

Influence of ACE Inhibitors on Frailty, Cardiac Contractile Function and
Calcium Homeostasis in Middle-Aged Male and Female Mice

by

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Table of Contents

List of Tables	iv
List of Figures	v
Abstract	vii
List of Abbreviations and Symbols Used	viii
Acknowledgements	x
Chapter 1: Introduction	1
1.1 Broad Overview	1
1.2 Frailty	2
1.2.1 <i>The Concept of Frailty</i>	2
1.2.2 <i>Measuring frailty in humans</i>	4
1.2.3 <i>Measuring frailty in animal models</i>	7
1.3 Cardiovascular System in Ageing	13
1.3.1 <i>Microscopic Changes Associated with Cardiac Ageing</i>	13
1.3.2 <i>Macroscopic Changes in Cardiac Morphology with Ageing</i>	17
1.3.3 <i>Impact of Age on Cardiac Function</i>	18
1.3.4 <i>Age-related change in heart rate and conduction</i>	18
1.3.5 <i>Age-related change in atrial remodelling</i>	19
1.3.6 <i>Age-related change in systolic and diastolic function</i>	20
1.4 Mechanisms of Dysfunction in the Ageing Heart.....	22
1.4.1 <i>Changes in Intracellular Homeostasis with Age</i>	22
1.4.2 <i>Chronic Activation of Renin-Angiotensin Axis in Cardiovascular Aging</i> ...	24
1.5 Potential Role of ACE Inhibitors in Attenuating Frailty.....	27
1.6 Objectives and Hypothesis	28
Chapter 2: Methods	30
2.1 Animals	30
2.2 Administration of enalapril	30
2.3 Quantification of Frailty.....	31
2.4 Measuring Blood Pressure	36
2.5 Measuring <i>in vivo</i> heart function.....	38
2.6 Ventricular Myocyte Isolation	39
2.7 Field Stimulation: Intracellular Ca ²⁺ and contraction measurements	41

2.8	Chemicals	42
2.9	Statistics	42
Chapter 3: Results		44
3.1	Weight and enalapril dose	44
3.2	Impact of enalapril administration on development of frailty.....	49
3.3	Impact of enalapril on blood pressure	52
3.4	Impact of enalapril on <i>in vivo</i> cardiac function.....	52
3.6	Impact of enalapril on ventricular myocyte contractions and Ca ²⁺ transients....	59
Chapter 4: Discussion		70
4.1	Overview of Key Findings	70
4.2	Enalapril attenuates frailty in middle-aged mice.....	71
4.3	Effects of enalapril on blood pressure.....	74
4.4	Effects of enalapril on <i>in vivo</i> cardiac function.....	75
4.5	Changes in ventricular myocyte contractile function in enalapril and control-treated animals.....	77
4.6	Limitations	80
4.7	Summary	81
4.8	Future Work	82
References		84

List of Tables

Table 2.1. Mouse frailty assessment form.....	32
Table 2.2. Clinical assessment of deficits in ageing mice to create a frailty index.....	33
Table 3.1. Baseline characteristics animals.....	45
Table 3.2. Average weights (g) of male and female enalapril-treated and control mice over 3 mos.....	47
Table 3.3. Average enalapril dose (mg/kg/day) in male and female mice over the 3 mos	48
Table 3.4. Frailty Index scores measured at baseline and monthly intervals.....	51
Table 3.5. Systolic and diastolic blood pressure measured at baseline and monthly intervals during drug treatment.....	54
Table 3.6. Morphological and Functional cardiac parameters measured via echocardiography.....	58
Table 3.7. Contractile properties measured in isolated ventricular myocytes.....	63
Table 3.8: Intracellular Ca ²⁺ transient parameters measured from isolated ventricular myocytes.....	68

List of Figures

Figure 2.1. General timeline of experimental interventions.	37
Figure 3.1. Weight was similar between male and female enalapril-treated and control mice, with a few exceptions.....	46
Figure 3.2. Enalapril treatment attenuated frailty in females, but not in males.	50
Figure 3.3. Blood pressure did not change with the administration of enalapril.	53
Figure 3.4. Representative M-mode and Doppler images of in vivo cardiac function	55
Figure 3.5. Enalapril had no effect on physical parameters of the left ventricle in males or females.....	56
Figure 3.6. Heart rate was reduced in male mice compared to females.	57
Figure 3.7. Cell length, width and surface area were not affected by either drug treatment or sex.....	60
Figure 3.8. Representative traces of ventricular myocyte contractions and from female and male control and enalapril-treated animals	61
Figure 3.9. Enalapril-treatment enhanced myocyte contractile function in females, but not males and male mice had larger, faster contractions compared to female controls.	62
Figure 3.10. Representative traces of Ca^{2+} transients in male and female enalapril-treated and control cardiomyocytes	65
Figure 3.11. Enalapril had no effect on diastolic or systolic intracellular Ca^{2+} in isolated ventricular myocytes, but males had higher intracellular calcium levels compared to females	66

