Effects of Acarbose on Age-Related Declines in Motor and Cardiac Function

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ABSTRACT

Age is a primary risk factor for chronic conditions including cardiovascular diseases. With inflation in the aged population over the last century, there is a growing need for interventions that directly target aging. Acarbose, an alpha-glucosidase inhibitor, is a promising aging intervention that increases lifespan in male and female mice by 22% and 5%, respectively (Harrison, 2013). Acarbose is an FDA approved anti-diabetic drug with a limited side effect profile. However, it has not been established if Acarbose has beneficial effects on healthspan or measures of functional health. We hypothesized that, in addition to lengthening lifespan, Acarbose would also diminish the effects of aging on motor and cardiac function.

Using an outbred mouse model with young control, old control, and old Acarbose treated mice, motor function testing demonstrated a decline in functional health with age that was ameliorated by Acarbose treatment. Echocardiography showed concentric left ventricular hypertrophy with age in both males and females that was abrogated with Acarbose treatment in males. However, no significant declines in cardiac function were detected with age nor were any effects of Acarbose observed on cardiac function.

Preliminary mass spectrometry analysis suggested that metabolism could play a role in the cardiac aging process, but no evidence suggested a role of Acarbose in reversing these age-related changes. An ELISA based assay (PROCISE) also demonstrated a reduction in constitutive proteasome activity in the heart with age, but this was not affected by Acarbose.

The findings that Acarbose ameliorates some functional declines seen with age and abrogates left ventricular hypertrophy in males furthers its promise as an aging intervention, but more research is needed into its effects in the heart and other physiological systems before it could serve as a clinically relevant drug for non-diabetic patients.

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INTRODUCTION

Burdens of an Aging Population – Healthspan and Lifespan

Advancements in public health as well as medical interventions have significantly increased the median lifespan of individuals in society (Wright and Weinstein, 1998). The United States in particular is experiencing drastic growth in its population of people 65 years and older, to the extent that these individuals are anticipated to comprise 20% of the overall population by the year 2023 (Arias, 2014). Aging is a risk factor for many chronic diseases including neurodegenerative diseases, cancer, and cardiovascular diseases (Partridge, 2010), the latter representing the leading causes of death around the world (World, 2014). Therefore, with more people living longer lives, chronic diseases and their burden will become increasingly prevalent, necessitating advancements in healthcare treatment strategies. Maintenance of cardiac health is a key focus of these healthcare strategies, as it is an essential component to lifespan (Quarles, 2015) and also healthspan (Lakatta, 2003), which refers to the period in one's life when general health and function is maintained. Extending healthspan, instead of just focusing on lifespan, has become a greater clinical and research focus, as delaying or preventing disease and disability can facilitate a healthier life with aging (Living, 2015).

Motor Function

One of the most obvious declines in healthspan for an elderly patient is impairments in motor function. Not being able to perform everyday tasks like getting out of bed, walking to the bathroom, and preparing meals can pose a large burden on someone's life. There are numerous tests that physicians and physical therapists utilize in order to assess the levels of functional disability in an aged patient. One commonly used test referred to as the "timed up and go test" requires the individual to stand from sitting in a normal arm chair, walk to an imaginary finish

line 10 feet away, walk back to the chair, and sit back down as quickly as possible. Longer times on this test are predictive of a higher risk of falling for the patient. A similar test also used for geriatric patients is the "sit-to-stand test," which measures the time required for the individual to stand from a normal arm chair and return to a sitting position a total of 5 consecutive times. Another assessment of disability involves asking the patient to perform a squat and observing the smoothness of the movements and muscle contractions and relaxations. Together, these kinds of tests model everyday movements and abilities that are important for an individual's quality of life. They all show marked declines in performance with age, and reduced ability at these skills are predictive of other comorbidities associated with aging such as falling (Gellert).

The Cardiac Aging Phenotype

Healthspan parameters also often include elements of cardiac health, and changes in the heart with age that are indicative or predictive of dysfunction have been established. These kinds of variables are essential in not only diagnosing impairments in the heart, but also in measuring the effectiveness of treatment strategies at ameliorating age-related changes. There are several key features, both structural and functional, that typically distinguish an old from a young heart. In terms of cardiac structure, aging is associated with hypertrophy of the left ventricle, the largest chamber of the heart that is responsible for pumping blood throughout the body. More specifically, cardiac aging usually leads to concentric hypertrophy, in which there is thickening of the walls of the left ventricle and the cavity size is either reduced or shows no significant changes. When it comes to age-related functional changes in the heart, there are often impairments in diastolic function, which is the ability of the heart to properly relax following a contraction. However, the contractile ability of the heart, referred to as systolic function, is typically maintained with age (Fleg, 2012).

Directly Targeting Aging

Motor function and cardiac health are important measures of healthspan that decline with age, but aging-associated comorbities are countless and occur all throughout the body. This means that treatment strategies targeting individual physiological systems could grow impractical as more and more markers of dysfunction appear. Therefore, there is an increasing amount of research going into interventions that target aging directly instead of targeting individual diseases or symptoms. The ideal intervention would not only extend lifespan, but also improve a variety of healthspan measures that are critical to overall quality of life.

Two of the most well characterized aging interventions are caloric restriction, which is typically defined as consuming approximately 70% of one's normal caloric intake, and rapamycin, an immunosuppressant compound. Both caloric restriction (Flurkey, 2010) and rapamycin (Harrison, 2010) have been shown to significantly extend lifespan in male and female mice. It has also been demonstrated that both interventions lead to improvements in motor function (Salvatore, 2016; Zhang, 2014) in addition to reversing several cardiac structural and functional changes seen with aging (Dhahbi, 2006; Dai, 2014). The mechanism of lifespan extension for both interventions involves inhibition of the kinase mTOR, or mammalian target of rapamycin. mTOR signaling integrates intracellular and extracellular signals and serves as a key regulator of cell proliferation, growth, metabolism, and survival (Laplante, 2009). While the lifespan and healthspan improvements of caloric restriction and rapamycin are well documented in model organisms, the clinical applicability of them as potential aging interventions in humans is limited. Rapamycin can be accompanied by several adverse effects, including metabolic complications, immunosuppression and infectious complications, and pulmonary and renal toxicities (Somers, 2015). Caloric restriction may be considered a safer intervention, albeit with

potential for nutritional deficiencies in older adults (Miller, 2008), but adhering to such a regimen is exceedingly difficult and considered impractical (Johnson, 2011). An exploration of new potential interventions in the aging process that have greater clinical relevance is therefore needed.

Acarbose as a Promising Aging Intervention

A new compound entered the conversation of potential aging interventions in 2014 when Acarbose was shown to significantly increase lifespan in male (22%) and female (5%) mice, with greater improvement in males than females (Harrison, 2014). Importantly, Acarbose is a well-tolerated drug with minimal gastrointestinal side effects prescribed for diabetic patients (Hollander, 1992). Acarbose therefore arguably has greater translational value than caloric restriction or rapamycin as an aging intervention, yet the effects of Acarbose on healthspan have not been assessed. The compound is an intestinal alpha-glucosidase inhibitor, preventing the breakdown of polysaccharides into glucose and limiting glucose absorption from the GI tract. Acarbose was first theorized to potentially work as an aging intervention based on its ability to prevent postprandial hyperglycemia, which has been suggested to contribute to aging (Archer, 2003). Furthermore, based on its gastrointestinal role of limiting glucose absorption, Acarbose could also be thought of as a plausible calorie restriction mimetic (Harrison, 2014). This implies that Acarbose may also inhibit mTOR signaling and extend lifespan through a similar molecular mechanism to caloric restriction and rapamycin. The possible connection between Acarbose and mTOR signaling could also be explained based on the impact of Acarbose on insulin-like growth factor (IGF) signaling. It has been demonstrated that Acarbose reduces IGF-1 levels (Harrison, 2014) and that IGF signaling is interconnected with mTOR complex 1 (mTORC1) through

several molecular mediators including FOXO transcription factors phosphatidylinositol-3-OH

kinase (PI(3)K) and Akt kinase (Johnson, 2013). Therefore, if Acarbose is extending lifespan

through similar molecular processes to rapamycin and caloric restriction, it can be hypothesized

that Acarbose may also lead to similar improvements in healthspan measures of motor and

cardiac function. Determining if Acarbose can successfully improve healthspan is essential in

analyzing its potential as an aging intervention in humans.   This ultimately leads to the overall

goal of this thesis research: to investigate the effects of Acarbose on age-associated declines in

motor and cardiac function and dissect the molecular mechanisms through which Acarbose
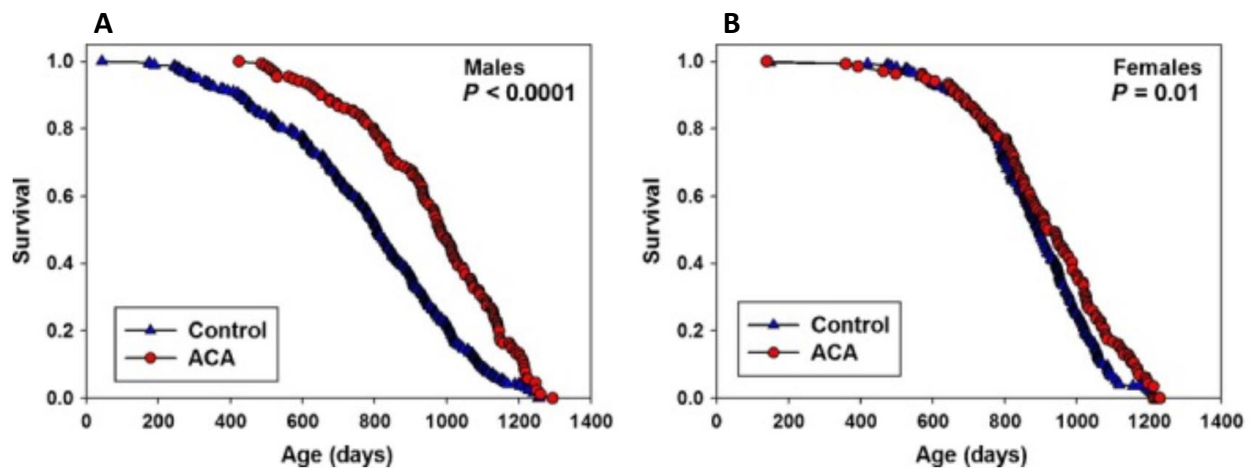
impacts the aging process.

