). There was also an interaction between strain and time point. C57Bl/6 mice at 30 week time point were significantly heavier than ApoE-KO mice at 30 week time point. Moreover, mice on diet for 30 weeks had a significantly higher weight compared to mice on diet for 15 weeks ($p<0.01$).

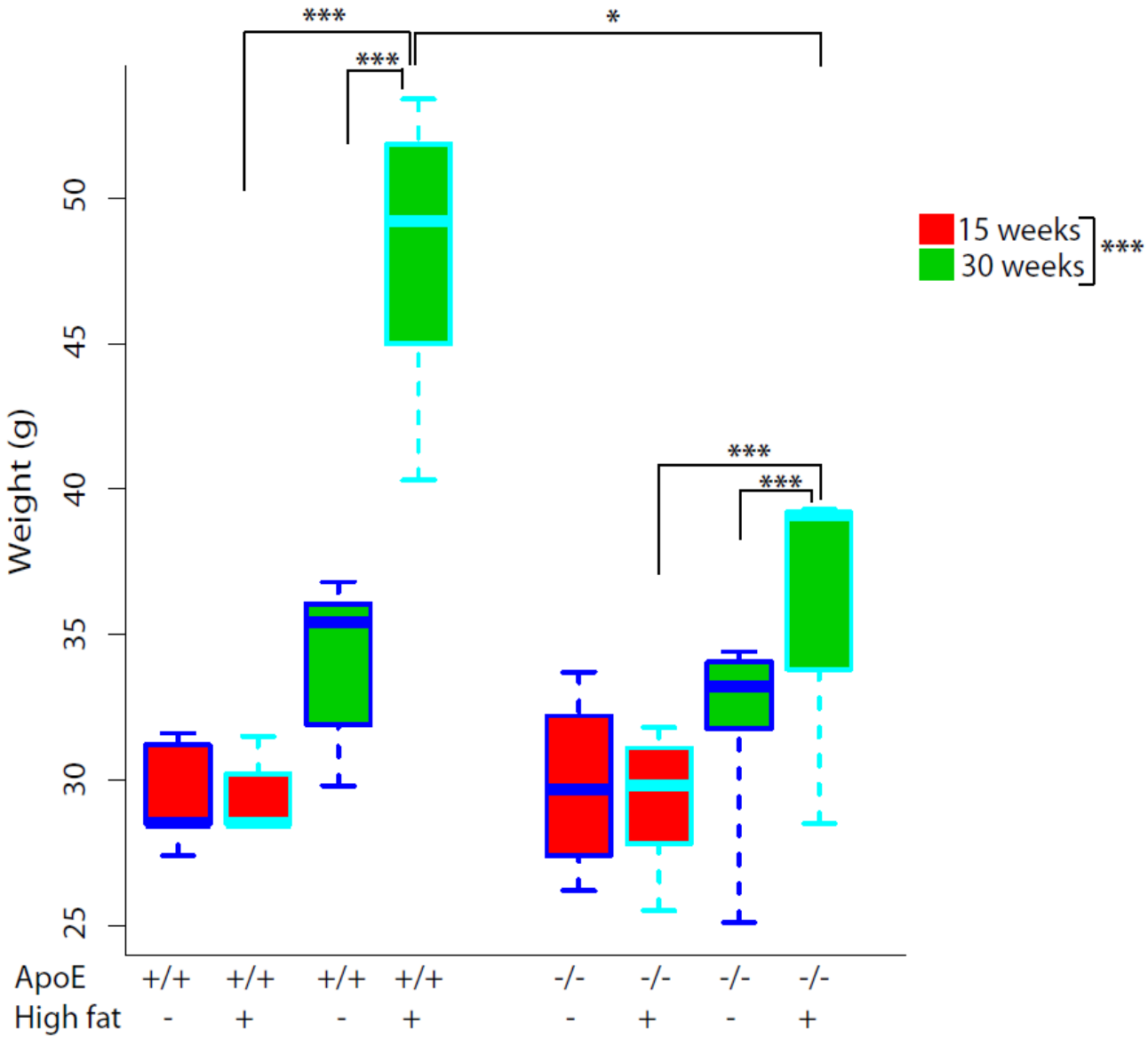

Figure 3.1 Effect of diet and strain on weight. C57Bl/6 and ApoE-KO mice are denoted as "+/+" and "-/-," respectively. Regular chow and high fat diet are symbolized as "-" and "+" respectively. The bars represent range of values between 25th and 75th percentile. The horizontal line inside the bar is the median, and the whiskers represent minimum and maximum in the data set. The differences between strain, diet, and time points were all significant. At 30 week time point, high fat diet showed a significant difference compared to regular chow diet. Similarly, on high fat diet, 30 week time point was significantly different than 15 week time point. On high fat diet, weights of C57Bl\6 mice were significantly different. Also, high fat diet resulted in significant differences compared to regular chow diet. At 30 week time point, weights of C57Bl\6 mice were also significantly different than ApoE-KO mice. Left to right: N=5, 5, 7, 8, 4, 5, 7, 3.*P<0.05, ***P<0.001

3.3 Histology

There were no appreciable changes between C57Bl/6 and ApoE-KO mice observed in the tendon morphology as assessed on H&E stained cryosections of Achilles tendon (Figure 3.2). The tenocytes typically had an elongated shape and no obvious difference in quantity or density was seen; they were predominantly normal in shape and size, and collagen fibers were aligned in parallel orientation. However, morphology was challenging to assess due to small size of the samples, and due to folding artifact that remained a problem, despite extensive re-sectioning by trained histology technicians. There was no obvious evidence of inflammatory cell infiltration, foam cell accumulation or metaplastic change.

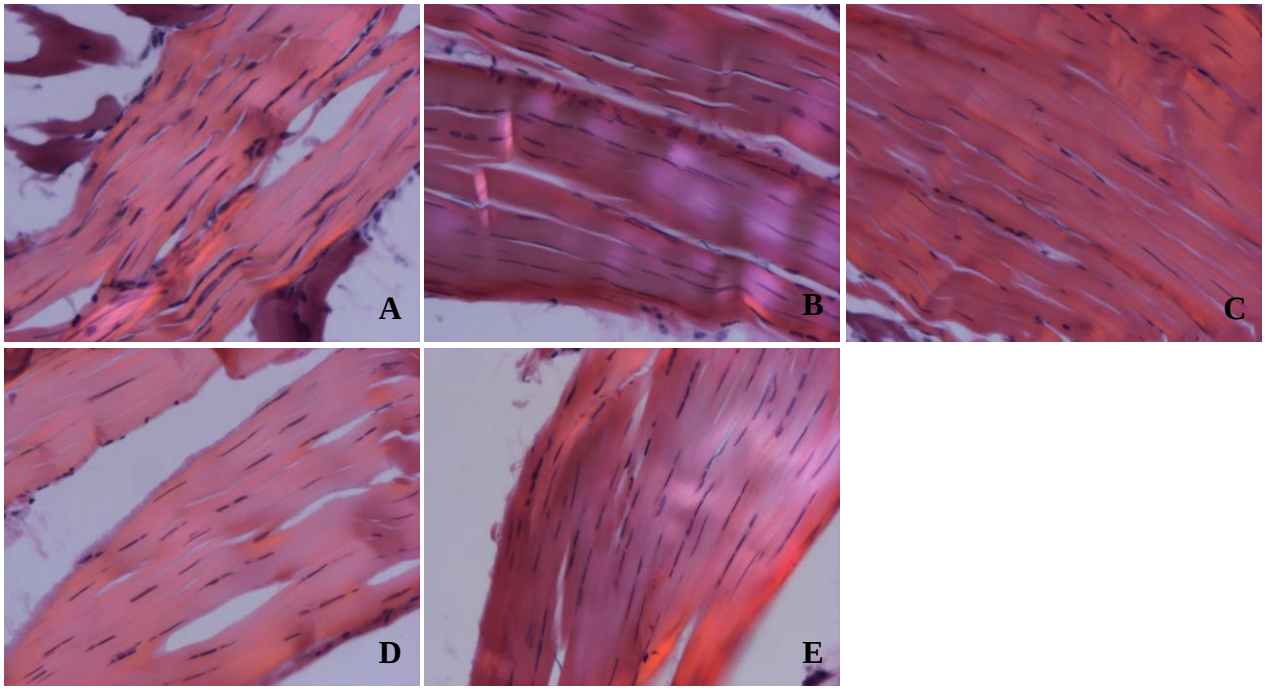

Figure 3.2 Tendon morphology of ApoE-KO mice Achilles tendon. (A) 0 week, no diet (B) 15 week, regular chow (C) 15 week, high fat (D) 30 week, regular chow (E) 30 week, high fat. Original magnification x 20. No difference is observed in collagen organization, cellularity, or shape of tenocytes.

Oil Red O staining on the tissue sections was typically concentrated in and around the tendon tissue, whereas muscle tissue was typically devoid of staining. Oil Red O stained a large number of adipocytes, particularly in the peritendinous and vascular regions, but was also occasionally observed within tenocytes and also within the collagenous extracellular matrix. The Positive Pixel Count Algorithm used in ImageScope software selected the tendon areas stained with Oil Red O, and quantified them in two ways: number of strong positives (red), and positivity ratio. The number of strong positives is a result of number of red pixels in the image (Figure 3.3). It is normalized by total area in square millimeters of all tissue within the selected area, including both positive and negative pixels but not blank spaces. Positive pixels refer to Oil Red O staining on tendon; however the algorithm further classified these pixels into weak, medium, and strong. The positivity ratio is the total number of positive pixels divided by total number of pixels (N_{total}-N_n)/(N_{total}), where N_{tota}l is number of total pixels (positive and negative, N_n).