Figure 3.12. Enalapril had no effect on Ca^{2+} handling in cells from male and female mice, although male animals had increased Ca^{2+} transient amplitude, velocity and time to peak..... 67

Abstract

Angiotensin Converting Enzyme (ACE) inhibitors were chronically administered to middle-aged male and female mice to determine whether treatment with ACE inhibitors attenuates frailty and whether this is accompanied by changes in cardiac function. In females, enalapril-treated mice had lower frailty scores compared to controls after 3 mos. Frailty scores were not different between male ACE inhibitor-treated and control groups after treatment. Echocardiography showed no changes in *in vivo* cardiac structure or function between enalapril-treated and control animals of both sexes. Individual ventricular myocytes showed improved contractile function in enalapril-treated females compared to controls. Interestingly, this was not associated with changes in intracellular Ca^{2+} handling. Males showed no difference in contractile function or Ca^{2+} handling between enalapril-treated and control mice. Males had improved contractile function and Ca^{2+} handling compared to females. These results suggest that enalapril-treatment may attenuate frailty in females, at least in part, by improving myocyte contractile function.

List of Abbreviations and Symbols Used

°C	Degree Celsius
ACE	Angiotensin Converting Enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance
ATPase	Adenosine triphosphate enzyme
bpm	Beats per minute
Ca ²⁺	Calcium ion
CaCl ₂	Calcium chloride
CICR	Calcium induced calcium release
CVD	Cardiovascular disease
DAG	Diacylglycerol
ECG	Electrocardiography
EGTA	Ethylene glycol tetraacetic acid
FI	Frailty Index
FS	Fractional Shortening
g	Gram
Hz	Hertz
I _{Ca,L}	L-type Ca ²⁺ current
IL-6	Interleukin-6
IL-10	Interleukin-10
I _{TO}	Transient outward K ⁺ current
IP ₃	Inositol-1,4,5-triphosphate
IVS	Interventricular septum
K ⁺	Potassium ion
KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
L	Litre
LVID	Left ventricular interior diameter

LVPW	Left ventricular posterior wall
mg	milligram
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulfate
Min	Minute
ml	Milliliter
MLCK	Myosin light chain kinase
mM	Millimolar
mos	Months
ms	Millisecond
mV	Millivolt
nm	Nanometer
Na ⁺	Sodium Ion
NaCl	Sodium chloride
NaH ₂ PO ₄	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NCX	Na ⁺ /Ca ²⁺ exchanger
NS	Not significant
O ₂	Oxygen
PLB	Phospholamban
PTI	Photon Technology International
RyR	Ryanidine receptor
SD	Standard deviation
SEM	Standard error mean
SERCA	Sarcoplasmic endoplasmic reticulum Ca ²⁺ -ATPase
SR	Sarcoplasmic reticulum
μm	Micrometer
μM	Micromolar

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Chapter 1: Introduction

1.1 Broad Overview

There is often a disparity between chronological and biological age, where people of the same age can vary in health status and level of fitness. To account for this diversity the concept of frailty was developed (Mitinski *et al.*, 2001). Frail individuals are vulnerable to adverse clinical outcomes and use more health care services compared to their fit counterparts (Rockwood *et al.*, 2012). Frailty may be a modifiable risk factor, however little is known about interventions that alter its development. Angiotensin converting enzyme (ACE) inhibitors are usually prescribed to treat high blood pressure or other cardiovascular diseases. Previous studies that investigated the effects of administration of an ACE inhibitor in older adults without cardiovascular disease showed that it improved their exercise capacity as well as their quality of life (Huchon *et al.*, 2002; Sumukadas *et al.*, 2007). In addition, studies in aged rodents showed that 2-3 months of ACE inhibitor treatment prevented the age-dependent decline in physical performance and actually improved exercise performance (Carter *et al.*, 2011; Habouzit *et al.*, 2009). This suggests that the renin-angiotensin pathway may be a target for attenuating frailty, but little research has been done.