Figure 1. Kaplan-Meier survival plot demonstrating that Acarbose significantly extends lifespan in both
male and female mice. Lifespan extension in males (A) is more significant than in females (B). Adapted
from Harrison et al., 2014.

Outbred Mouse Model Study Design

A plethora of model organisms have been used in aging research, but some of the most ubiquitous include yeast, roundworms, fruit flies, and mice. While they have disadvantages regarding cost and time investment, mice were used for this study based on the ease and clinical relevance of obtaining measures of motor function, cardiac function, and cardiac tissue for molecular analysis from the model. More specifically, an outbred mouse strain was utilized to best model human genetic heterogeneity. Control 4-month-old mice, control 22-month-old mice, and Acarbose treated 22-month-old mice were used for this study to delineate the effects of both age and Acarbose on measures of healthspan and relevant molecular pathways.

Motor Function Testing in a Mouse Model

To evaluate the ability of Acarbose to improve elements of motor function in mice, a series of mouse-specific functional tests were performed that demonstrate age-related declines in a mouse model and that are translationally relevant measures in humans. One such measure is forelimb grip strength, which has been shown to significantly decline with age in mice (Justice, 2013). Grip strength is also considered a clinically useful parameter, as poorer grip strength has been shown to be associated with increased mortality from all-causes, from cardiovascular disease, and from cancer in men (Gale, 2007). Several protocols for measuring functional health in mice have also been established using a rotarod, which is an electrically controlled spinning rod on which mice walk or run. A rotarod acceleration test, which serves as a measure of balance and coordination, has shown significant declines in performance in aged mice, similar to grip strength (Justice, 2013). A rotarod endurance test, which represents a form of exercise performance, has also demonstrated reduced endurance in mice with aging (Justice, 2013). Both coordination and exercise performance are important in human functional health. Therefore, grip

strength and rotarod testing serve as clinically relevant measures of healthspan that show age-associated declines in mice and can be used to test the effects of Acarbose on functional health.
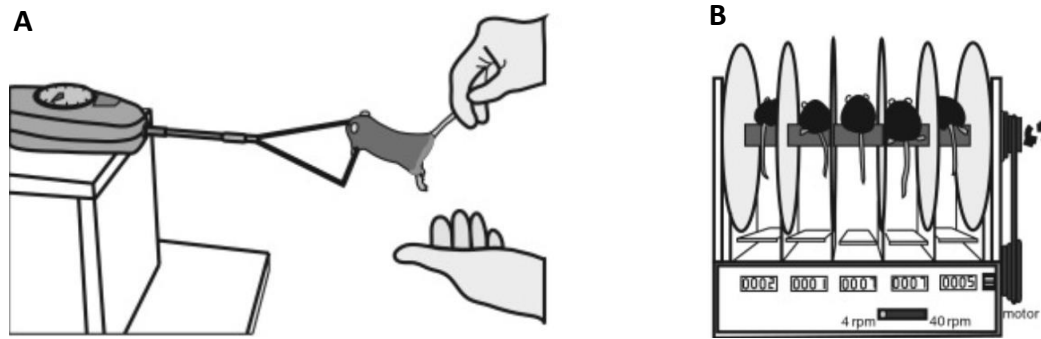

Figure 2. Depictions of grip strength (A) and rotarod (B) tests performed on experimental mice. Adapted from Justice et al., 2013.

Mouse Echocardiography

Measuring changes in cardiac structure and function in human patients is often performed using echocardiography, a form of ultrasound imaging. This technique can also be utilized with mice and therefore serves as a valuable tool to assess the impacts of age and Acarbose on cardiac health. One form of echocardiography, often referred to as conventional echocardiography, can provide structural measures such as left ventricular mass and chamber wall thicknesses in addition to some functional parameters like ejection fraction (a systolic measure of the percentage of blood leaving the heart each time it contracts). Additional forms of ultrasound imaging include Pulsed Wave Doppler and Tissue Doppler echocardiography which measure velocities of the valves and muscles of the heart as well as functional time intervals during contraction and relaxation. One frequently analyzed Doppler measurement is isovolumic relaxation time (IVRT), which is the time between the closure of the aortic valve and the opening

of the mitral valve. This interval reflects the time between the end of a contraction and the beginning of relaxation of the heart, therefore making it an indicator of cardiac diastolic function. Another useful diastolic variable is the ratio of early to late left ventricular filling, or E'/A'. The early filling variable E' reflects the velocity of blood as it passively flows into the left ventricle during diastole. The late filling variable A' is the velocity of blood that is forced into the left ventricle at the beginning of systole prior to ventricular contraction. This parameter is therefore affected by changes in the heart's diastolic ability to allow blood to passively flow into the ventricle. An additional variable often measured with Doppler echocardiography is Myocardial Performance Index (MPI), a combinatorial measure of systolic and diastolic function that considers the times required for left ventricular contraction, relaxation, and ejection.

Echocardiography in mice has revealed that many of the key features of human cardiac aging can be recapitulated in a mouse model. In mice, increased left ventricular mass has been demonstrated with age (Dai, 2009). Increased IVRT, indicating diastolic dysfunction, has also been shown in an aged mouse model (Qin, 2013). Mice have also been shown to have significantly reduced E'/A' ratios, signifying a decline in diastolic function, in addition to increased MPI with age, indicative of a worsening of overall cardiac function (Dai, 2009). This suggests that conventional and Doppler echocardiography can detect age-related declines in cardiac structure and function in mice and could potentially also detect any Acarbose-induced changes as well.

Another form of imaging that has been suggested to be a more sensitive measure of cardiac function is strain echocardiography. Strain uses motion tracking to detect subtle changes in the length of the left ventricular wall during contraction and relaxation, serving as a measure of tissue deformation throughout the cardiac cycle. The outputs of strain echocardiography are

measures of strain, in percent change of left ventricular wall length, and strain rate, in percent change of wall length per second. Measures are obtained from 2 different planes of view: short axis (from the left to right side of the heart) and long axis (from the base to the apex). Short axis imaging provides measures of radial and circumferential strain and strain rate, whereas long axis provides measures of radial and longitudinal strain and strain rate. Radial strain measures tissue deformation in the left-to-right orientation of the heart, longitudinal strain measures deformation in the base-to-apex orientation, and circumferential strain measures rotational deformation of the ventricle.

Strain echocardiography has also been performed in mice and demonstrated age-associated impairments. Slightly decreased measures of strain and strain rate were found in the radial, circumferential, and longitudinal directions, suggesting an overall decline in contractile function. The aging intervention rapamycin was also tested in these mice, and treatment led to selective improvements in some strain parameters. Age-related declines in circumferential strain and strain rate were ameliorated with rapamycin, while all other measures were unaffected (Flynn, 2013). Being a more sensitive measure of cardiac function, strain serves as an additional tool to detect functional changes with age or Acarbose that may be too subtle to detect with conventional or Doppler echocardiography.
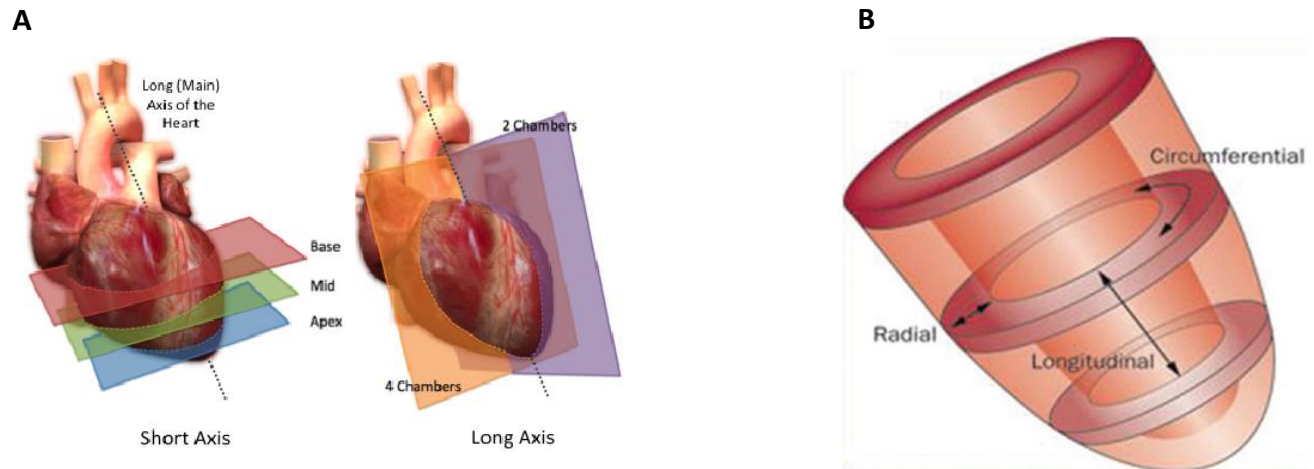
A

B

Figure 3. Diagrams to help illustrate the short-axis and long-axis views of the heart in strain echocardiography (A) in addition to the orientations of radial, longitudinal, and circumferential strain of the left ventricle of the heart (B). Adapted from Gonzalez, 2009 (A) and Malik et al., 2016 (B).

Investigating Molecular Pathways with Mass Spectrometry

As previously discussed, there is reason to believe that Acarbose could interact with mTOR signaling to impact the aging process. However, it is also possible that the link between Acarbose and aging could be explained by changes in metabolic efficiency. It is known that in the normal adult heart, fatty acid oxidation is responsible for the majority of ATP production. However, under pathological conditions such as hypertrophy, the main fuel for energy production in the heart shifts to glucose (Ma, 2015), demonstrating a reduction in metabolic efficiency. Based on the hypertrophic signature of the aged heart, it would be expected that this shift in energy metabolism would also occur with aging.