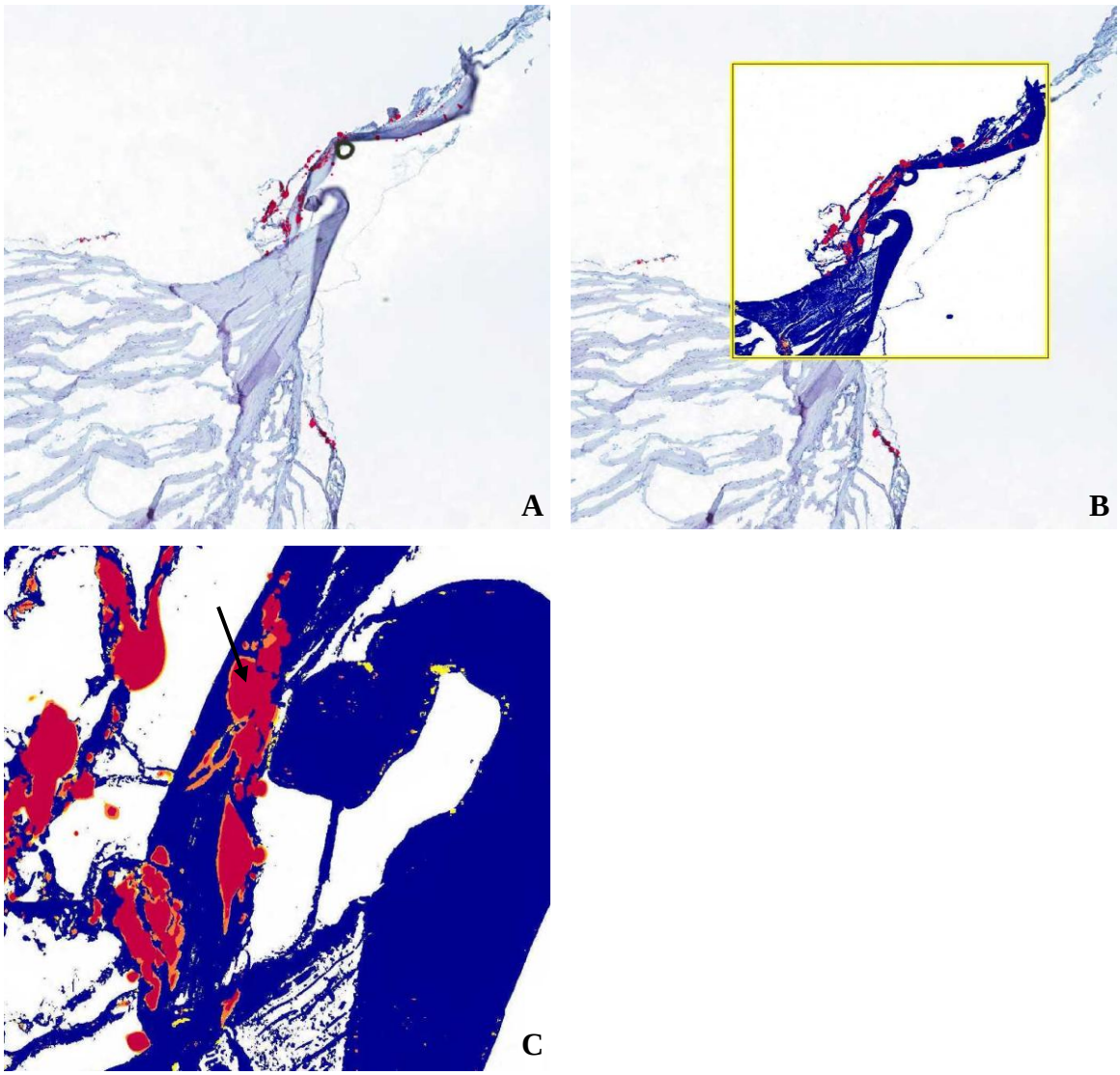

Figure 3.3 Analysis of Oil Red O staining before and after use of Positive Pixel Count Algorithm. (A) Achilles tendon stained with Oil Red O stain at (original) 2X digital magnification. (B) Achilles tendon stained with Oil Red O stain and analyzed by Positive Pixel Count algorithm(C) Boxed area in "B" shown at higher magnification (original 20X digital magnification). Strong positive pixels are in red as shown by the arrow. Medium positive pixels and weak positive pixels are shown in orange and yellow, respectively. Negative pixels are shown in blue.

Oil Red O stained slides showed differences in staining of tendons between C57Bl/6 and ApoE-KO Achilles mice. The number of strong positives were significantly lower overall in ApoE-KO ($1.52x10^6 \pm 2.4x10^5$) mice compared to C57Bl/6 ($3.1x10^6 \pm 2.9x10^5$; $p<0.001$). Between the two time points, 30 week time point showed a significantly lower number of strong positives than 15 week time point ($p<0.05$). However, there was no interaction between strain and time points. Diet had no effect on number of strong positives. The positivity ratio also showed significantly less staining in ApoE-KO (0.32±0.04) than C57Bl/6 mice (0.53±0.05$p<0.01$), as seen in Figure 3.4. The side of the tendon used, i.e. right or left, had no effect on the positivity ratio, therefore, only the results from right tendons are shown in the figure below. Regular chow and high fat diet, or the two time points had no significant effect on the positivity ratio of Achilles tendons.

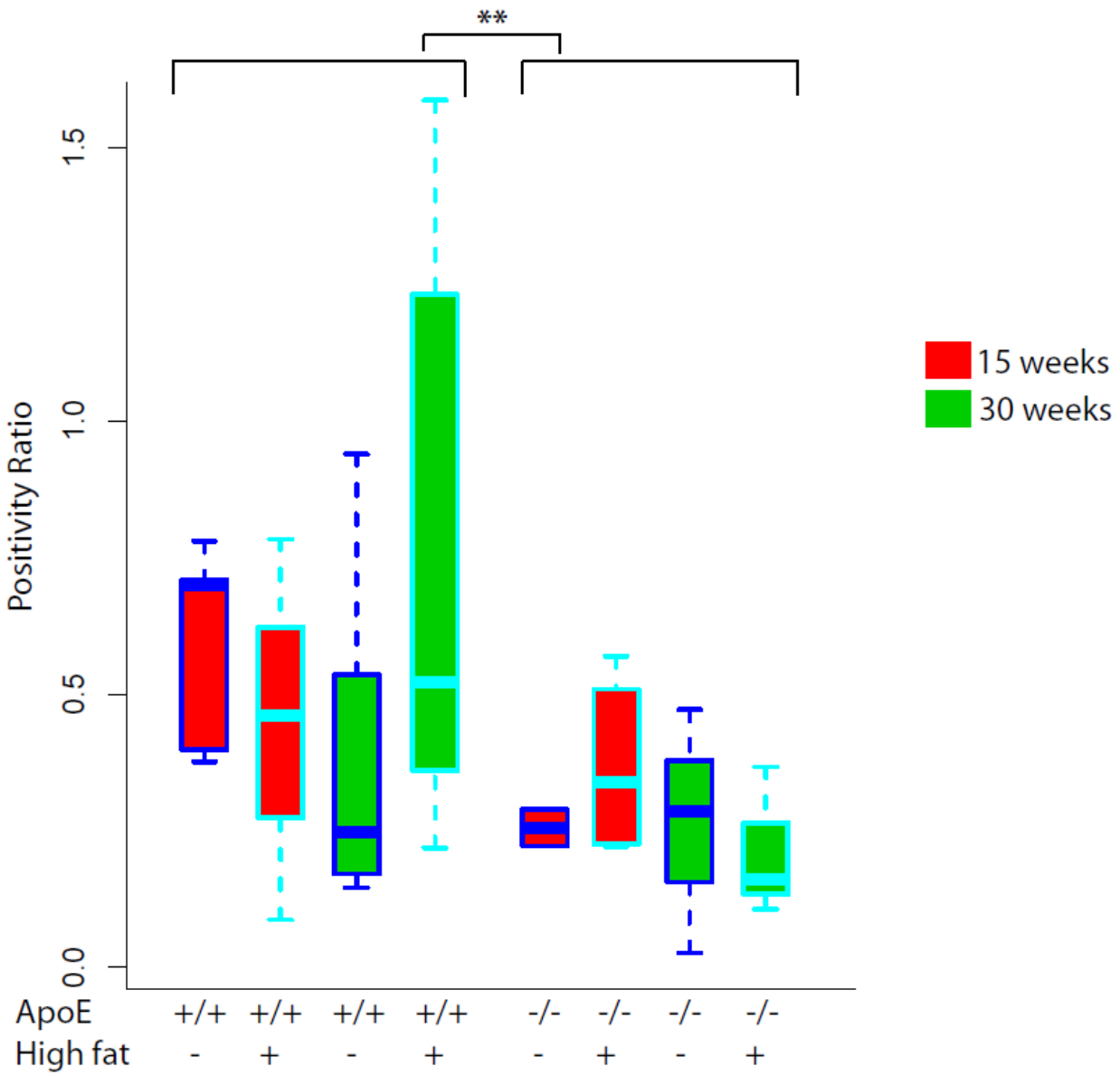

Figure 3.4 Effect of strain and diet on tendon lipid content. The lipid content was quantified as positivity ratio of Oil Red O staining in tendon. C57Bl/6 and ApoE-KO mice are denoted as "+/+" and "-/-," respectively. Regular chow and high fat diet are symbolized as "-" and "+" respectively. The bars represent range of values between 25th and 75th percentile. The horizontal line inside the bar is the median, and the whiskers represent minimum and maximum in the data set. Graph shows results from staining of the right Achilles tendons. Lipid content was significantly different between strains, with increased lipid content in C57Bl/6 mice. Left to right: N= 7, 6, 10, 14, 6, 8, 9, 4. **P<0.01.

3.4 Cross-sectional area

The average CSA of the patellar tendons from mice sacrificed at 0 weeks was 0.72±0.18 mm^2 of C57Bl/6 and 0.77±0.22 mm^2 of ApoE-KO mice (Appendix D). After 15 or 30 weeks, there was a significant difference observed in the cross-sectional area of patellar tendons of ApoE-KO (0.84±0.03) and wild type C57Bl/6 mice (0.70±0.02). The patellar tendons of ApoE-KO mice had a significantly larger CSA than patellar tendons of the wild type mice ($p<0.001$) (Figure 3.5). When the left and right patellar tendons of all mice were compared, there was no significant difference between the two sides. Unexpectedly, there was a statistically significant interaction between the side of the tendon and two other factors (strain and diet); however, since the side of the tendon (left or right) is not an important factor in this study, the graph below shows CSA of right tendons only. Diet (regular vs. high fat) and time (15 vs. 30 weeks) did not significantly influence the tendon CSA.