It is well established that the cardiovascular system changes with age, and targeting the renin-angiotensin pathway may improve frailty, at least in part, via cardiovascular mechanisms. Cardiovascular changes observed with age include both microscopic changes to individual myocytes, as well as macroscopic changes in both the atria and ventricle. The changes in physical structure ultimately manifest as changes in the functional capacity of the heart. Many of these changes are detectable by middle-age and,

although changes in cardiovascular structure and function occur in both males and females, there are important differences in how cardiac ageing manifests between the sexes. The overall objective of this thesis is to determine if ACE inhibitor treatment attenuates frailty in male and female mice through improvements to cardiac function.

1.2 Frailty

1.2.1 The Concept of Frailty

It has been observed that people age at different rates, where two individuals of the same chronological age may not have the same health status. Evidence shows that older people can vary drastically in health status, from fit to frail (Mitinski *et al.*, 2001). In this way they are said to vary in their “biological age” (Rockwood *et al.*, 2000). Frailty is a term that has been used for over 20 years to describe a person’s biological age (Bergman *et al.*, 2007). Frailty was derived from French word *frêle*, which means little resistance, and was developed as a way to account for this heterogeneity in health status (Bergman *et al.*, 2007). Frail individuals have increased susceptibility to adverse health outcomes, have higher mortality and use more health care services compared to their less frail counterparts (Rockwood *et al.*, 2012). Frailty typically develops slowly within an individual (Theou & Rockwood, 2015).

For a long time, frailty was a term that remained undefined, however it was generally understood to be associated with a loss of independence to perform daily activities (Rockwood *et al.*, 1994). Some researchers have viewed frailty as a dynamic and multidimensional model, where there is a balance between assets and deficits, which determines an individual’s level of independence (Rockwood *et al.*, 1994). The deficits are generalized into three domains, physical, psychological and social (de Vries *et al.*,

2011). If an individual's assets outweigh their deficits, as is the case with most aged individuals, they are considered healthy and can maintain independence in their daily living. When deficits outweigh assets, frailty occurs and there is a loss of independence, where the individual will often require institutionalization. But when assets and deficits are closely balanced, you see frail individuals who are able to live within the community. It is important to note that frailty is not a permanent outcome, as it is possible for a person to become more or less frail over time (Rockwood *et al.*, 1994).

When a person's deficits become greater than their assets, they enter a state of reduced physiological reserve and, when faced with stressors, are more vulnerable. Stressors generally fall under the categories of acute or chronic illness (eg. myocardial infarction), or iatrogenic factors (eg. cardiac surgery) (Afilalo *et al.*, 2014), but can even be more minor events such as a medication error, influenza or a fall (Clegg & Trust, 2011). When frail patients experience such stressors, they are at a higher risk for adverse events, complications from procedures, prolonged recovery, functional decline, disability, and mortality (Shamliyan *et al.*, 2013).

Although research is ongoing, the mechanisms responsible for the development of frailty have yet to be identified, and are often debated. It has been suggested that the pathophysiological pathway of frailty is not identical to, but shares similar characteristics to aging (de Vries *et al.*, 2011). Some mechanisms being considered include dysregulation of immune, hormonal and endocrine systems. In particular, an upregulation of pro-inflammatory cytokines, reduction in testosterone levels, and/or an increase in insulin resistance have been implicated (Afilalo *et al.*, 2014). An overall decline of muscle mass and strength due to muscle breakdown exceeding muscle synthesis is also

thought to be involved (Afilalo *et al.*, 2014). In addition, it has been revealed that inflammatory biomarkers, notably interleukin-6 and C-reactive protein, become elevated with frailty (Afilalo *et al.*, 2014). Immune cells and cytokines can affect cellular senescence, influence body composition through the induction of sarcopenia, and lead to neuroendocrine dysregulation, all of which are thought to contribute to the development of frailty (Afilalo *et al.*, 2014; Visser *et al.*, 2002). Frailty ultimately arises from the dysregulation of multiple physiological systems, leading to vulnerability.

1.2.2 *Measuring frailty in humans*

Frailty is one of the greatest challenges for healthcare professionals faced with aging populations, as it is associated with adverse health outcomes, dependency, institutionalization and mortality (Rockwood *et al.*, 2012). Because some evidence suggests that there are opportunities to positively influence signs associated with frailty through intervention (Sumukadas *et al.*, 2007; Carter *et al.*, 2011), it is important to develop a measure of frailty which can accurately measure the multiple dimensions of frailty, reflecting the multiple risk factors involved. It is also important to be able to distinguish the level of frailty, which will allow changes over time to be observed (de Vries *et al.*, 2011). The development of a standardized frailty assessment tool is important for quantifying how interventions may affect frailty. There are more than 20 different tools used to quantify frailty clinically (de Vries *et al.*, 2011). There is a debate regarding the best tool or tools to use (Heuberger, 2011).