Because of the potential link between cardiac metabolism and aging, the impact of rapamycin on metabolic efficiency has been investigated in aged mice. Consistent with an improvement in the metabolic profile of the heart to a youthful state, rapamycin was shown to

decrease the levels of 4 glycolytic intermediates, indicative of a reduction in glycolysis. This finding was accompanied by an increase in TCA cycle intermediates that serve as entry points of anaplerosis (Chiao, 2016), also suggestive of an improvement in metabolic efficiency with rapamycin.

In addition to the possibility that Acarbose may function through a similar molecular mechanism to rapamycin, the direct role of Acarbose in carbohydrate metabolism suggests that it may also restore the heart to a healthier metabolic state. By acting as an alpha-glucosidase inhibitor, Acarbose decreases the levels of free circulating glucose in the body. It is possible that this would therefore lead to a decrease in glucose utilization through a reduction in glycolysis in various organ systems, including the heart, leading to greater metabolic efficiency.

The role of Acarbose in aging could be explained by an interaction with mTOR signaling, by improvements in cardiac metabolic efficiency, a combination of the two, or through any combination of previously unpredicted mechanisms. Therefore, unbiased mass spectrometry was performed in the hopes of elucidating the molecular changes seen in the heart with age and with Acarbose treatment.

Aging and Proteostasis

In addition to an unbiased study with mass spectrometry, a specific molecular process investigated was proteostasis, which refers to the maintenance of protein quality control mechanisms including synthesis, degradation, folding, and trafficking. Proteostasis underlies many forms of dysfunction throughout the body, and there are several diseases that are associated with deficits in proteostasis including Creutzfeldt-Jakob disease, Alzheimer's disease, Parkinson's disease, and many more. Impaired protein homeostasis is also a hallmark of aging (López-Otín, 2013). The induced synthesis of cytosolic and organelle-specific chaperones during

stress is greatly impaired with age (Calderwood, 2009). Long-lived mouse strains show a marked

upregulation of some heat-shock proteins (Swindell, 2009). Amyloid-binding compounds can

help maintain proteostasis during aging and extend lifespan (Alavez, 2011). The activities of the

authophagy-lysosomal system (Rubinsztein, 2011) and the ubiquitin-proteasome system

(Tomaru, 2012), two key proteolytic systems in protein quality control, also have been shown to

decline in aging. Given this, it is possible that impairments in proteostasis could underlie some

aspects of cardiac dysfunction seen with age.

       A key component to proteostasis is the controlled expression and activity of proteasomes,

which are protein complexes that function to degrade misfolded, damaged, or unneeded proteins

through proteolysis. Proteasomes are cylindrical complexes made up of stacked rings of protein

subunits that carry out two main functions. The rings at the end of proteasomes serve as gates

that only allow proteins tagged for degradation by ubiquitination to enter. The inner rings are

then composed of proteins with various proteolytic capabilities to break down the targeted,

unwanted protein.

       There are two primary kinds of proteasomes that exist in cells: constitutive and

immunoproteasomes. In terms of structure, the two kinds of proteasomes are identical except for

differences in 3 of the core subunits, leading to different catalytic activity. Most of what is

known about the immunoproteasome concerns its role in producing peptides for antigen

presentation by MHC class I proteins in the immune system. However, a number of studies have

started to suggest a greater diversity of roles of the immunoproteasome. Enhanced induction of

the immunoproteasome was demonstrated in neurons expressing a mutation for Huntington's

disease (Diaz-Hernandez, 2004). Studies of hematologic cancer patients showed greater mRNA

transcript levels of one of the immunoproteasome catalytic subunits compared to its subunit counterpart in constitutive proteasomes (Kuhn, 2007).

Immunoproteasomes have also been implicated in aging. It has been observed that longer lived species have increased immunoproteasome subunit expression (Pickering, 2015), suggesting that induction of immunoproteasomes could play a role in attenuating the aging process. In addition, there is reason to believe that Acarbose may alter immunoproteasome activity, which could partially account for the lifespan extension seen with treatment. mTOR complex I has been implicated in regulating elements of proteostasis (Quarles, 2015). One component of this is recent evidence also implicating mTOR complex I in the formation of immunoproteasomes (Yun, 2016). If Acarbose regulates mTOR, then it could also play a role in altering immunoproteasome levels and activity.

The impact of aging and Acarbose on both constitutive and immunoproteasome activity in the heart was therefore investigated using the Proteasome Constitutive Immuno-Subunit Elisa, or PROCISE method (Kirk, 2014). This assay measures the activity of one of the primary catalytic subunits in constitutive proteasomes, β5, and its immunoproteasome counterpart, β5i. Differential activity of these subunits could potentially suggest a role of proteostasis in the effects of aging and Acarbose treatment on the heart.

Overview

The experimental goal of this research is to assess the impact of Acarbose on age-associated declines in motor and cardiac function and delineate its molecular role in the aging process. It has been established that Acarbose improves longevity in mice, but the impact of Acarbose on healthspan has not been determined. To evaluate this, young control, old control, and old Acarbose treated mice underwent motor function testing, including measurements of grip

strength, balance/coordination, and exercise performance. These same mice then underwent conventional, Doppler, and strain echocardiography to detect any cardiac structural or functional changes due to age or Acarbose. Cardiac tissue was then harvested from these mice for molecular analysis using mass spectrometry and the PROCISE assay. The aim is for the outcomes of this project to help guide future research and applications of Acarbose as a potential aging intervention in humans.

METHODS

Mouse Model

      58 mice were provided for this study by the Glenn Foundation. The mice were an outbred strain (UMHET3) resulting from the following F1 cross: [BL/6 x Balb/c] females x [C3H x DBA] males. 17 mice, 9 males and 8 females, were aged 4 months on control, Purina 5LG6 diet. 17 mice, 9 males and 8 females, were aged 22 months on control diet. 24 mice, 12 males and 12 females, were aged 22 months on control diet for the first 8 months of life and on control diet supplemented with Acarbose at 1000ppm for the remaining 14 months.

Forelimb Grip Strength Testing

      Grip strength protocol was adapted from Justice et al., 2013 and was assessed using the Columbus Instruments Grip Strength Meter provided by the Frankel Cardiovascular Center Physiology and Phenotyping Core at the University of Michigan. Testing was randomized and performed blinded by Sean Louzon and Jonathan Herrera. All 58 mice had their forelimb grip strength measured with 5 trials per mouse. Testing occurred over a 5-day period between the hours of 8:00am and 12:00pm. Each mouse had all 5 of its trials occur in the same morning roughly 30 minutes apart. Grip strength was reported as kilograms of force and the average grip strength across the 5 trials for each mouse was analyzed.

Acceleration and Endurance Rotarod Testing

      Rotarod testing protocol was adapted from Justice et al., 2013 and was performed on the Columbus Instruments Dual Species Economex Rota-Rod provided by the Frankel Cardiovascular Center Physiology and Phenotyping Core at the University of Michigan. Testing was randomized and performed blinded by Sean Louzon and Jonathan Herrera. All 58 mice were

tested following an acceleration protocol with 3 trials per mouse. Acceleration testing took place

over a 5-day period between the hours of 1:00pm and 4:00pm. Each mouse had all 3 of its trials

occur in the same afternoon roughly 30 minutes apart. Mice were placed onto the rotarod set at

4rpm and accelerated at 0.1rpm/s until falling off the rod. The time was recorded from the start

of acceleration to the time the mice fell. The maximum trial for each mouse was used for

analysis.

All the mice also underwent an endurance protocol with 1 trial per mouse. Endurance

testing occurred over a 5-day period between the hours of 8:00am and 12:00pm. The average of

the max acceleration trials was used to calculate the average max speed for each of the following

groups: 4 month males, 4 month females, 22 month males, and 22 month females. Mice were

placed onto the rotarod set at 4rpm and accelerated at 0.1rpm/s until they reached 25% of their

group's max speed. They then maintained this speed for 2 minutes. The mice were then

accelerated again at 0.1rpm/s until they reached 50% of their max speed where they were held

for 5 minutes. They were then accelerated at 0.1rpm/s to 75% of their max speed and held for 10

minutes. They were then accelerated one final time at 0.1rpm/s to their max speed and

maintained at this final speed. The time was recorded from the start of the first acceleration and

ran continuously throughout the test. If a mouse fell prior to the start of the 10-minute period at

75% of max speed, it was placed back on the rotarod. If a mouse fell 10 times prior to the 10-

minute period at 75% of max speed, the mouse's test ended, and its time was recorded as the

time of its 10th fall. If a mouse fell once during the first 2 minutes of the 10-minute period at 75%

max speed, it was placed back on the rod. If a mouse fell twice during the first 2 minutes of the

10-minute period at 75% max speed, the mouse's test ended, and its time was recorded as the

time of its 2nd fall. If a mouse fell after the first 2 minutes of the 10-minute period at 75% max

speed, the mouse's test ended, and its time was recorded as the time of its fall. If a mouse reached 30 total minutes, its test ended and its time was recorded as 30 minutes.

Echocardiography

Echocardiography was performed randomized and blinded on all 58 mice by research echocardiography sonographer Kimber Converso-Baran at the University of Michigan Frankel Cardiovascular Center Physiology and Phenotyping Core. The following protocol description was provided by Kimber Converso-Baran.

Induction of anesthesia was performed in an enclosed container filled with 5% isoflurane. After induction, the mice were placed on a warming pad to maintain body temperature. 1 – 1.5% isoflurane was supplied via a nose cone to maintain a surgical plane of anesthesia. The hair is removed from the upper abdominal and thoracic area with depilatory cream. ECG is monitored via non-invasive resting ECG electrodes**.** Transthoracic echocardiography was performed in the supine or left lateral position. Two-dimensional, M-mode, Doppler and tissue Doppler echocardiographic images were recorded using a Visual Sonics' Vevo 2100 high resolution *in vivo* micro-imaging system. We measured LV ejection fraction from the two-dimensional long axis view. In addition, we measured systolic and diastolic dimensions and wall thickness by M-mode in the parasternal short axis view at the level of the papillary muscles. Diastolic function was assessed by conventional pulsed-wave spectral Doppler analysis of mitral valve inflow patterns (early [E] and late [A] filling waves). Doppler tissue imaging (DTI) was used to measure the early (E') and late (A') diastolic tissue velocities of the septal and lateral annuluses of the mitral valve in the apical 4-chamber view. Speckle-tracking strain was also utilized to evaluate subtle abnormalities in myocardial dysfunction.