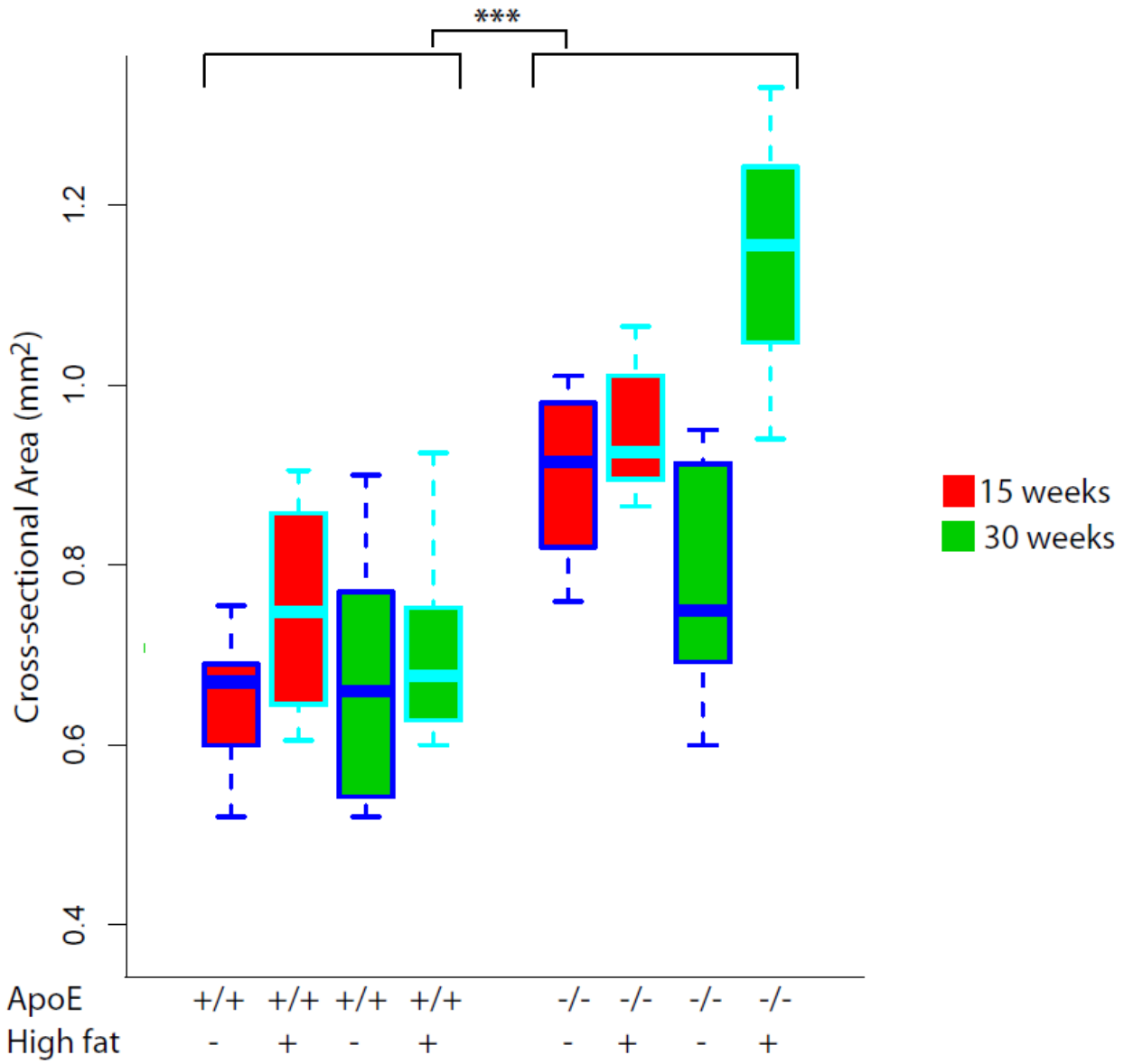

Figure 3.5 Effect of diet and strain on patellar tendon cross-sectional area. C57Bl/6 and ApoE-KO mice are denoted as "+/+" and "-/-," respectively. Regular chow and high fat diet are symbolized as "-" and "+" respectively. The bars represent range of values between 25th and 75th percentile. The horizontal line inside the bar is the median, and the whiskers represent minimum and maximum in the data set. Graph shows results of CSA from right patellar tendons. Cross-sectional area was significantly different between strains. Left to right: 10, 8, 14, 14, 8, 10, 14, 6. ***P<0.001.

3.5 Analysis of *Cpa3, TGF-β, Col1a1*, and *Col3a1* expression

Cpa3, Col1a1, *Col3a1* and *TGF-β* were expressed in both wild type and ApoE-KO mice (Figure 3.6). *Cpa3* expression was significantly decreased (1.6 times lower) in mice that were fed high fat diet (0.0021±0.0003) compared to those on normal diet (0.0034±0.0006; $p<0.05$). *Col1a1* expression was significantly decreased (5 times lower in log2 ratio) in ApoE-KO mice (0.48±0.13) compared to C57Bl/6 mice (1.08±0.16). *Col3a1* and *TGF-β* expression did not differ significantly between the strains, diets, or time points. There was one outlier among the samples observed during *TGF-β* qPCR analysis. Statistical tests were conducted both with and without this outlier; the outlier did not influence the results of the analysis and the RNA sample was not an outlier for any other gene examined. The *TGF-β* expression graph does not include this outlier.

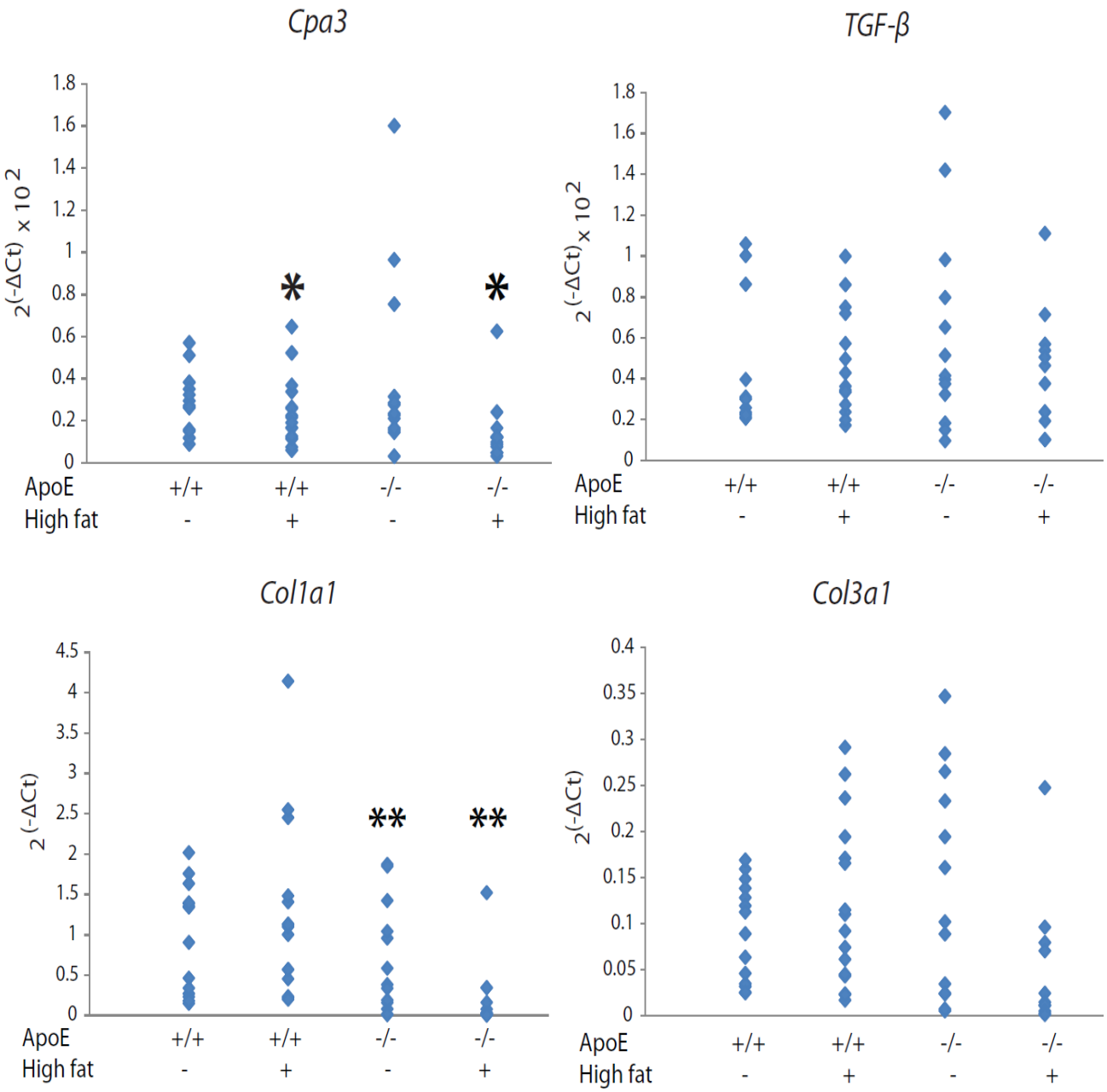

Figure 3.6 Effect of strain and diet on gene expression. C57Bl/6 and ApoE-KO mice are denoted as "+/+" and "-/-," respectively. Regular chow and high fat diet are symbolized as "-" and "+" respectively. Each diamond represents one of two samples taken from each animal. Linear mixed model with log 2 ratio showed significant differences in *Cpa3* and *Col1a1* expression due to strain. Cpa3 expression was significantly lower in mice fed high fat diet, compare to regular chow diet. Col1a1 expression was significantly lower in ApoE-KO mice, compared to C57Bl/6 mice. Left to right for *Cpa3*, *Col1a1*, *Col3a1*: 14, 16, 13, 11. Left to right for *TGF-β*: 14, 15, 13, 11. *P<0.05, **P<0.01.

Chapter 4: Discussion

In this study, we found that the tendons of Apo-KO mice were significantly altered compared to wild type controls. The patellar tendons demonstrated an increased CSA, and the tail tendons demonstrated reduced *Col1a1* expression levels. However, high fat diet did not influence any of the measured tendon-related variables (with the exception of a small decrease in *Cpa3* levels occurring in both ApoE-KO and wild type mice). We did not observe any evidence of increased lipid deposition in tendons, compared to control mice. Taken together, these findings indicate that tendons are negatively affected in ApoE-KO mice, however future work is required to determine the mechanism involved and the potential impact on tendon function.

ApoE is a lipoprotein known for its function in cholesterol transport and other lipids between peripheral tissues and the liver. It serves as a ligand for cellular lipoprotein receptors (Curtiss, 2000) and its deficiency leads to severe hypercholesterolemia and atherosclerosis. In this study, ApoE deficient mice on a high fat diet for 30 weeks showed frailty and increased morbidity compared to wild type C57Bl/6 mice. It is known that ApoE-KO mice have higher plasma cholesterol levels compared with wild type mice. Their plasma levels increase even more when fed a high fat diet, and mice start to develop skin lesions (Piedrahita, 1992), and these lesions were also observed in mice used in the present study. The lesions, mainly composed of lipids and extracellular matrix, develop in the form of xanthomas on the shoulder and back area due to extremely high plasma cholesterol (Moghadasian, 1999).

ApoE deficiency also results in the development of atherosclerotic lesions, whether the mice are fed a high fat or regular chow diet. These lesions develop and grow in size with age in the aortic

root and throughout the aorta, and also affect the peripheral vasculature, eventually leading to vascular senescence (Bonomini, 2010). Mononuclear cells adhere to the endothelial surface leading to the development of foam cell lesions in the intima of the artery, which further progress to fibrofatty lesions containing multiple layers of lipid-filled macrophages and smooth muscle cells, culminating in the formation of fibrous plaques (Nakashima, 1994). These lesions are commonly identified with Oil Red O staining, which stains both intra- and extra-cellularly.