The components that should be present for a comprehensive frailty quantification system are debated. Gobbens *et al.* (2009) suggest that a quality frailty assessment tool should measure a number of changes that fall under physical, psychological and social

domains. Within each domain, there are a number of risk factors that can determine an individual's frailty. It has been suggested that parameters included in an effective tool should be associated with aging, be associated with adverse outcomes, span across multiple systems and not saturate too early in life, meaning that they are not present in all older adults (Rockwood & Mitnitski, 2011; Searle *et al.*, 2008)

One popular method of assessing frailty is Fried's scale, which treats frailty as a phenotype (Fried *et al.*, 2001). This was one of the earlier studies that looked at frailty not just within hospitalized or nursing home populations, but also in individuals living within the community (Fried *et al.*, 2001). This approach views frailty as a physiologic syndrome, and is based on a cluster of signs and symptoms that commonly occur in vulnerable older adults. It takes into consideration five categories; shrinking (unintentional weight loss), weakness (measured by grip strength), poor endurance (self-reported exhaustion), slowness (walk test) and low physical activity level (Fried *et al.*, 2001). A combination of physical tests and self-reporting via questionnaires can be used to determine if a person is frail. If an individual presents with three or more characteristics, they are considered to be "frail". If one or two characteristics are present, subjects are "pre-frail", and if none are present they are considered "fit" (robust) (Fried *et al.*, 2001). This method is easy to administer and its reproducibility and consistency have led to its use to assess clinical risk in a variety of settings (Bergman *et al.*, 2007). Although this method provides an important instrument to measure frailty, it does have its limitations. For example, Fried's method is a one-dimensional approach, in that it only measures declines in physical performance and weight loss (Hogan *et al.*, 2003). On the other hand, frailty is a multi-dimensional problem that sees changes in physiological,

psychological and social domains, as well as the physical domain (de Vries *et al.*, 2011). In addition, Fried's model of frailty as being a "wasting disease" means that obesity cannot be associated with frailty in this model. This is confounding, as obese people are likely to have low physical activity and experience exhaustion. In fact, when evaluated statistically, obesity could not be eliminated from its association with frailty, suggesting that obese people can be frail (Rockwood, 2005).

Another notable approach, often referred to as a "frailty index" (FI), quantifies frailty by considering deficit accumulation (Rockwood *et al.*, 2011; Mitnitski *et al.*, 2001). This view considers that frailty is a multidimensional risk state (Theou & Rockwood, 2015). It centralizes around the idea that, as people age, the number of deficits that they acquire increases (Rockwood *et al.*, 2011). The FI measures the quantity of health problems, rather than focusing on their nature. The overall result is that the more things an individual has wrong, the more likely they will be frail, and the greater their risk for adverse health outcomes (Theou & Rockwood, 2015).). This index includes deficits related to signs, symptoms, diseases, disabilities, and laboratory abnormalities (Singh *et al.*, 2014; Rockwood and Mitnitski, 2007 & 2011). Deficits included in a FI can be variable, as long as they are age-related, associated with adverse outcomes and cover several organ systems (Searle *et al.*, 2008). The FI score is calculated as a proportion of deficits present in an individual. The goal of the index was to create a tool that was graded, conceptually simple and capable of accommodating changes within the individual (Rockwood & Mitnitski, 2007). Some tests that create a FI based on deficit accumulation report on up to 40 items, as is the case with a standard Comprehensive Geriatric Assessment (Singh *et al.*, 2014; Rockwood & Mitnitski, 2007). Deficits are

obtained through tests, clinical evaluation, laboratory measurements, or they are self-reported (Singh *et al.*, 2014; Rockwood & Mitnitski, 2007). Each of the individual deficits are scored, with a 1 representing the presence of a deficit, a 0 if it is absent, and a 0.5 if it is present, but to a limited extent. The total number of deficits observed are then summed and divided by the total number of parameters measured. This will result in an FI score between 0 and 1, with 0 meaning there are no deficits present and 1 meaning all deficits are present to the fullest extent. For example, if a patient has 15 of 40 deficits ($15/40 = 0.375$), they would receive a score of 0.375. So, even if two individuals have the same FI score, the composition of deficits that led to that score will likely differ (Rockwood & Mitnitski, 2007). Although some may be concerned about the specific nature of the items making up the FI, when a large number of variables (>30) are included, they can be randomly selected and still accurately predict the risk of adverse outcomes (Rockwood *et al.*, 2006).