Mass Spectrometry

Mass spectrometry was carried out by the Proteomics Resource Facility through the Department of Pathology at the University of Michigan. Protein sample preparation from frozen left ventricular heart tissue was performed by Sean Louzon using RIPA Buffer and Roche Complete Mini protease inhibitor cocktail tablets. Proteomic analysis was performed using Thermo Scientific TMT10plex Mass Tag Labeling Kits and Reagents. 27 samples were analyzed across 3 independent experiments, with each experiment containing 9 samples and 1 master-mix sample containing a small amount of all 27 samples. All samples sent for mass spectrometry were from male mice: 9 young controls, 8 old controls, and 10 old Acarbose treated. The following summarized protocol was adapted from the instructions manual for Thermo Scientific TMT10plex Mass Tag Labeling Kits and Reagents (a more detailed protocol can be found in the instructions manual).

Protein extracts were provided to the core and were then reduced, alkylated, and digested overnight. Samples are labeled with the TMT reagents and then mixed before sample fractionation and clean-up. Labeled samples are analyzed by high resolution Orbitrap LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundance.

A preliminary analysis of the mass spectrometry data was performed with independent sample t-tests to identify differentially expressed proteins with age and Acarbose treatment. Significance threshold for differential expression with age was set at $p<0.001$ and at $p<0.01$ for differential expression with Acarbose. More robust data analysis that accounts for multiple comparisons is currently being performed.

PROCISE Assay

Frozen left ventricular heart samples from all mice were sent to Ping Wang MD at the Feinstein Institute for Medical Research to perform the PROCISE assay. The protocol for the assay was adapted from Kirk et al., 2014.

RNA Sequencing

RNA was prepared from left ventricular cardiac tissue from young control, old control, and old Acarbose treated mice using RNeasy Fibrous Tissue Mini Kit from Qiagen. RNA samples were sent to the DNA Sequencing Core at the University of Michigan for quality checks. All samples that had an RIN value greater than 6.5, a 260/280 ratio between 1.7 and 1.9, and a 260/230 ratio greater than 1.5 were subsequently sent for RNA sequencing at the core.

Statistical Analysis

The overall effects of age and Acarbose treatment on each parameter were calculated with 2-way ANOVA's using SPSS. When sex specific differences were present, individual group comparisons were calculated using independent samples t-tests.

RESULTS

Acarbose Ameliorates Declines in Motor Function with Age

In addition to weighing the mice before testing began, the first set of healthspan measures conducted on the mice included grip strength, acceleration testing, and endurance testing. For both males and females, body weight was significantly increased with age and reduced with Acarbose (Figure 4A). Grip strength, which was normalized to body weight, was markedly impaired in 22 month controls compared to 4 month controls, and this was partially rescued by Acarbose treatment (Figure 4B). Significant effects of age and Acarbose on grip strength were still present without normalization to body weight (data not shown).

Acceleration testing, a reported measure of balance and coordination, was also performed in all mice. Fall latency in young controls was found to be greater than in old control males and females, indicating a decline in performance with age (Figure 4C). Acarbose treated old mice displayed greater fall latency than old controls in both sexes, again indicating a partial rescue in performance with Acarbose.

The final motor function test conducted was the endurance rotarod protocol which is suggested to represent a measure of exercise performance. While no statistically significant effects were found related to age or Acarbose treatment, there was a trend toward a reduction in fall latency with age and improvement with Acarbose in males and females (Figure 4D). Together, these tests help verify age-associated impairments in motor function and demonstrate that Acarbose can successfully restore functional ability to a more youthful state in mice.
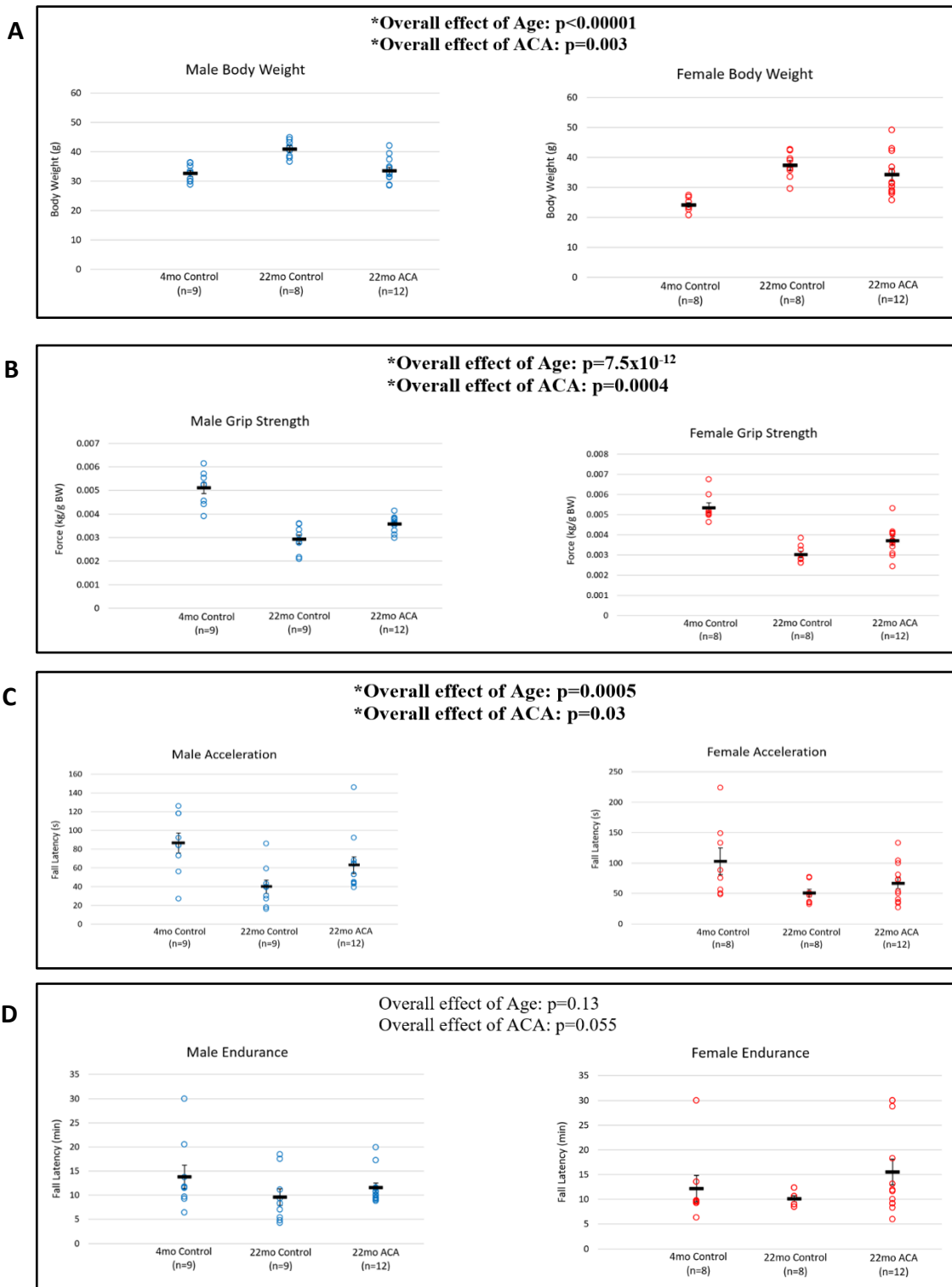
Figure 4. Dot plots for body weight (A), grip strength (B), acceleration testing (C), and endurance testing (D). Circles represent data points for individual animals, horizontal black bars indicate the mean of the group, and error bars are ± standard error of the mean. No sex specific effects were found. Overall effects of age and Acarbose for each parameter are displayed above the corresponding dot plots with significance threshold set at p<0.05.

Male Specific Reduction in Concentric Left Ventricular Hypertrophy by Acarbose

Following motor function testing, all mice underwent conventional echocardiography to measure any changes in cardiac structure that occurred with age or Acarbose treatment. Left ventricular mass was found to be significantly increased in both males and females, but to a greater extent in males (Figure 5A). This is indicative of age-related ventricular hypertrophy, one of the hallmarks of the cardiac aging phenotype. Acarbose was successfully able to abrogate hypertrophy, but only in males. The effects of age and Acarbose on left ventricular mass are diminished when normalized to body weight (data not shown).