We hypothesized that ApoE-KO mice would demonstrate increased extracellular or intracellular lipid deposition in the Achilles tendons as gauged by Oil Red O staining, however, the opposite was found: ApoE-KO mice Achilles tendons demonstrated *decreased* lipid content compared to C57Bl/6 mice, as determined both by the normalized number of pixels strongly stained with Oil Red O, as well as by the Oil Red O positivity ratio. Oil Red O is a non-specific lipid soluble dye that stains cholesterol esters and other hydrophobic lipid red, both intracellularly and extracellularly. The deposits of cholesterol byproducts have been implicated in the formation of tendon xanthomas. Detailed lipid analysis in previous studies has showed that lipid composition of tendon xanthomas is mainly free cholesterol, with some cholesterol esters and phospholipids. Staining of these tissue sections demonstrated that unesterified cholesterol accumulated predominantly extracellularly, and esterified cholesterol accumulated both extra and intracellularly (Adams, 1974; Kruth, 1985). To determine the presence of intracellular and extracellular cholesterol, Kruth et al.'s study also used Oil Red O staining on human Achilles tendon sections. Similar to their results, in our study lipids were stained in ApoE-KO mice tendons both intracellularly in fibroblasts and adipocytes, and in the extracellular space along the

collagen fibers. Much of the staining was concentrated in the paratenon, rather than in the tendon proper.

Control tendons in Kruth et al.'s study demonstrated small amounts of Oil Red O staining in the extracellular space and within fibroblasts. In our study, there was more lipid deposition in controls at both time points, (Figure 3). Adam et al. also showed lipid deposition in normal tendons, mainly esterified cholesterol, using Oil Red O staining (Adams, 1974).

It is uncertain whether the lipids detected in control mice were a result of excessively high plasma cholesterol levels, or whether it is physiological for fat to be stored around tendons. The wild type mice did not develop any skin lesions or xanthomas, however traces of lipids were observed macroscopically around tendons when they were harvested from C57Bl/6 mice, especially those on high fat diet for 30 weeks. These findings suggest that there may be a normal, cell-mediated process that relies on the LDL-receptor in tendon cholesterol deposition. Furthermore, ApoE-KO weighed less overall than C57Bl/6 mice. Similar results were also found in Pereira et al.'s study where ApoE-deficient mice had a lower body weight compared to C57Bl/6 mice. In that study, ApoE-KO fed a high fat diet had smaller adipocytes compared to wild type controls. Since the adipocyte size was smaller, the mice may have been less likely to gain weight with a high fat diet (Pereira, 2012). Adipocyte size was not studied in this present study but this could also be a reason why ApoE-KO mice had lower lipid content in and around the Achilles tendon. The adipocytes in C57Bl/6 may have expanded and adipose deposition around tendons may be the reason that we saw higher lipid content in the Achilles tendons of these wild type mice.

Another way to quantify Oil Red O staining would be to choose an area of interest, and count the number of stained and unstained tenocytes. We were unable to use this method because 1) the tendon tends to fold on itself during cryosectioning and staining between these folds could be an artifact, 2) during the staining process (which is conducted on unfixed tissue), the lipids might have been displaced as in some Oil Red O stained slides, lipids were seen around the tendons as well.

We did not see any significant morphological changes in tendon structure between ApoE-KO and wild type mice or between the two diets, as gauged by H&E staining. There may have been some changes in the paratendon of ApoE-KO mice, but due to limited number of samples it was not possible to confirm if this was true. A few sections of ApoE-KO Achilles tendons showed some changes in the paratendon including the presence of inflammatory cells such as macrophages, and an increase in cellularity compared to wild type controls seemed to suggest that there might be some ongoing inflammation in the paratendon contributing to these changes. Possible inflammation in the paratendon may have led to changes in the patellar tendon cross-sectional area. However it could not be assessed as this change was only observed in a small number of samples.

In support of our hypothesis, ApoE-KO mice were found to have thicker patellar tendons than C57Bl/6 mice. The thicker tendons of ApoE-KO mice could be due to inflammation in paratendon (as mentioned above) and aging. A previous study has shown that tendons of older people are thicker, compared to younger people (Stenroth, 2012). With aging, there may be a decrease in the loading of the tendon, which could increase the extracellular water content,

resulting in an increased tendon CSA. Studies on tendon xanthomas have measured the cross-sectional area of the Achilles tendon, as xanthomas predominantly occur in Achilles tendons or in the extensor tendons of the hands (Dussault, 1995), but our study measured cross-sectional area on patellar tendons. Therefore, it cannot be concluded that tendon thickness was due to xanthoma formation. Another explanation for a larger patellar tendon CSA is related to high levels of circulating cholesterol.

Lipid droplets can be deposited along collagen fibers and disrupt the interaction of the fiber network, making it more prone to tendon rupture or injury (Jozsa, 1984). Biomechanical testing of ApoE-KO and C57Bl/6 patellar tendons would give further explanation and clarification of the effect of lipid accumulation on tendon function. It can be hypothesized that ApoE-KO patellar tendons would have reduced biomechanical properties, or fail at a lower load, compared to C57Bl/6 patellar tendons. This can be tested in a materials testing device that gives information on load and displacement when tendons are tested to failure (Figure 1.4).

ApoE-KO mice also demonstrated decreased expression levels of *Col1a1*, which suggests that these tendons were not thicker due to increased type I collagen content, but that the thickening resulted from other substances or factors. As mentioned earlier, tendon thickening could be due to inflammation, and the tendency of tendinopathic or aging tendon tissue to increase in size. To determine if tendon thickness in ApoE-KO mice is due to aging and/or inflammation, changes in pro-inflammatory cytokines can be examined. Pro-inflammatory cytokines IL-6 and iNOS are induced during the aging process due to age-related redox imbalance that activates many signaling pathways (Chung, 2009). Compared to controls, ApoE-KO mice had increased IL-6

and iNOS expression in the renal cortex and medulla, and cytoplasm of hepatocytes. In addition, IL-6 expression was also increased in cardiomyocytes of Apo-KO mice (Bonomini, 2010). mRNA levels of these pro-inflammatory cytokines, along with IL-1β, can be measured with qPCR. It is hypothesized that ApoE-KO mice would have increased levels of IL-6, iNOS, and IL-1β in the tendon.

Collagen is the most important component of tendons, with type I collagen accounting for 65-80% of the total tendon mass. Expression of *Col1a1* was seen to be lower in ApoE-KO mice. A decrease in *Col1a1* could be related to aging. Couppe and his colleagues showed that collagen concentration was lower in older men compared to younger men. This may represent the reduced size or density of collagen fibrils that is known to occur with aging (Couppe, 2009). Inflammation, mediated by prostaglandin E2 (PGE2), has been to shown to affect collagen concentration. In human tendon fibroblasts, high levels of PGE2 have been shown to decrease collagen synthesis (unpublished data, Scott laboratory). PGE2 is a product of COX2 gene, so measuring gene expression levels of COX2 can further provide information on the potential mechanism of decreased *Col1a1* expression in ApoE-KO mice.

The mRNA expression levels of *TGF-β* and *Col3a1* showed no differences between ApoE-KO and wild type mice or between diets. *TGF-β* expression was measured as it plays an important role in fibroblast migration, neovascularization, and secretion of ECM proteins such as procollagen type I and III (Kashiwagi, 2004). It is normally expressed in the process of tendon and repair and remodeling. However, no such difference in its gene expression was seen therefore, it may imply that the ApoE mice did not experience an increase in collagen repair and

remodeling. This is also in agreement with the other finding that *Col1a1* expression was decreased in ApoE-KO mice. In a healing tendon, TGF-βincreases expression of collagen I and III, however, none of the mRNA levels of these genes were seen to be increased in ApoE-KO mice.

Mast cells play a role in connective tissue remodeling, including repair after direct mechanical injury or overuse (Dumont, 2007). Mast cells degranulate as a reaction to physical trauma and release a number of different factors that contribute to the first inflammatory stage of healing process (Moyer, 2004). Carboxypeptidase A3 (CPA3), also known as mast cell carboxypeptidase A, is an enzyme secreted from mast cells which functions together with endopeptidases secreted from mast cells to degrade proteins and peptides (Abe, 2000). The strain of mice did not affect the expression of *Cpa3*, but it was lower in high fat fed mice in both strains. No significant changes between wild type and ApoE-KO mice may suggest that mast cells were equally present in the tendons of both mouse strains.

ApoE-KO mice have been considered as a model of aging in a few other studies. These mice are used in cardiovascular research because ApoE deficiency leads to accumulation of cholesterol enriched particles in the blood. On a high fat diet, these mice develop fatty streaks and lesions as they age. As mentioned in Ang et al.'s study, ApoE-KO mice develop age-related phenotypes regardless of what diet they are fed, even though high fat diet accelerates these phenotypes. This is in agreement with our results as we observed that these mice had hair loss and graying earlier than C57Bl/6 mice, and shortened life spans (Ang, 2008).

It is possible that the results presented may not be directly due to ApoE deficiency, but could be because of ApoE induced oxidative or inflammatory stress. ApoE not only regulates cellular and systemic cholesterol metabolism, but it also has anti-inflammatory, anti-atherogenic, and anti-oxidant properties (Tarnus, 2009). High fat diet in ApoE-KO mice causes an increase in oxidative stress and inflammation in adipose tissue. Moreover, fatty acid oxidation is increased with high fat diet intake compared to regular chow diet. The oxidative status in these animals is responsible for inflammation in adipose tissue, which in turn is a risk factor for a disease such as atherosclerosis (Pereira, 2012). Aging, a biological process, is characterized by time-dependent, progressive, and physiological declines, accompanied by an increased incidence of age-related diseases. According to the oxidative stress hypothesis, oxidative damage is not only caused by the uncontrolled production of oxygen species (Herman, 1956), but also by other oxidants such as reactive nitrogen species and reactive lipid species. Atherosclerosis is now considered to be an age-related disease that occurs due to chronic inflammation. Inflammatory mediators increase with aging due to redox imbalance induced by oxidative stress. This redox imbalance is most likely caused by the net effect of weakened anti-oxidative defense systems, which deficiency of ApoE provides, and increasing production of reactive species (Chung, 2009).

The pathogenesis of tendon degeneration, such as decreased collagen synthesis by tendon cells and increased degradation of synthesized collagen in the tendon matrix, may be due to changes induced by oxidative stress. Furthermore, oxidative stress could also be the reason for hair loss and lack of weight gain in ApoE-KO mice, along with development of xanthomatic skin lesions. Collagen synthesis by human tendon cells has been shown to decrease under oxidative stress

(Yuan, 2004). Tendon matrix degradation can be due to increased MMP1 expression, which is upregulated by phosphorylated JNK induced via oxidative stress (Wang, 2007). Last but not least, oxidative stress can also induce apoptosis which has also been proposed to play an important role in tendon degeneration (Yuan, 2003).