1.2.3 Measuring frailty in animal models

The older adult population is continuing to grow, and utilization of health care services is increasing. Because of this it is important to develop interventions that can prevent or reduce frailty. Performing biological studies in frail, aging humans is difficult, ethically challenging and time consuming. In order to get a better understanding of this topic, developing animal models of frailty is essential. These models will help improve our understanding of basic mechanisms of cellular dysfunction in aging and hopefully translate this into effective treatments. It is important that animal models exhibit signs and symptoms of human frailty, meaning that they should develop later in life (Theou & Rockwood, 2015). In this section, relevant animal models of frailty will be reviewed.

Because of its connection to inflammation, Walston et al. (2008) proposed that an interleukin-10 (IL-10) knockout model could be used as a mouse model of frailty. These mice have a homozygous deletion of IL-10, meaning the animal cannot produce the anti-inflammatory cytokine IL-10. Studies have shown that IL-10 knockout mice develop muscle weakness with age more rapidly than sex-matched controls (Walston *et al.*, 2008). At 50-weeks of age, IL-10 knockout mice also have higher serum levels of the pro-inflammatory cytokine IL-6 compared to controls (Walston *et al.*, 2008). This study also showed an up-regulation of several muscle genes that are related to apoptosis and mitochondrial function at older ages (Walston *et al.*, 2008). This suggests that this model can be an important tool in studying the biology of human frailty (Walston *et al.*, 2008). Although this is an important development, there are some limiting factors. The first of these is that, because the mice have a complete loss of IL-10, they may differ from the inflammation observed in natural ageing (Walston *et al.*, 2008). In addition, because of their compromised immune system, these IL-10 knock-out animals must be maintained in barrier conditions to prevent the development of Crohn's disease (Theou & Rockwood, 2015). In fact, this model was originally developed for and is widely used to study inflammatory bowel disease (Yuan *et al.*, 2013). Therefore, it is not clear if this mouse model experiences normal characteristics of ageing.

Another notable method developed to measure frailty in a mouse model is the phenotype method developed by Liu et al. (2014). This model is largely based on Fried's clinical model that is used in humans. The criteria for this method were selected based on similarity to Fried's method, however the animal model identifies four physical indicators of frailty instead of five (Liu *et al.*, 2014). They are weakness, slow walking speed, low

activity level and poor endurance. Weakness is measured using a grip test, walking speed by a rotarod test. Physical activity was determined by measuring voluntary wheel running and endurance by measuring the length of the grip and rotarod tests (Liu *et al.*, 2014). Interestingly, weight loss, one of the categories in Fried's model, was not included. For each criterion an average and standard deviation are calculated. Each criteria had a cut point of 1.5 SD and if animals fell below in three of the categories they were identified as frail (Liu *et al.*, 2014). If animals only fell below the cut-off in two of the categories they were designated as mildly frail (Liu *et al.*, 2014). This method provides a non-invasive, clinically relevant approach to quantifying frailty. However, because this method only evaluates 4 criteria it is more conservative than some others developed. This means that it could potentially underestimate the true number of frail or mildly frail animals (Liu *et al.*, 2014).

Based on FI concepts developed for use in humans, Parks *et al.* (2012) developed an approach to quantify frailty in a mouse model with a FI based on deficit accumulation. In order to do this, a large number of health-related variables linked to the function of different systems that are known to change with age in both human and animal models are measured. These investigators used C57BL/6 mice, allowing them to age naturally. Thirty-one specific variables are chosen to provide information about activity level, hemodynamic status, body composition, and basic metabolism and organ function. This model was tested in adult (12 mos) and aged (30 mos) mice and a unique FI score was assigned to each animal (Parks *et al.*, 2012). Animals are placed in an open-field and activity levels are measured using a video tracking system. This provides information about distance moved, duration of movement, meander, velocity and rearing frequency.