To further characterize the hypertrophy seen with age, measurements were obtained of the thickness of the posterior (Figure 5B) and septal (Figure 5C) walls of the left ventricle. Both parameters were shown to be significantly increased with age in males, but not in females. This demonstrates that, in at least males, the hypertrophy seen in the left ventricle is concentric, meaning it is due to increased thickness of the chamber walls. This is the same kind of hypertrophy that is typically reported in elderly patients. However, Acarbose did not significantly affect either wall thickness measurement. It is therefore possible that in males, the reduction in left ventricular mass with Acarbose is due to subtle declines in muscle thickness throughout the ventricle that are not significant individually but together result in significantly reduced left ventricular mass. Overall, this represents the first evidence that Acarbose ameliorates age-related concentric hypertrophy of the left ventricle in male mice.
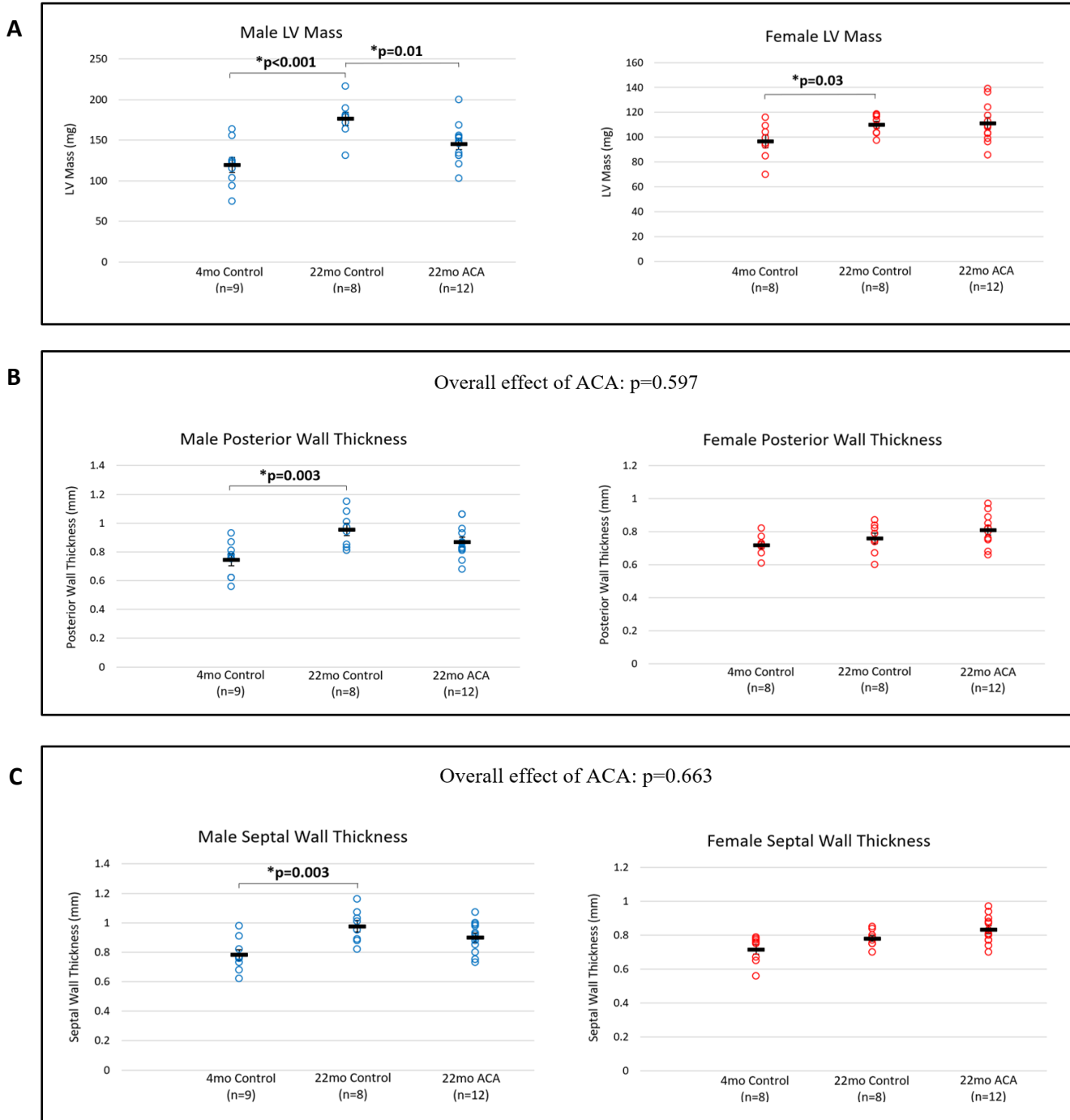
Figure 5. Dot plots for left ventricular mass (A), posterior wall thickness (B), and septal wall thickness (C). Circles represent data points for individual animals, horizontal black bars indicate the mean of the group, and error bars are ± standard error of the mean. Sex specific effects are shown within the graphs. Where effects are not sex specific, overall effects for each parameter are displayed above the corresponding dot plots with significance threshold set at p<0.05.

Age-Associated Cardiac Functional Declines Not Detected

Given the left ventricular hypertrophy detected with age in our mice, our next question was whether these structural changes resulted in any cardiac functional declines. Functional parameters were assessed with both conventional and Doppler echocardiography.

Several measures of diastolic function, or the heart's ability to properly relax following contraction, were measured including isovolumic relaxation time (Figure 6A) and the ratio of early to late ventricular filling (Figure 6B). However, no diastolic parameters were found to be significantly affected by either age or Acarbose treatment. This was also found to be true for myocardial performance index (Figure 6C), a combined measure of the left ventricle's systolic (contractile) and diastolic function. The only parameter that demonstrated significant change was ejection fraction (systolic measure), which was shown to be increased with age in males (Figure 6D). This change in ejection fraction however is not indicative of cardiac dysfunction. Based on the cardiac aging phenotype hallmarks, we expected to see age-related declines in diastolic function with preserved systolic function. However, we were not able to detect any cardiac functional deficits in our mice or any Acarbose-induced functional changes.
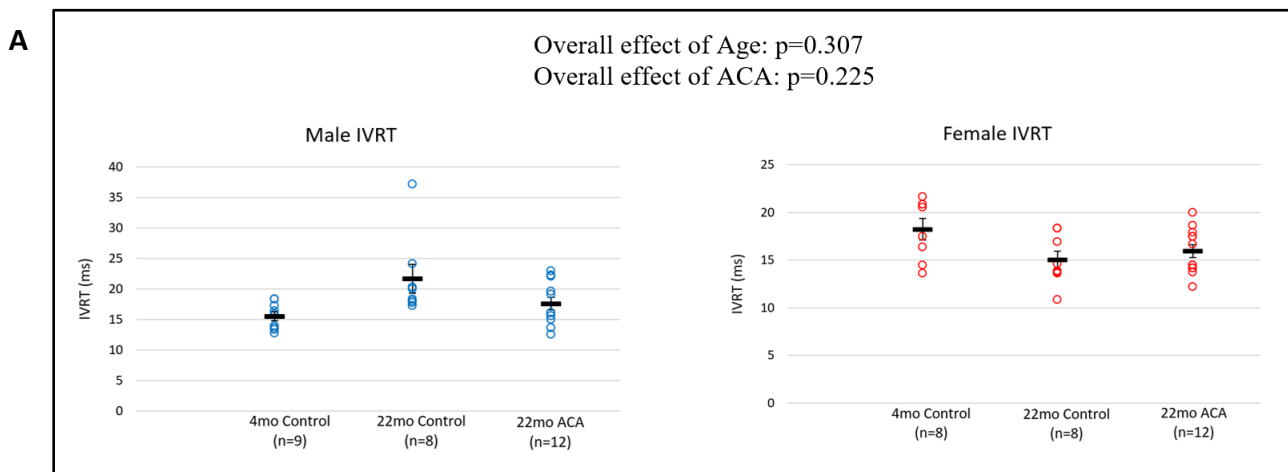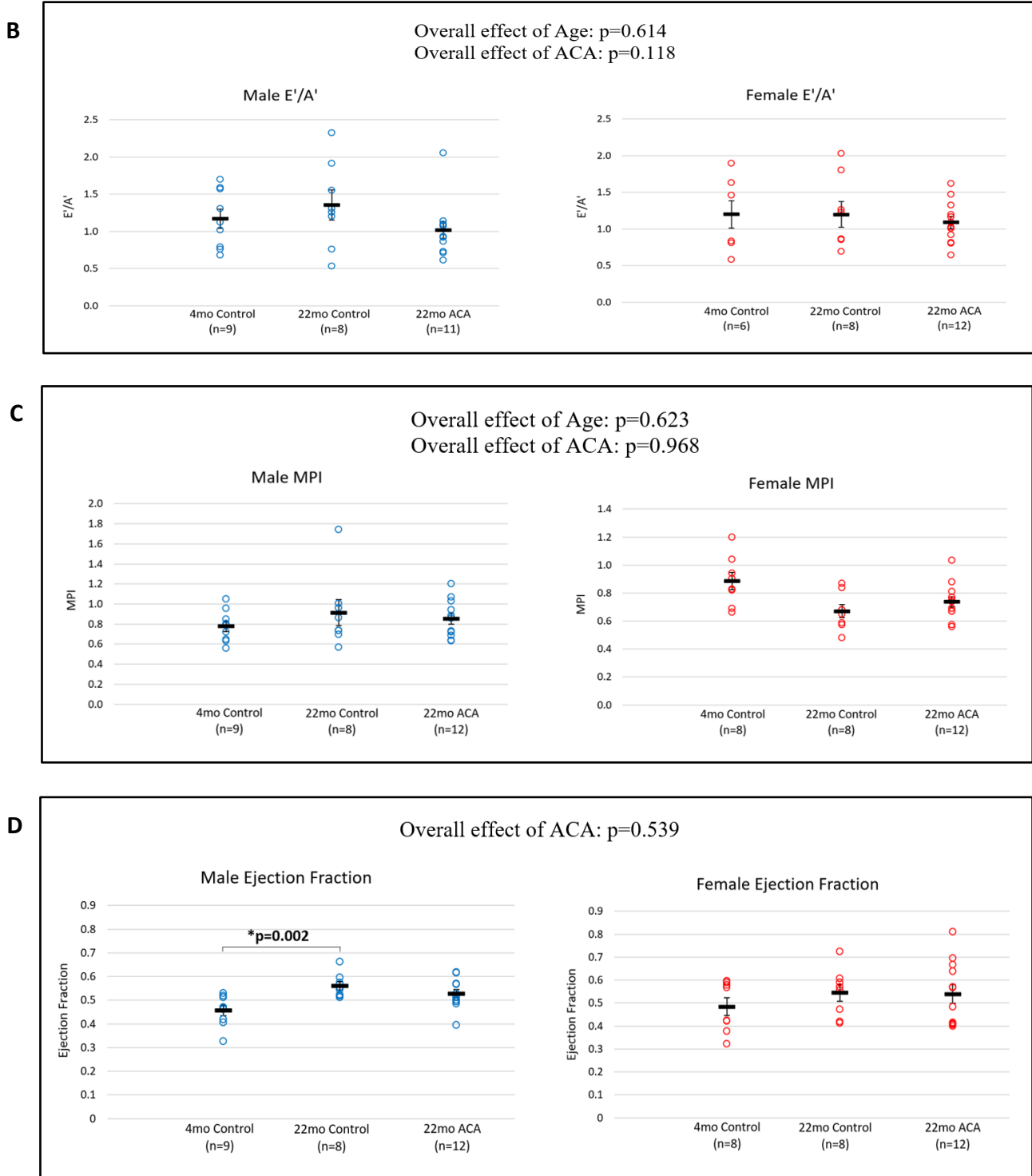
Figure 6. Dot plots for IVRT (A), E'/A' (B), MPI (C), and ejection fraction (D). Circles represent data points for individual animals, horizontal black bars indicate the mean of the group, and error bars are ± standard error of the mean. Sex specific effects are shown within the graphs. Where effects are not sex specific, overall effects for each parameter are displayed above the corresponding dot plots with significance threshold set at p<0.05.