Chapter 5: Conclusion

ApoE-KO mice fed a high fat diet can be used as a model to study tendon pathology related to hypercholesterolemia. Our study demonstrated that the tendons of ApoE-KO mice were thicker and displayed reduced *Col1a1* expression levels, possibly making them more susceptible to injury. The thickness in tendon could possibly be due to inflammation in paratendon, aging, or lipid accumulation. Further experiments using biomechanical testing, qPCR of pro-inflammatory genes, and measures of collagen density and organization, could provide more information about the changes in the tendons of ApoE-KO mice and potentially the mechanisms involved. The link between hypercholesterolemia and tendon pathology can lead to studies on mechanisms involved, which may be used for therapeutic interventions. These studies can aid in establishing a suitable model that can be used to examine the potential reversibility of tendinopathy associated with high cholesterol levels.

5.1 Limitations

Because this study was conducted with animals, it has limitations as information obtained with mice cannot always be directly translated into data that would be clinically relevant for humans. ApoE-KO mice are known to demonstrate hypercholesterolemia with systemic effects, but this may not be clinically applicable as most patients have mixed hypercholesterolemia. The total cholesterol to HDL-C ratio gives is a good indicator for determining if an individual is hypercholesterolemic. ApoE-KO mice are known to have high total cholesterol and low HDL-C, which increases their total cholesterol to HDL-C ratio. However, lipid tests were not carried out on ApoE-KO mice in our study to confirm this. For cross-sectional area measurements, the study

seemed to be underpowered. As seen in results (Fig 3.4), ApoE-KO at 30 week time point showed an increase in cross-sectional area; however it was not significant. Low number of samples could be a reason why this was not statistically significant. The histology on frozen sections was very challenging and may have limited our ability to detect morphological differences. The genes were evaluated at defined time points, therefore, the study does not give information about when the gene expression levels started to decline, or if the expression patterns observed were uniform for the whole duration of the experiment. Gene expression also does not always correlate to protein levels. At a given point in time, often they have no relationship and sometimes the reverse. Furthermore, gene expression experiments were done on tail tendons, which are not load-bearing. Carrying out this experiment on a tendon, such as patellar or Achilles, may have been more useful as those tendons are used for load-bearing and could give us more relevant information.

5.2 Future directions

Further studies are planned using biomechanical testing of the ApoE-KO mice patellar tendons. Due to the increased cross-sectional area and the reduced type I collagen mRNA, it is expected that patellar tendons of these mice would rupture at a lower stress than the wild type mice. Studies on gene expression of pro-inflammatory cytokines or other markers of aging could give further insight into the mechanisms underlying the observed changes. Second harmonic generation (SHG) imaging could also be conducted to determine if there is an influence of mouse strain or diet on collagen density and organization. SHG is a non linear optical process in which photons interact with non linear material and combine to form new photons with twice as energy,

twice the frequency, and half the wavelength of the initial photons.The association between high cholesterol and tendon injuries can be studied in larger studies, and cholesterol levels can be checked in people with tendon injuries. Information gained from this study and similar studies on ApoE deficiency and hypercholesterolemia can give further insight on intrinsic risk factors that affect tendon biology and biomechanics. The effects of cholesterol on the cardiovascular system have been studied, but not much has been examined on the musculoskeletal system. Studies like these can give a better understanding of how hypercholesterolemia affects the long-term structure and function of tendons. Furthermore, it can provide orthopaedic patients and clinicians information on musculoskeletal health and preventative treatments.

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Appendices

Appendix A Initial weight of mice at 7 weeks of age and final weights at their respective time points.

Some mice were sacrificed prior to their experimental time points, labeled as "Sick." Animals with an "*" were sacrificed prior to their planned time point as well, but their tendons were harvested and included in the qPCR analysis.

Strain	Cage	Animal number	Diet	Initial weight (g)	Final weight (g)	Time point
ApoE-KO	C	43	Normal	20.1	20.1	Sick
	B	8	High fat	23	26.8	Sick
	B	2	High fat	27.6	31.8	15 weeks
	B	3	High fat	25.1	25.5	15 weeks
	B	5	High fat	25.1	27.8	15 weeks
	A	9	High fat	25.6	29.8	15 weeks
	A	10	High fat	26.4	31.1	15 weeks
	B	4	High fat	26.4	28	Sick
	A	7	High fat	27.3	32.9	Sick
	B	1	High fat	26.3	28.9	Sick
	D	24	Normal	24.7	30.7	15 weeks
	D	25	Normal	25	28.6	15 weeks
	F	26	Normal	28.3	33.7	15 weeks
	E	31	Normal	28	26.8	Sick
	C	42	High fat	27.9	30.1	Sick
	E	30	Normal	26.1	26.2	15 weeks
	A	6	High fat	25.6	39.1	30 weeks
	A	8	High fat	25.4	28.5	30 weeks
	F	27	High fat	26	26.9	Sick
	F	29	High fat	23	23.6	Sick
	A	15	Normal	29	34.4	30 weeks
	A	16	Normal	27.6	34.3	30 weeks
	A	17	Normal	26.8	25.1	30 weeks
	A	18	Normal	25.8	33.8	30 weeks
	F	51	High fat	25.6	23.9	Sick *
	D	21	Normal	26.2	33.2	30 weeks
	D	22	Normal	24.3	31.7	30 weeks
	D	23	Normal	26.3	31.8	30 weeks
	F	28	High fat	27.2	39.3	30 weeks
	C	41	High fat	27.9	31	Sick *
	F	53	High fat	21.9	23.4	Sick *

Strain	Cage	Animal number	Diet	Initial weight (g)	Final weight (g)	Time point
C57Bl/6	C	1	Normal	23	31.2	15 weeks
	C	2	Normal	23.2	31.6	15 weeks
C57Bl/6	C	3	Normal	23	28.5	15 weeks
	C	4	Normal	21	27.4	15 weeks
	C	5	Normal	21.5	28.5	15 weeks
	B	6	High fat	23.7	28.5	15 weeks
	B	7	High fat	25	30.2	15 weeks
	B	8	High fat	24.3	31.5	15 weeks
	B	9	High fat	23.6	28.5	15 weeks
	B	10	High fat	23.6	28.5	15 weeks
	A	1	High fat	23.2	47.6	30 weeks
	A	2	High fat	23.9	40.3	30 weeks
	A	3	High fat	21.8	45	30 weeks
	A	4	High fat	23.2	51.1	30 weeks
	A	5	High fat	21.5	45	30 weeks
	D	6	Normal	23.1	35.4	30 weeks
	D	7	Normal	23.7	30.1	30 weeks
	D	8	Normal	21.7	35.4	30 weeks
	E	9	Normal	22.2	36.8	30 weeks
	E	10	Normal	22.6	36.7	30 weeks
	F	11	Normal	22.5	29.8	30 weeks
	F	12	Normal	22	33.7	30 weeks
	G	54	High fat	27.6	52.6	30 weeks
	G	55	High fat	27.6	53.4	30 weeks
	G	56	High fat	24.7	50.8	30 weeks

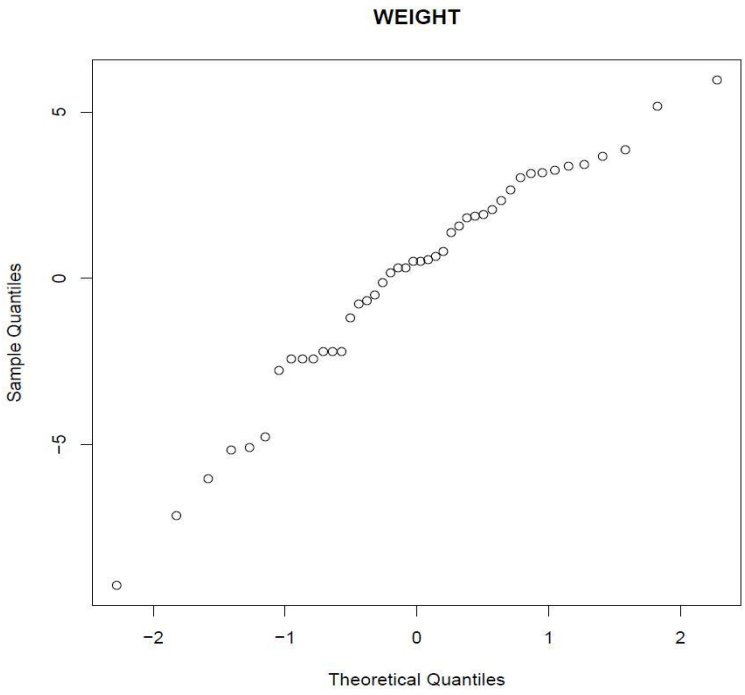
Appendix B ApoE monitoring sheet

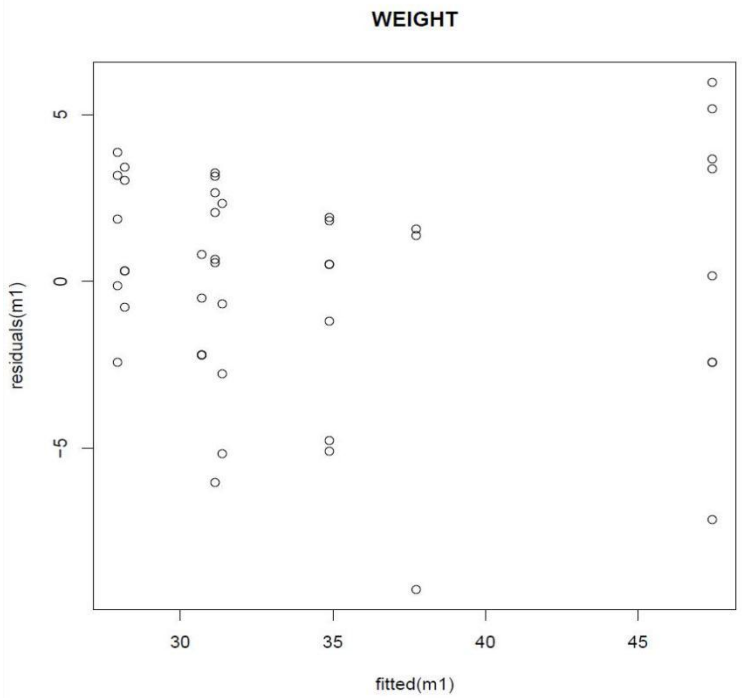
ApoE monitoring sheet		Week	1	1	2	2	3	3	4	4
Animal number		Date and initial when checked								
Assigned end-point		Weight								
DOB		Xanthoma size (diameter)								
High fat or normal										
Date started on high fat or control		If any distress, note below								
Initial weight										
Critical weight (85% weight)										