To measure hemodynamic properties, a tail cuff blood pressure techniques are used. This provides information about systolic and diastolic blood pressure, pulse pressure, average blood pressure, heart rate, and tail blood flow and volume. A whole body scan of anesthetized mice provides details of body composition, including weight, bone mass density, body mineral content, body surface area, lean and fat body mass, percent body fat, and total body tissue. To assess basic metabolic status, blood samples are obtained and analyzed for electrolytes, pH, glucose, hematocrit, bicarbonate, hemoglobin and urea. To determine the FI, references values are obtained from a sample of young adult male and female mice to create standard reference values. Values obtained from animals within the study are then compared to those reference values (Parks et al., 2012). A graded scale is used to determine frailty. Values 1 standard deviation above or below the reference value are given a score of 0.25. Values that differed by 2 SD received a score of 0.5. If they differed by 3 SD, they are given a score of 0.75, and any scores that exceeded that are given a 1. The values are summed and divided by the total number of parameters measured. An animal with no deficits receives a score of 0, and if all deficits are present to their full extent the animal scores 1. This study showed that aged animals of both sexes had significantly higher FI scores than younger mice (Parks *et al.*, 2012). This study demonstrated that it is possible to use a deficit accumulation approach to model frailty in animals (Parks *et al.*, 2012). However, due to the invasive nature of many of the tests and the specialized equipment required to measure many of the parameters, it can't be applied to longitudinal studies and may be hard to execute in other labs (Whitehead *et al.*, 2014).

To address these limitations, a simplified, non-invasive method of quantifying frailty based on the accumulation of clinically-apparent deficits in health was developed

by Whitehead et al (2014). This method measures a broad range of more than 30 different parameters selected based on previously published clinical signs of deterioration in mice. Changes in the integument, musculoskeletal system, the ocular and nasal systems, the respiratory system, the urogenital system, the digestive system, the vestibulocochlear/auditory system, as well as signs of discomfort, body temperature and weight are considered. If the deficit is not present, a score of 0 is denoted. If the deficit is present, it receives a score of 0.5 if it is mild, or 1 if severe. The deficits are then added and divided by the total number of parameters measured, which yields a FI score between 0 and 1. The results of the study demonstrated that frailty scores increased in age in female C57BL/6 mice (similar results were obtained in a small sample of male mice as well). To further validate this method, Whitehead et al. (2014) compared it to frailty index data in humans, finding that the FI values obtained from mice at varying age groups are similar to those in humans of comparable age. For example, in young adults, previous studies observed an average FI of 0.04, which is comparable to the value of 0.02 observed in young adult mice from this study. In older adult mice, individuals have FI scores that ranged between 0.3 and 0.4, which are comparable to the average frailty value of 0.329 measured in this study. In addition, the rate of deficit accumulation in mice is similar to the rate in people (Whitehead et al., 2014). These findings show that this simple, non-invasive method can be used to assess frailty in a mouse model. The non-invasive nature of this approach makes it suitable for use in longitudinal studies. There is also potential for this method to be used in genetically-modified mice and other disease models.

The reliability of the mouse clinical FI was tested for inter-rater reliability by Feridooni et al. (2015), where two different raters independently measured FI in a large cohort of aging C57BL/6 mice. Both raters produced overall FI scores that were comparable, showing a high inter-rater reliability. Kane et al. (2015) further validated this by showing that inter-rater reliability remains the same across individuals with varying experience with the tool. This group found that this method of measuring frailty was fast and convenient, as tools were easy to access, even when the test was being performed at a different facility than where it was originally developed (Kane *et al.*, 2015)

Now that there is an established, non-invasive method of measuring frailty in a mouse model, there is potential for it to be used in interventional experiments, to determine whether various interventions can impact frailty over time. For example, Kane et al. (2015) noted that a number of factors are known to affect the lifespan of a mouse, such as diet and pharmaceutical interventions. In research, there has been recent shift to focus more on improvement of health span and quality of life. Frailty provides a way to measure this in animal studies. Resveratrol, and antioxidant, is a compound that has previously been shown to improve health span in mice, while caloric restriction improves lifespan (Kane *et al.*, 2015). To investigate how these interventions would impact the frailty of aged adult mice (19 and 24 mos), Kane et al. (2015), placed separate cohorts of male and female mice on caloric restriction, resveratrol-enriched diet, or control *ad libitum* diet for 6 mos. In male mice, both caloric restriction and resveratrol treatment reduced FI scores compared to controls. Interestingly, FI scores in females are not affected by either intervention (Kane *et al.*, 2015). Thus, the mouse FI provides a new

measure of healthspan that can be used as an outcome in interventional longevity studies (Kane *et al.*, 2015). In addition to this, Huizer-Pajkos *et al.* (2015) looked at how polypharmacy affected frailty in the mouse model. Although this study did not find any effect of polypharmacy on frailty, it is worth noting that the treatment period was only two weeks (Huizer-Pajkos *et al.*, 2015). Overall, the FI allows the assessment of the effects of interventions in mice of the same chronological age on overall health.