Strain Echocardiography Reveals no Cardiac Dysfunction with Age

With no indications of cardiac dysfunction in our aged mice or any impacts of Acarbose treatment from conventional and Doppler echocardiography, we then looked to our strain data, which could potentially reveal more subtle abnormalities that would be otherwise undetected. We obtained measures of radial strain and strain rate in short axis and long axis orientations in addition to circumferential strain and strain rate in the short axis and longitudinal strain and strain rate in the long axis.

Of all the parameters analyzed, the only ones that showed any significant changes were circumferential strain and strain rate (Supplemental Figure 1). Both measures demonstrated no effect of Acarbose but were significantly increased with age in females. An increase in strain or strain rate in any orientation is indicative of improved contractile function, where a decrease suggests systolic dysfunction. We were therefore still not able to detect any cardiac dysfunction in our aged mice or any effect of Acarbose on cardiac functional parameters.

	Effect of Age	Effect of Acarbose
LV Mass	Increased (males: P<0.001; females: P=0.03)	Decreased in males (P=0.01)
Septal Wall Thickness	Increased in males (P=0.003)	No effect (P=0.663)
Posterior Wall Thickness	Increased in males (P=0.003)	No effect (P=0.597)
IVRT	No effect (P=0.307)	No effect (P=0.225)
MPI	No effect (P=0.623)	No effect (P=0.968)
E'/A'	No effect (P=0.614)	No effect (P=0.118)
Ejection Fraction	Increased in males (P=0.002)	No effect (P=0.539)
SAX Radial Strain	No effect (P=0.126)	No effect (P=0.497)
SAX Radial Strain Rate	No effect (P=0.228)	No effect (P=0.321)
SAX Circumferential Strain	Increased in females (P=0.002)	No effect (P=0.651)
SAX Circumferential Strain Rate	Increased in females (P=0.009)	No effect (P=0.551)
LAX Radial Strain	No effect (P=0.571)	No effect (P=0.433)
LAX Radial Strain Rate	No effect (P=0.759)	No effect (P=0.39)
LAX Longitudinal Strain	No effect (P=0.717)	No effect (P=0.714)
LAX Longitudinal Strain Rate	No effect (P=0.856)	No effect (P=0.471)

Figure 7. Summary of findings from all echocardiography measurements. Significant parameters are bolded with significance threshold set at p<0.05.

A Potential Role of Metabolism in Cardiac Aging

Even though we were not able to directly identify any deficits in cardiac function with age or any functional effects of Acarbose, we did detect age-related left ventricular hypertrophy that was ameliorated with Acarbose treatment in males. This suggests that there could potentially be molecular cardiac changes that precede overt dysfunction.

Mass spectrometry on left ventricular cardiac tissue was therefore performed to detect differentially expressed proteins between 4 month control and 22 month control samples and between 22 month control and 22 month Acarbose treated samples. As a preliminary analysis of the data, independent sample t-tests were performed on each identified protein to determine if age or Acarbose treatment resulted in differential expression. Differentially expressed proteins were then categorized based on molecular function into one of four categories: metabolism, structural, cell signaling, or cell homeostasis (Figure 8). The proteins that demonstrate age-related changes in expression are involved in all four functional categories, but the largest group of differentially expressed proteins are metabolic. Very few proteins that were upregulated with age were downregulated with Acarbose treatment, and no proteins were identified that were downregulated with age and upregulated with Acarbose. This preliminary analysis is being followed up by more robust statistical testing that accounts for multiple comparisons, yet this initial evaluation of the data suggests a potential role of metabolism in cardiac aging. No evidence currently exists to suggest that Acarbose might reverse any of these age-related metabolic changes.

Figure 8. Summary of the preliminary analysis of mass spectrometry data by independent sample t-tests. Significance threshold for differential expression with age was set at p<0.001 and at p<0.01 for differential expression with Acarbose treatment.

Catalytic Activity Levels of the Constitutive Proteasome Decline with Age

To further investigate the molecular effects of Age and Acarbose treatment on the heart, the PROCISE assay was performed to assess the activity levels of a catalytic subunit of the constitutive (β5) and immunoproteasomes (β5i). Catalytic activity of the constitutive proteasome subunit, β5, was found to be reduced in 22 month controls compared to 4 month controls. No effect of Acarbose was detected on β5 activity. In addition, neither age nor Acarbose treatment demonstrated a significant effect on activity of the immunoproteasome catalytic subunit, β5i. These findings indicate that a decline in proteostasis in the heart with age could be taking place in the form of reduced proteasome activity, but no evidence suggests a role of Acarbose in abrogating this process.
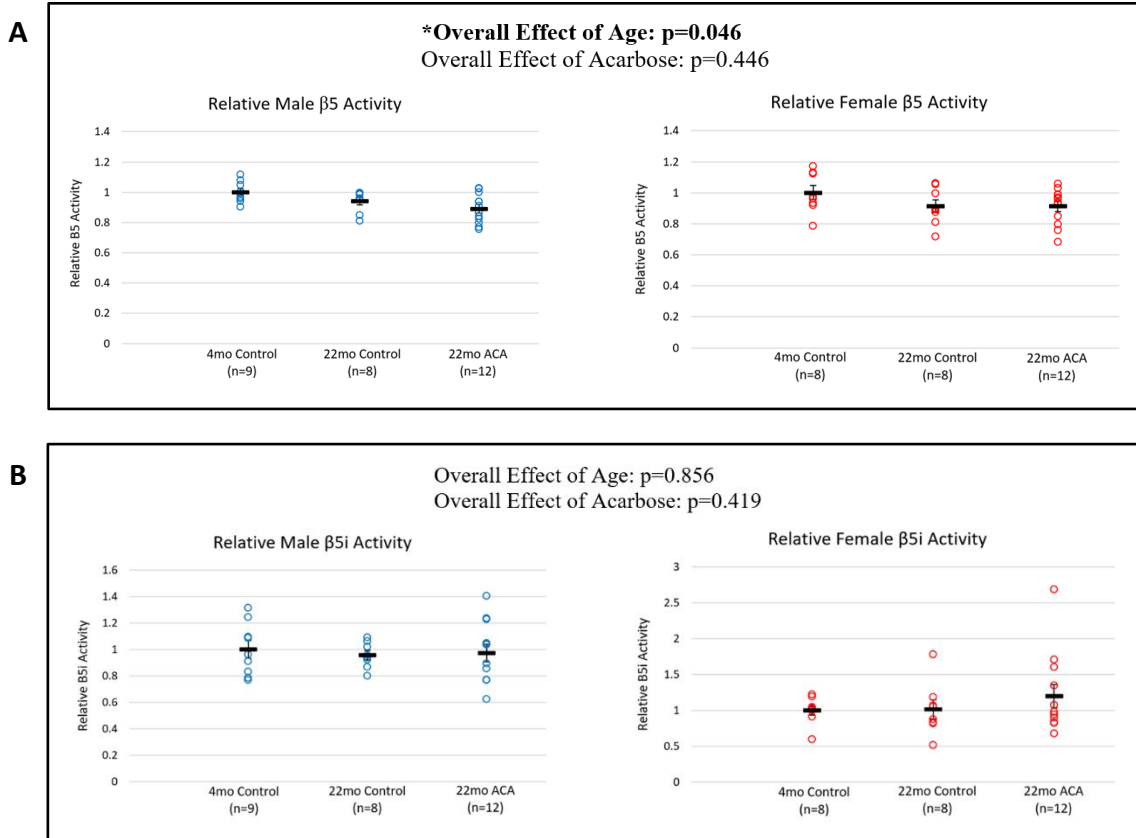
Figure 6. Dot plots for relative β5 (A) and β5i (B) activity. Circles represent data points for individual animals, horizontal black bars indicate the mean of the group, and error bars are ± standard error of the mean. Overall effects for each parameter are displayed above the corresponding dot plots with significance threshold set at p<0.05.

DISCUSSION

Conclusion

This thesis has demonstrated that Acarbose, in addition to extending lifespan, also ameliorates age-associated deficits in motor function and reduces left ventricular hypertrophy in males. These age-related effects could potentially be due to changes in cardiac metabolism with aging. However, no evidence indicates that the beneficial effects of Acarbose are a result of changes in cardiac metabolism or proteasome activity. The findings of this study further the promise of Acarbose as a potential aging intervention, but a lot of research is still needed before it could become a prescribed drug outside of diabetic patients.

Functional Healthspan Testing

The results of the motor function tests were promising in that they demonstrated abrogation in age-associated deficits with Acarbose treatment. Each of the performed tests measure a specific parameter, such as balance and coordination for the acceleration test and exercise performance for the endurance test. It is important to recognize that both rotarod protocols and grip strength testing can be heavily influenced by the motivation of the animal to perform the tasks. However, we observed a significant decrement in performance with aging, consistent with published literature. Improvement in rotarod performance with Acarbose treatment demonstrates that Acarbose enhances functional health.