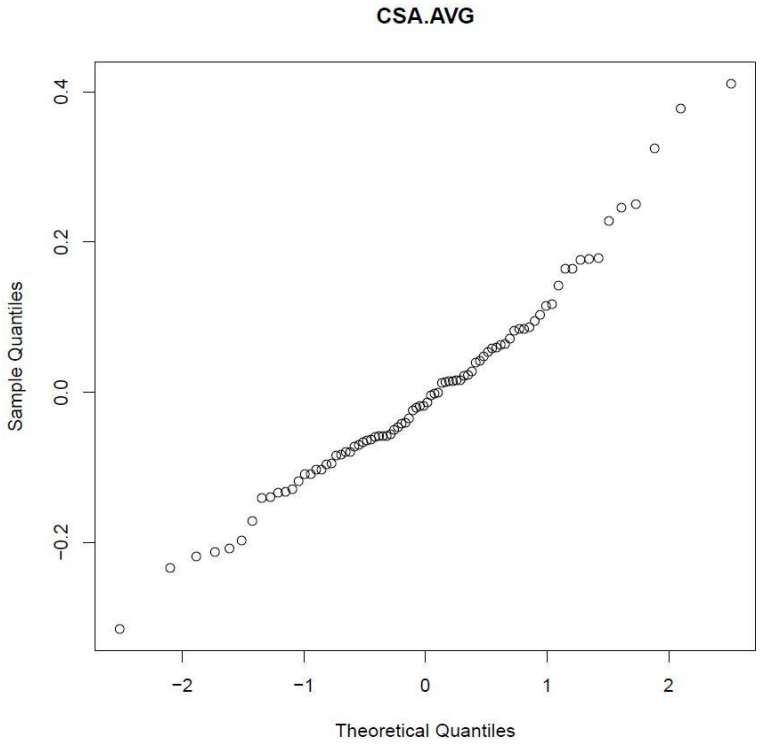
While on diet, animals to be checked twice per week, and weighed once per week. Weight loss > 15% will require euthanasia
Food (normal or high fat) can be added *ad libitum*
Distress:

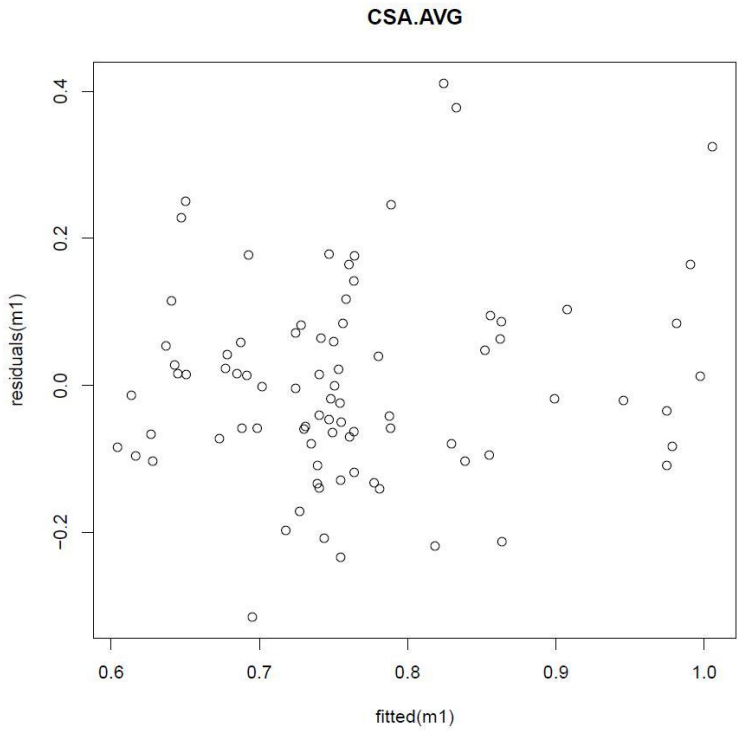
Animals will be checked twice per week and general behaviour will be followed. For those animals where a change in behaviour is noted then the animal will be examined for the following: 1) posture, hunching posture would indicate pain in the abdomen; 2) gait and movement, do the animals exhibit normal gait and movement (circling, excessive activity, wobbly, etc.); 3) behaviour, do animals exhibit normal behaviour when stimulated (ie. being picked up or fleeing); 4) respiration, do the animals exhibit laboured respiration, noisy breathing, wide stance, or open-mouth breathing. Animals that fall into the 4th category will be immediately sacrificed. Animals that exhibit signs in 1-3 will be closely monitored (checked several times per day) or sacrificed.
In the case of the appearance of cutaneous xanthomas appearing, mice will be monitored closely. If the xanthomas worsen (area greater than 1 cm2) or are causing obvious discomfort, the mouse will be sacrificed. Discomfort will be assessed based on gait, attitude, appearance, behavior, appetite and weight.

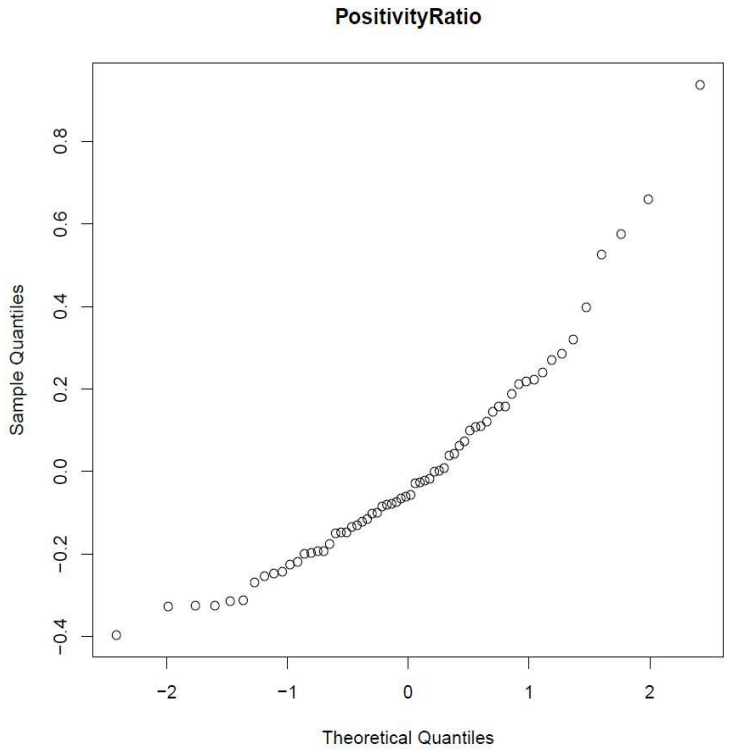
Appendix C Normal quantile and residual plots for all variables studied.







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Sample Quantiles
Theoretical Quantiles
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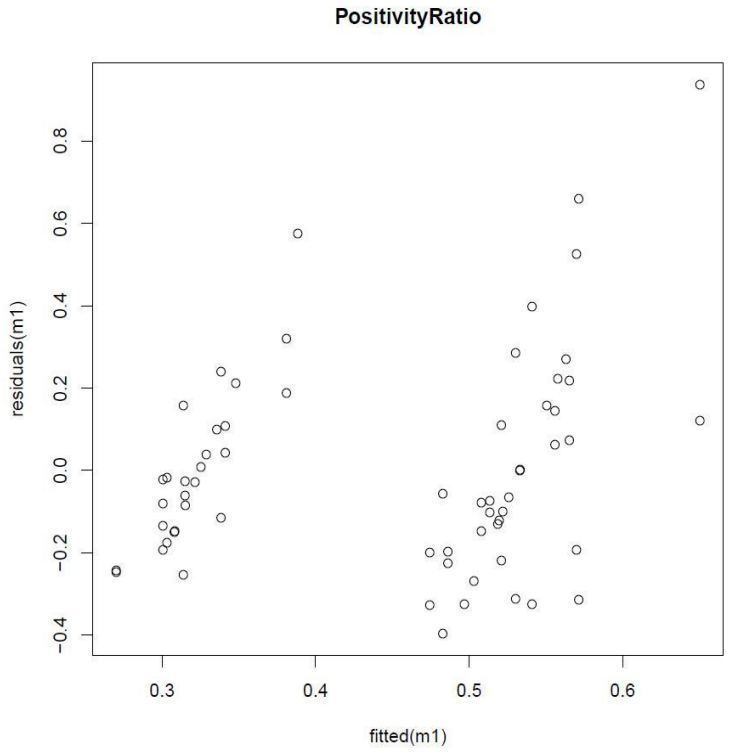
0.2
0.4
0.6
0.8
−2
−1
0
1
2