Because frailty has only recently been quantified in animal models, the development of frailty and its relationship with the function of various organ systems has not been well investigated. Recently, Parks *et al.* (2012) discovered that hypertrophy and contractile dysfunction in cardiomyocytes was observed in cardiomyocytes from 30 month-old mice with the highest frailty scores when compared to mice of the same age with low FI scores. This suggests that deleterious changes in cardiomyocytes that have traditionally been associated with an increase in chronological age are more closely linked to frailty. This newly discovered link has sparked interest in the relationship between cardiovascular function and frailty development. Still, little is known about links with frailty and cardiovascular function. To better understand cardiovascular function and how it changes with age, the next section will outline basic mechanisms of cardiac function in ageing, as well as some of the major micro- and macroscopic physical and functional changes observed in the ageing heart.

1.3 Cardiovascular System in Ageing

1.3.1 Microscopic Changes Associated with Cardiac Ageing

The heart is an important component of the cardiovascular system and is composed of contractile cells known as cardiomyocytes. Ventricular myocytes vary in

size and generally range from 60-140 μm in length and 17-25 μm in width, but share a striated appearance arising from the presence of myofilaments (Loscalzo et al., 2012). Ventricular myocytes make up approximately 75% of the hearts mass (Loscalzo et al., 2012). Myocytes are an important component of the heart as they translate the electrical impulse, known as the action potential, into mechanical movement of the heart.

Intracellular ion concentrations differ from the extracellular concentrations. Na^+ , K^+ and Ca^{2+} ion concentrations inside the cell are 10 mM, 135 mM, and 0.1 mM respectively, while extracellular concentrations are 145 mM, 4 mM, and 2 mM (Ten Eick *et al.*, 1981). The resting permeability of the membrane to K^+ ultimately gives rise to a resting membrane potential of approximately -80 mV (Bers, 2001). When the membrane potential reaches a threshold of -65 mV, an action potential is generated (Berne & Levy, 1997). In humans, distinct action potential stages are clearly identifiable. Once the threshold is reached, voltage-gated Na^+ -channels are activated, allowing the influx of Na^+ , which causes a rapid upstroke (Berne & Levy, 1997). This is considered phase 0. Almost immediately, Na^+ channels inactivate and K^+ channels open to allow an efflux of K^+ , creating a transient outward current (I_{to}) that causes the slight repolarization seen in phase 1 (Birkeland *et al.*, 2005). As Na^+ channels move from an inactive to closed state, voltage-gated Ca^{2+} -channels open resulting in Ca^{2+} influx. There are two types of voltage-gated Ca^{2+} channels, L-type and T-type. L-type Ca^{2+} channels are the primary channel found on cardiomyocytes (Bers et al., 2002). The Ca^{2+} influx and simultaneous K^+ efflux cause the plateau characteristic of phase 2. Phases 3 and 4 are a result of the continuing efflux of K^+ , bringing the membrane potential to rest. To restore ion

concentrations to values observed at rest, the Na⁺-K⁺ ATPase pumps 3 Na⁺ out of the cell in exchange for 2 K⁺ that enter the cell (Apell & Karlish, 2001).

Mice have a significantly higher heart rate than humans, and they have an action potential that is much shorter in duration (Knollmann *et al.*, 2006). This means that, unlike humans, the phases of the action potential are not as clearly identifiable. Although the upstroke observed in phase 0 is clearly visible, phases 1, 2 and 3 are not as easy to differentiate. The increased speed of repolarization is mostly due to the rapid activation of outward K⁺ currents, primarily the transient outward current. Although there are differences between mouse and human action potentials, ultimately they both trigger cardiac contraction (Knollman *et al.*, 2006).