All 3 of the functional tests performed in this study are affected by multiple variables, but the one that is arguably the most controlled is grip strength. Since this test demonstrated the most robust effect of Acarbose treatment, a logical follow-up experiment would be to look at the impact of Acarbose on skeletal muscle. Our lab is currently planning to investigate this through RNA sequencing of the quadriceps or gastrocnemius from Acarbose treated mice.

Along with functional health, another important quality of life measure that declines with age that this study did not address is learning and memory. The effect of Acarbose on cognition has not been described, and this is an interesting area of future research that would further characterize the role of Acarbose at ameliorating declines in healthspan with age.

Lack of Age-Related Cardiac Dysfunction

Echocardiography was used in this study partially because it had previously been demonstrated to show age-related declines in a mouse model. It was therefore a surprise when we were not able to detect cardiac diastolic dysfunction in our aged mice. One possible explanation for this is that our mice may not have been old enough to develop any cardiac dysfunction. Previous studies reporting declines in cardiac function with age in mice have used mice at 21 months of age (Qin, 2013), while others have used mice at 24 months (Dai, 2009) or even older. The decision to have 22 months serve as the "old" timepoint in this experiment was based on considerations of attrition of the cohort. It is possible that echocardiography performed at a later timepoint may have found significant declines in cardiac function that could potentially have been impacted by Acarbose treatment. However, it is notable that all our testing was performed in a blinded fashion, and this rigorous methodology could also explain the discrepancy between our results and others previously published.

Another variable that could have caused us not to detect any age-related cardiac dysfunction is the strain of mice used in this study. Studies previously reporting cardiac dysfunction in aged mice have used inbred strains, where this project utilized an outbred mouse model. If changes in cardiac function with age significantly vary across strains, then it is possible that outbred mice could be less likely to detect age-related declines in function.

Male-Specific Cardiac Effects of Acarbose

Even though the effects of Acarbose on the heart were limited to reduction of left ventricular hypertrophy, it is notable that this effect was seen only in males. The reason behind this sexual dimorphism is not completely established, but a recent study has demonstrated that male-specific effects of Acarbose are associated with increased insulin sensitivity and greater glucose tolerance. It is possible that this differentiation can be explained by gonadal hormones, as castrated males show fewer metabolic responses to drug treatment than intact males (Garratt, 2017). If Acarbose has limited beneficial effects in females, this could potentially hinder its translation value as an aging intervention, but more research is needed to address this.

Cardiac Metabolism and Aging

The preliminary finding that changes in cardiac metabolism occur with age are promising, as this was expected based on previously published literature. It is also intriguing because of the role of Acarbose in glucose metabolism, which suggests that Acarbose could potentially alter the age-associated metabolic changes seen in the heart. This effect of Acarbose was not detected through our preliminary analysis, but more robust statistical analysis is being performed that will potentially provide us with a more definitive description of the role of metabolism in cardiac aging.

RNA Seq to Further Explore the Molecular Roles of Aging and Acarbose in the Heart

Even before changes at the protein level, one of the first markers indicative of future dysfunction can be differential gene expression. RNA sequencing was performed on cardiac tissue from young control, old control, and old Acarbose treated mice. This sequencing is completed and is in the process of being analyzed. Detecting changes in gene expression with

aging or Acarbose treatment would offer further insight into the molecular mechanisms of cardiac aging.

Proteostasis in Cardiac Disease and Beyond

While no effects of Acarbose on proteasome activity were found, it does not mean that Acarbose has no impact on proteostasis. Additional experiments need to be conducted to explore the effect of Acarbose on other elements of protein homeostasis such as synthesis and folding.

The decline in constitutive proteasome activity in the heart with age also needs to be further investigated to understand its role in cardiac health. It is possible that reduced proteasome activity could lead to an accumulation of misfolded or unneeded proteins in the heart. This could therefore result in protein aggregation that could cause age-related cardiac disease, but this cannot be concluded from the current study.

Understanding the role of proteostasis with age is critical beyond the scope of the heart. Neurodegenerative diseases including Alzheimer's Disease are age-dependent and are often characterized by plaques or other forms of pathologic protein aggregation. Continued research into the connections between age and impairments in protein homeostasis is therefore essential.

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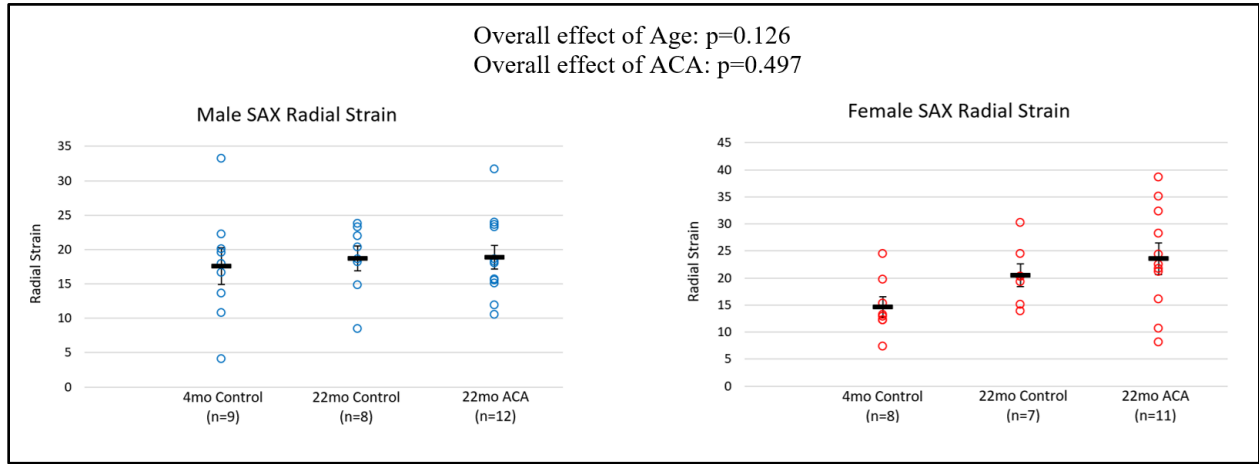
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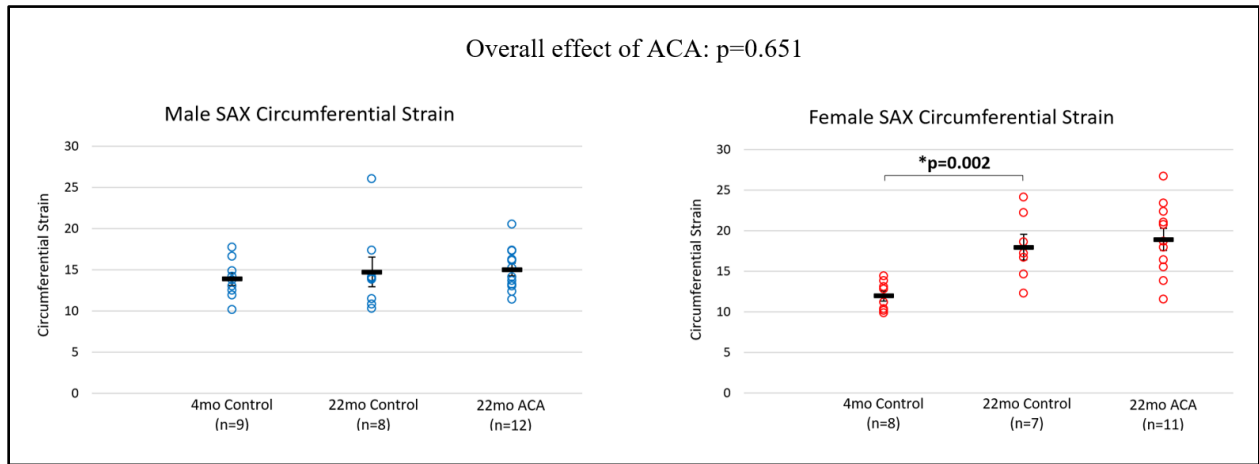
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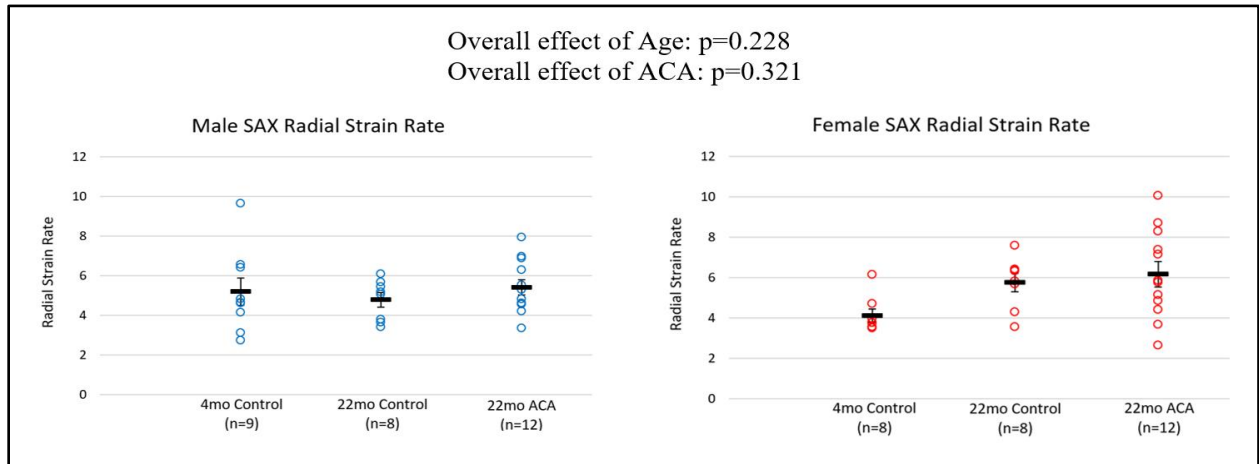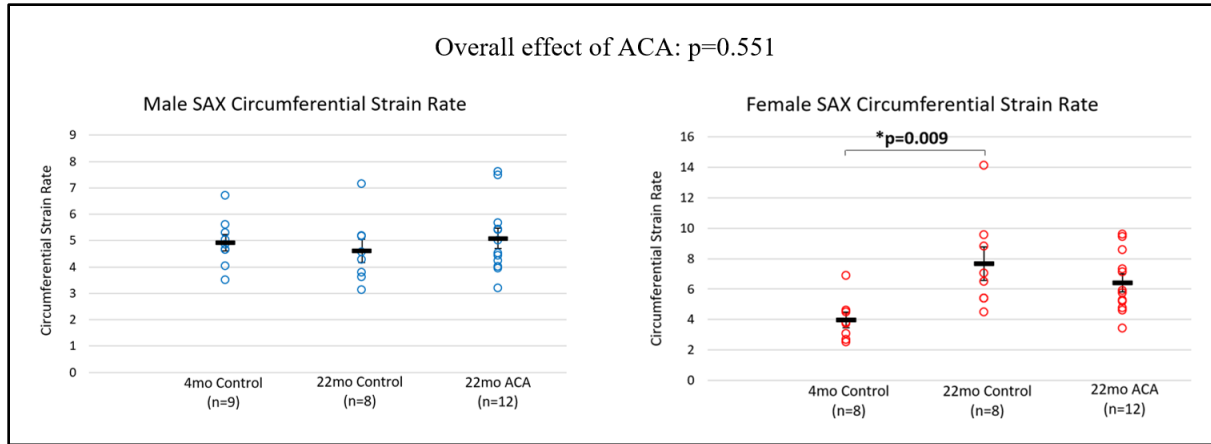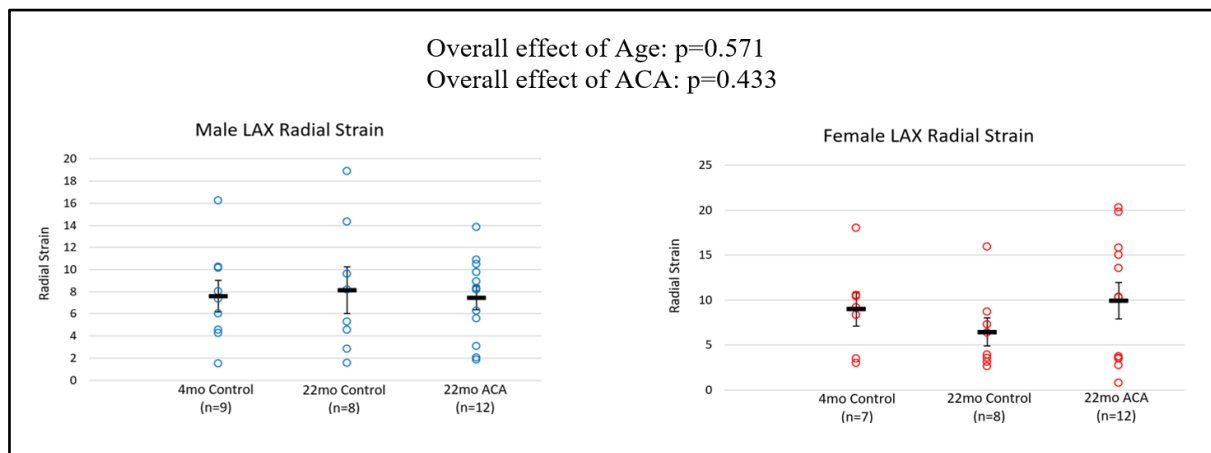
APPENDIX