PositivityRatio
residuals(m1)
fitted(m1)
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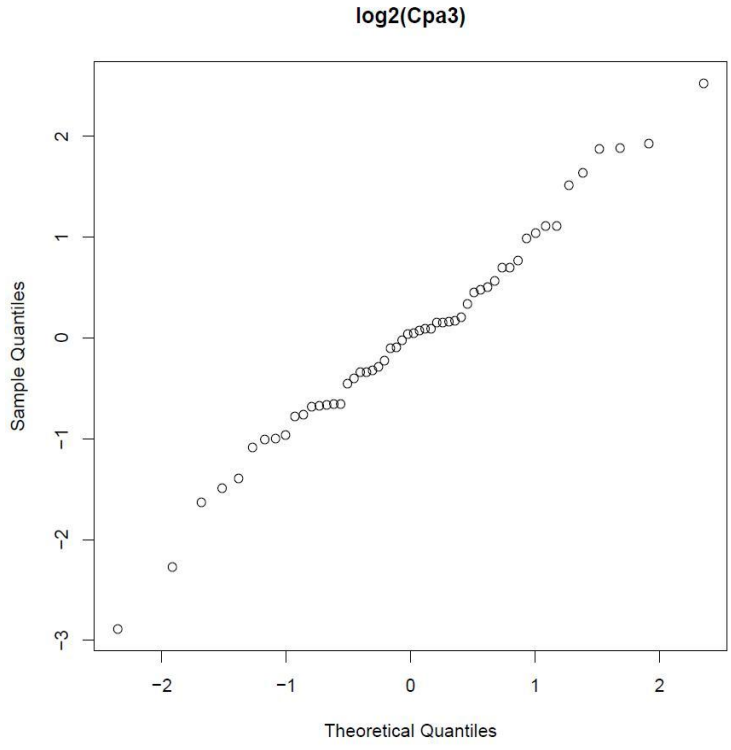
−0.2
0.0
0.2
0.4
0.6
0.8
0.3
0.4
0.5
0.6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Theoretical Quantiles
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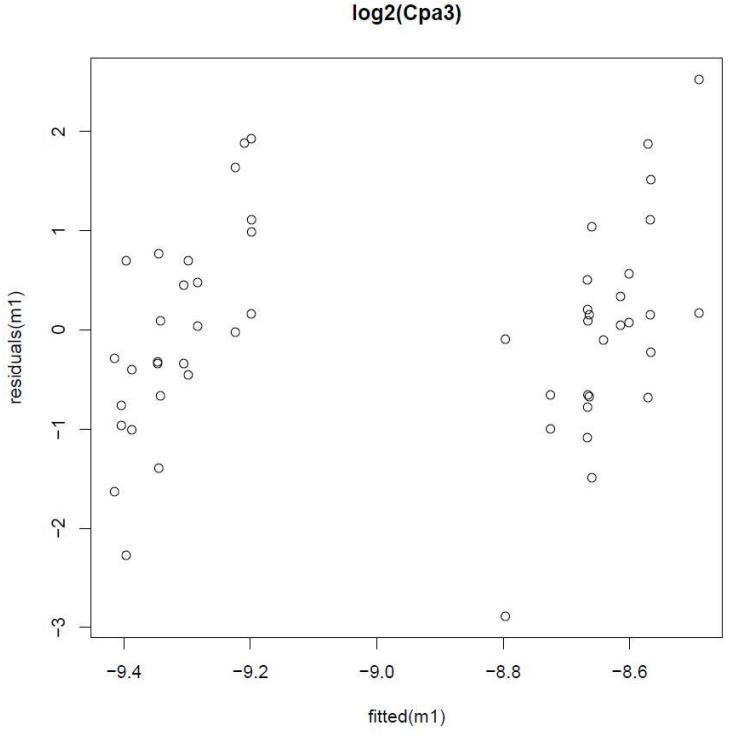
−1
0
1
2
−2
−1
0
1
2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residuals(m1)
fitted(m1)
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1
2
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−9.2
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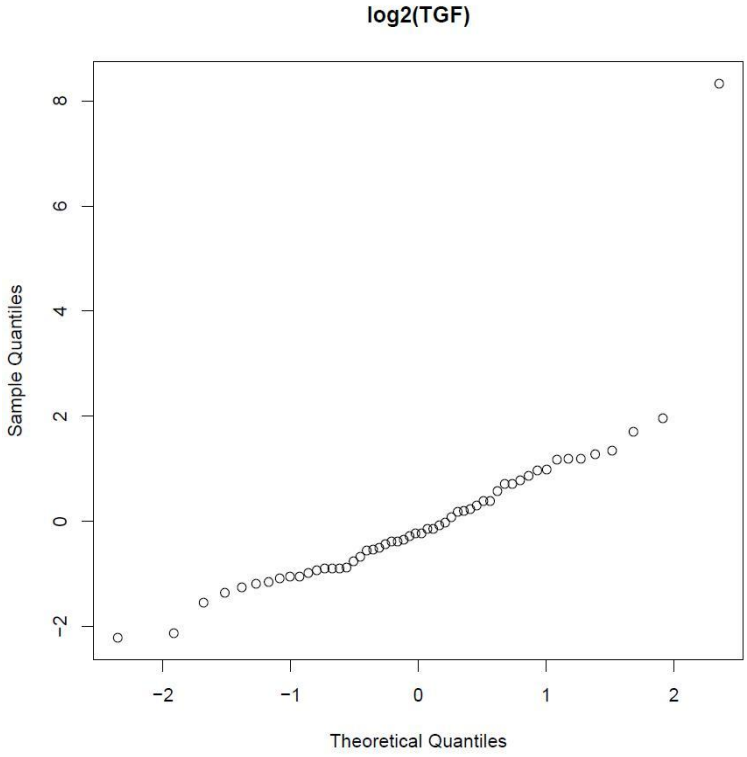
−8.8
−8.6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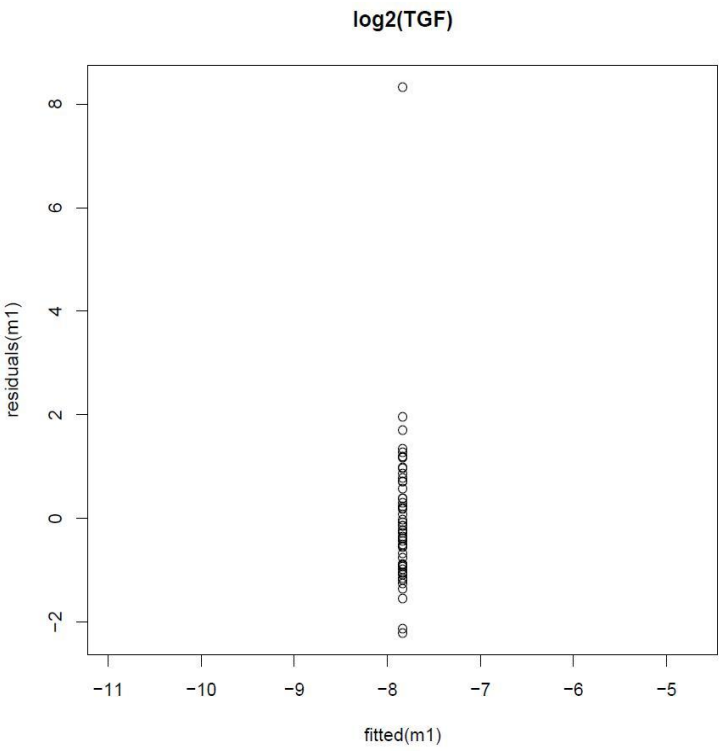
2
−2
0
2
4
6
8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−5
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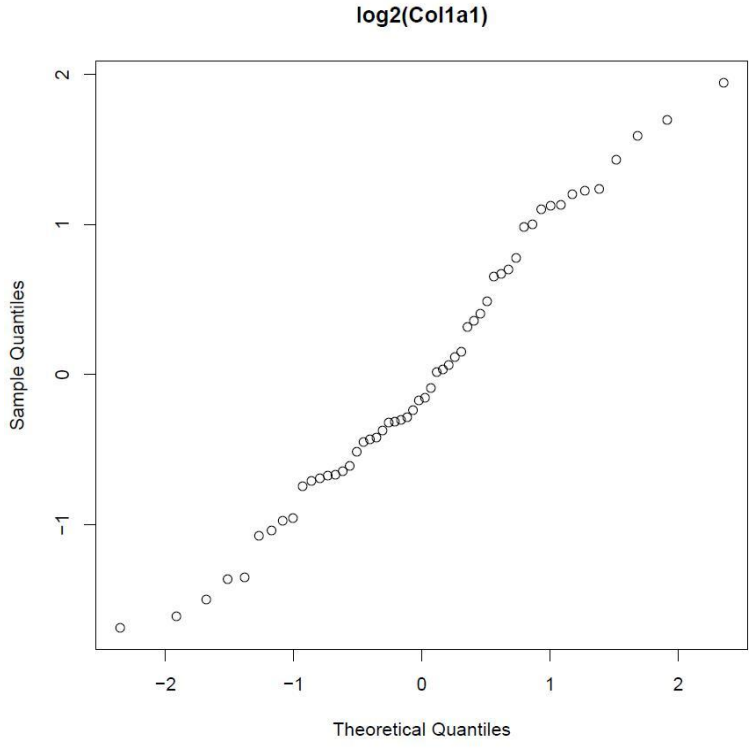
0
2
4
6
8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Sample Quantiles
Theoretical Quantiles
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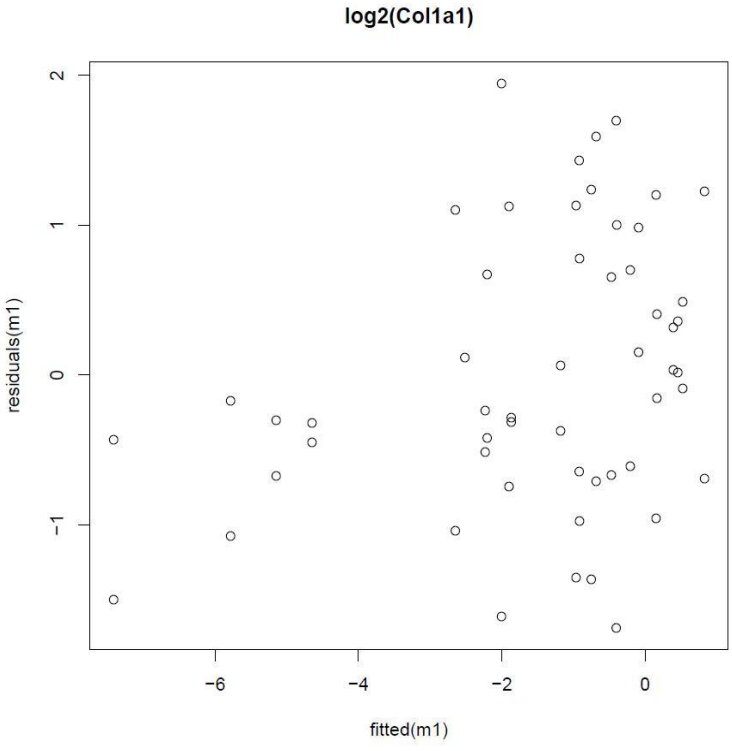
−1
0
1
2
−1
0
1
2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residuals(m1)
fitted(m1)
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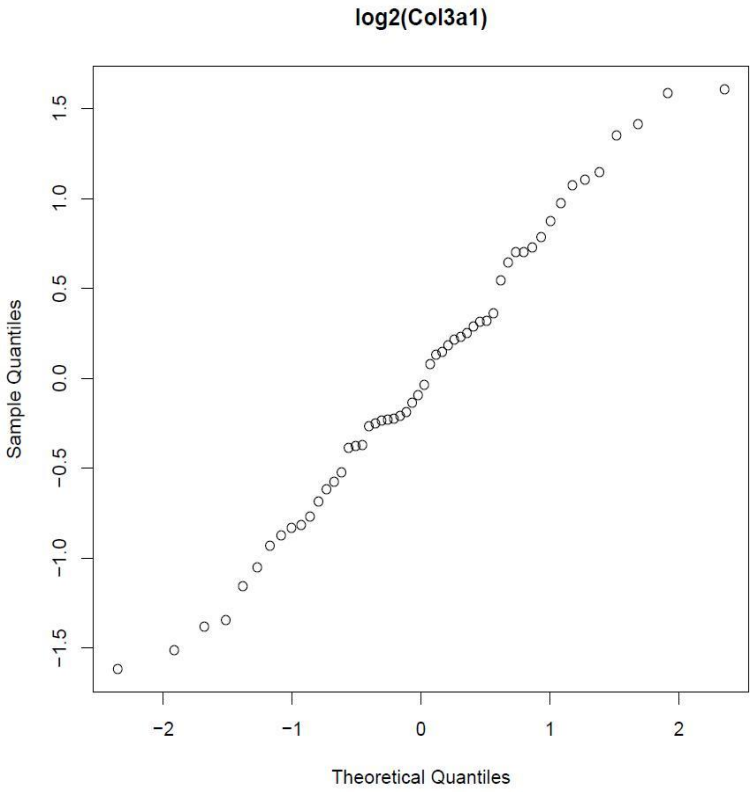
−1
0
1
2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Theoretical Quantiles
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−0.5
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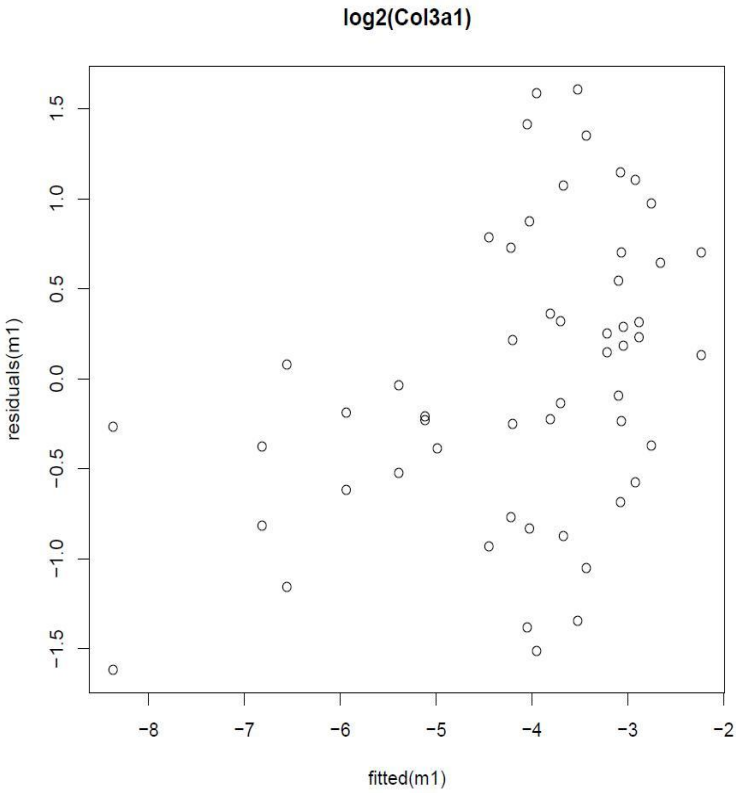
0.5
1.0
1.5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residuals(m1)
fitted(m1)
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−7
−6
−5
−4
−3
−2
−1.5
−1.0
−0.5
0.0
0.5
1.0
1.5