A

Overall effect of Age: p=0.126
Overall effect of ACA: p=0.497

B

Overall effect of ACA: p=0.651

C

Overall effect of Age: p=0.228
Overall effect of ACA: p=0.321

D

Overall effect of ACA: p=0.551

Male SAX Circumferential Strain Rate

Female SAX Circumferential Strain Rate
*p=0.009

E

Overall effect of Age: p=0.571
Overall effect of ACA: p=0.433

Male LAX Radial Strain

Female LAX Radial Strain

F

Overall effect of Age: p=0.717
Overall effect of ACA: p=0.714

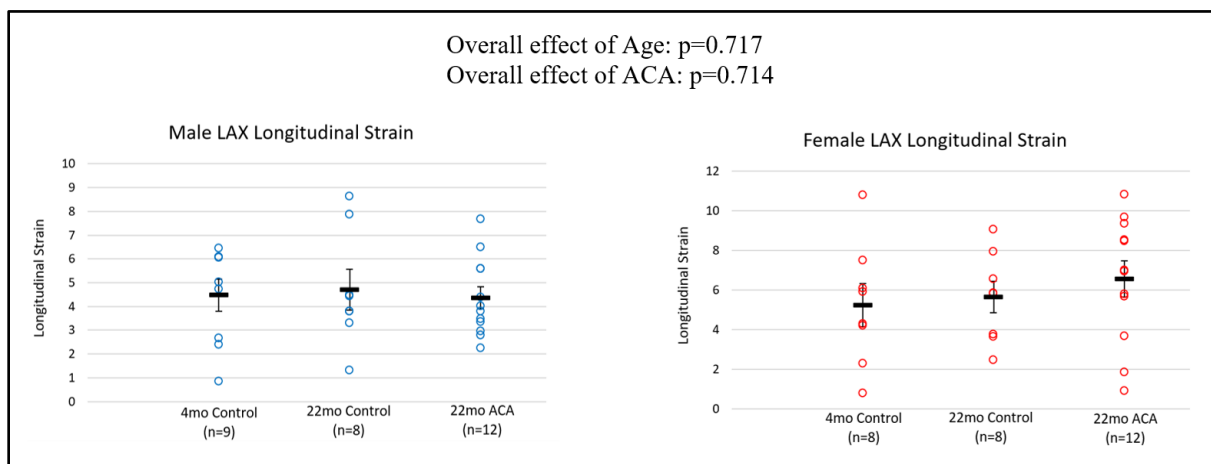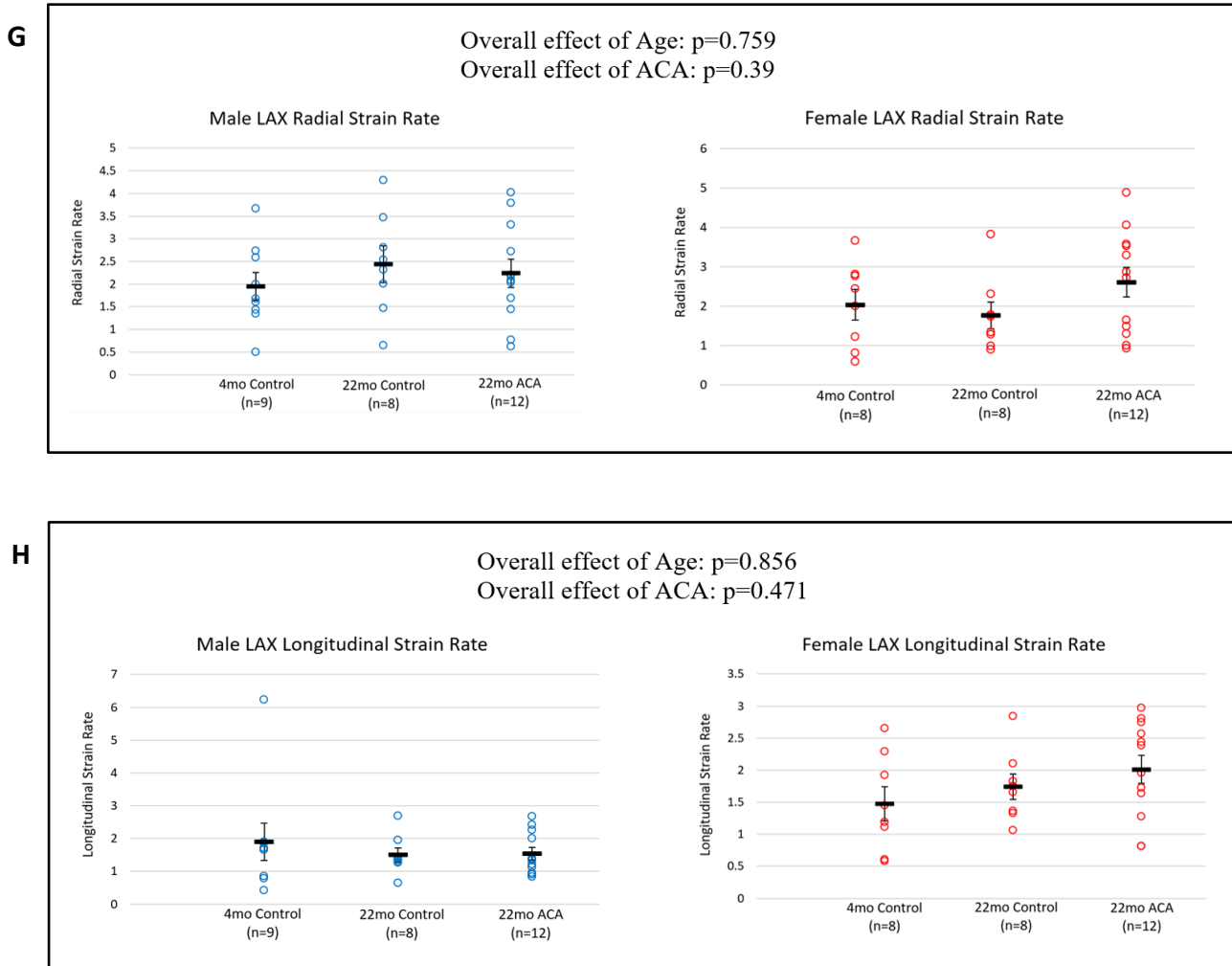Male LAX Longitudinal Strain

Female LAX Longitudinal Strain

Supplemental Figure 1. Dot plots for strain echocardiography data. Circles represent data points for individual animals, horizontal black bars indicate the mean of the group, and error bars are ± standard error of the mean. Sex specific effects are shown within the graphs. Where effects are not sex specific, overall effects for each parameter are displayed above the corresponding dot plots with significance threshold set at p<0.05.