Appendix D Cross-sectional area (CSA) measurements for animals sacrificed at 0 weeks on diet.

Strain	Animal Number	Side	CSA_1	CSA_2	$CSA_{average}$
ApoE-KO	A41	L	0.85	0.79	0.82
	A41	R	0.97	1.01	0.99
	A42	L	1	1.2	1.1
	A42	R	0.68	0.64	0.66
	A43	L	0.8	0.88	0.84
	A43	R	0.72	0.8	0.76
	A44	L	0.96	0.88	0.92
	A44	R	0.72	0.71	0.715
	A45	L	0.5	0.46	0.48
	A45	R	0.74	0.77	0.755
	F52	L	0.48	0.56	0.52
	F52	R	0.5	0.52	0.51
	F54	L	0.61	0.57	0.59
	F54	R	1.15	1.26	1.205
	F55	L	0.78	1.06	0.92
	F55	R	0.65	0.77	0.71
	F56	L	0.63	0.61	0.62
	F56	R	0.54	0.58	0.56
	F57	L	1.29	1.13	1.21
	F57	R	0.68	0.72	0.7
	B50	L	0.84	0.9	0.87
	B50	R	0.58	0.57	0.575
C57Bl/6	H13	L	1.26	0.91	1.085
	H13	R	0.56	0.53	0.545
	H14	L	0.68	0.71	0.695
	H14	R	1.13	1.08	1.105
	H15	L	0.64	0.61	0.625
	H15	R	0.46	0.52	0.49
	H16	L	0.75	0.7	0.725
	H16	R	0.52	0.42	0.47
	H17	L	0.66	0.63	0.645
	H17	R	0.48	0.56	0.52
	I18	L	0.86	1.04	0.95
	I18	R	0.67	0.85	0.76
	I19	L	0.78	0.86	0.82
	I19	R	0.63	0.56	0.595

Strain	**Animal Number**	**Side**	**CSA_1**	**CSA_2**	**$CSA_{average}$**
C57Bl/6	I20	L	0.76	0.86	0.81
	I20	R	0.72	0.71	0.715
	J21	L	0.74	0.82	0.78
	J21	R	0.61	0.61	0.61
	J22	L	NA	NA	NA
	J22	R	0.66	0.56	0.61
	J23	L	0.91	0.93	0.92
	J23	R	0.61	0.71	0.66

Appendix E $2^{(-\Delta Ct)}$ and standard errors of each gene in qPCR analysis.

Strain	Diet	*Cpa3*		*TGF-β*		*Col1a1*		*Col3a1*	
		$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE
ApoE-KO	Regular chow	0.007521	9.6E-05	0.017007	0.000196	0.380416	0.006045	0.088516	0.001796
		0.002248	4.17E-05	0.006509	0.000144	1.872218	0.032047	0.284101	0.002615
		0.001544	1.05E-05	0.00395	3.81E-05	0.957784	0.004769	0.26478	0.001985
		0.002743	4.34E-05	0.004139	3.14E-05	0.081414	0.004065	0.034235	0.00504
		0.000304	1.84E-06	0.001819	1.8E-05	0.008616	0.000142	0.005027	0.000124
		0.0021	4.76E-05	0.007959	0.000134	0.016055	0.002024	0.006839	0.000253
		0.015995	0.0003	0.009807	0.000153	1.042111	0.028968	0.232863	0.008133
		0.003133	5.36E-05	0.005129	9.22E-05	1.851165	0.022592	0.346608	0.004033
		0.001435	4.63E-05	0.003735	5.13E-05	0.33765	0.001252	0.101467	0.001012
		0.002836	0.000125	0.003222	3.87E-06	1.424363	0.013353	0.19399	0.005513
		0.001641	4.86E-05	0.001485	9.23E-06	0.159671	0.000145	0.023215	0.000244
		0.009633	0.000411	0.014194	0.000344	0.583848	0.013812	0.1606	0.016483
		0.002327	5.41E-05	0.000944	1.99E-05	0.189724	0.000164	0.024097	7.91E-05
	High fat	0.006231	0.000125	0.011086	0.000129	1.521569	0.025739	0.247219	0.002582
		0.000968	3.28E-05	0.001914	4.04E-05	0.077625	0.000888	0.023992	0.000248
		0.001642	9.06E-05	0.003741	8.46E-05	0.34229	0.005538	0.078916	0.000906
		0.001224	1.91E-05	0.002354	2.9E-05	0.161848	0.002218	0.070059	0.000708
		0.001215	9.7E-06	0.004628	4.47E-05	0.346059	0.001217	0.095839	0.00142
		0.00087	3.6E-05	0.005677	0.00016	0.031915	9.45E-05	0.014337	0.000646
		0.000756	7.7E-05	0.005373	0.000484	0.029187	0.000526	0.010623	0.000281
		0.001196	1.88E-05	0.005038	1.05E-05	0.004336	0.000101	0.002509	7.45E-05
		0.000471	4.95E-06	0.001003	1.03E-05	0.00207	3.33E-05	0.000985	2.09E-05
		0.000306	6.09E-06	0.002368	2.05E-05	0.022794	0.000328	0.011187	5.7E-05
		0.002398	7.51E-05	0.00712	0.000103	0.01762	0.000321	0.004767	0.000303

Strain	Diet	*Cpa3*		*TGF-β*		*Col1a1*		*Col3a1*	
		$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE	$2^{(-\Delta Ct)}$	SE
C57Bl/6	Regular chow	0.002927	0.000254	0.003945	6.7E-05	1.758513	0.027494	0.158885	0.001707
		0.005687	0.000169	0.008605	7.82E-05	1.385522	0.013515	0.16863	0.008517
		0.002611	0.000188	0.002567	2.29E-05	0.905743	0.005371	0.088723	0.002981
		0.001563	6.22E-05	0.002344	1.11E-05	0.269309	0.003312	0.031452	0.000579
		0.002714	0.0001	0.00309	4.83E-05	2.016183	0.007365	0.148018	0.00143
		0.003807	0.000284	0.002285	9.37E-06	1.352272	0.012257	0.137844	0.006542
		0.005092	5.27E-05	0.010007	0.000291	1.34176	0.01243	0.12789	0.002659
		0.000881	0.000101	0.002999	5.8E-05	1.63475	0.02409	0.119203	0.003212
		0.003492	6.72E-05	0.002986	0.00015	1.395496	0.008216	0.112273	0.002844
		0.001157	4.75E-05	0.002095	0.000125	0.230305	0.003522	0.034454	0.000167
		0.00264	2.4E-05	0.002101	2.02E-05	0.461644	0.009213	0.045608	0.000318
		0.003224	2.14E-05	0.010581	9.96E-05	0.339928	0.004962	0.063194	0.001093
		0.001178	3.21E-05	0.002052	2.28E-05	0.149241	0.00163	0.02457	0.000202
		0.001494	1.27E-05	0.002219	3.49E-06	0.180776	0.001223	0.02489	0.000452
	High fat	0.001644	8.9E-05	0.003335	0.00012	1.407218	0.010205	0.091568	0.001393
		0.002232	6.11E-05	0.002357	3.48E-05	0.567587	0.004038	0.06099	0.000905
		0.000585	1.59E-05	0.002721	2.04E-05	1.482445	0.084526	0.17058	0.003822
		0.002621	0.000116	0.007493	0.000223	1.003434	0.010405	0.109771	0.002702
		0.003362	5.2E-05	0.005707	0.00021	1.097189	0.004365	0.114541	0.004231
		0.003663	5.12E-05	0.00718	0.000237	4.144638	0.094375	0.291129	0.009019
		0.001128	4.1E-05	0.003611	4.04E-05	0.224442	0.022591	0.023199	0.000185
		0.00074	2.4E-05	0.0017	3.18E-05	0.220457	0.00069	0.016586	0.000191
		0.00257	4.64E-05	0.003435	7.8E-05	1.135021	0.015156	0.165259	0.001677
		0.001159	1.06E-05	1.407218	0.010205	0.453147	0.002873	0.042826	0.001162
		0.001648	2.88E-05	0.004945	5.31E-05	2.452028	0.071061	0.236093	0.004044
		0.00521	5.37E-05	0.008587	0.000179	0.234771	0.001072	0.044685	0.000269
		0.001247	2.85E-05	0.001968	9.58E-06	0.201071	0.003024	0.022647	0.000188
		0.002156	1.49E-05	0.003358	7.48E-05	1.124138	0.017357	0.193857	0.005553
		0.006454	7.2E-05	0.009979	7.53E-05	0.572248	0.026145	0.07376	0.000542
		0.001898	2.18E-05	0.004273	0.000225	2.550851	0.04075	0.261861	0.001675