As an individual ages, there is some evidence that ventricular myocyte numbers may decline, and that this may be more prominent in men than in women (Olivetti *et al.*, 1991; Olivetti *et al.*, 1995). The loss of myocytes may be due to apoptosis, necrosis and/or autophagy (Sheydina *et al.*, 2011; Dai *et al.*, 2012; Leon & Gustafsson, 2016) although limited regenerative ability of stem cells also may contribute (Hariharan & Sussman, 2015). Age-related cell loss can, in theory, increase the mechanical burden on surviving myocytes and lead to compensatory hypertrophy. Morphometric analysis suggests that ventricular myocyte volume increases with age and that this may be more pronounced in men than women. These changes in myocyte number and size are evident as early as middle age (Olivetti *et al.*, 1991; Olivetti *et al.*, 1995). Still, the question of whether age-dependent myocyte loss and hypertrophy occurs at different rates and via different mechanisms in male and female hearts has not been firmly established and additional studies would be helpful in resolving this issue. Although the number of cardiomyocytes

may decline with age, there is marked proliferation of cardiac fibroblasts, the cells that produce extracellular matrix and collagen (Horn & Trafford, 2016). The accumulation of collagen leads to interstitial fibrosis in the atria, sinoatrial node (SAN) and ventricles of older adults (Mirza et al., 2012; Dzeshka et al., 2015). Both the gross and the cellular changes in heart structure with age are believed to adversely affect myocardial function.

Ventricular myocyte loss through apoptosis, along with an increase in the cross-sectional area of surviving cells occurs in ageing male non-human primates, although this is not seen in older females (Zhang et al., 2007). There also is growing consensus that ventricular myocyte hypertrophy (increased length, width and cross-sectional area) occurs in ageing male rodents as well as in guinea pigs and rabbits (reviewed by Feridooni et al., 2015). Whether ventricular myocyte hypertrophy occurs in myocytes from females is less clear, as some studies have reported increases cell length, width and area while others have not (Feridooni et al., 2015; Howlett, 2010; Grandy & Howlett, 2006; Dibb et al., 2004). This suggests that both concentric hypertrophy, associated with lateral growth of individual myocytes, and eccentric hypertrophy, linked to longitudinal cell growth, may occur in the ageing male heart. As with humans, additional studies that investigate whether age-dependent myocyte loss and hypertrophy occur at the same rates and via the same mechanisms in both sexes would be interesting. Other age-dependent cellular changes reported in ageing rodents and in larger animals include fibroblast proliferation, collagen accumulation and interstitial fibrosis in both the atria and the ventricles (Horn & Trafford, 2016; Lindsey et al., 2005; Horn et al., 2012).

In addition to changes in myocytes, remodelling also occurs to conduction cells of the heart. In humans, the number of SAN pacemaker cells declines markedly with age

(Mirza et al., 2012). Microscopic changes observed in ageing human hearts are also seen in older animals. Whether the number of pacemaker cells decreases with age in animals is unclear, but the expression of ion channels involved in SAN function declines with age in rats (Tellez et al., 2011).

1.3.2 *Macroscopic Changes in Cardiac Morphology with Ageing*

Normal cardiac ageing is characterized by structural changes at both macroscopic and microscopic levels. Studies in humans have shown that epicardial adipose tissue deposition increases markedly with age (Silaghi *et al.*, 2008). Calcification of specific regions, including the aortic valve leaflets, also occurs in older adults (New & Akiawa, 2011). In addition, there are changes in the gross morphological structure of the heart. Atrial remodelling, characterized by a larger atrial size and volume, occurs although not until the eighth decade unless underlying cardiovascular disease is present (Boyd *et al.*, 2011). Left ventricular wall thickness increases in healthy older adults, with age-related increases in thickness becoming obvious as early as middle-age left ventricular systolic and diastolic volumes appear to decline with age in both sexes (Straight & Lakatta, 2012). Whether left ventricular mass is affected by age is controversial, although left ventricular mass/volume ratios increase with age in both men and women (Fleg & Straight, 2012; Straight & Lakatta, 2012).

Animal models have been used to explore many aspects of cardiac ageing. Rats and mice have a 50% mortality rate at 24-months of age (Turturro *et al.*, 1999), comparable to 85-year-old humans (Grundy *et al.*, 2003), so most studies use 24-month-old rodents to model human ageing. Many of the macroscopic changes characteristic of ageing human hearts also occur in older animals. For example, epicardial fat deposition

and aortic valve calcification are seen in older animals (Swifka *et al.*, 2008; Roosens *et al.*, 2012). There is also evidence for atrial hypertrophy and dilation in older rodents, and left ventricular wall thickness increases with age in older rats and mice (Lindsey *et al.*, 2005; Dai *et al.*, 2012; Lau *et al.*, 2013).

1.3.3 Impact of Age on Cardiac Function

Many structural changes associated with normal ageing are maladaptive. For example, age-related increases in epicardial adipose tissue are associated with a higher prevalence of various cardiovascular diseases (Sacks & Fain, 2011), although whether pericardial adipose tissue increases with age is unclear. This could be important, as pericardial fat is a known risk factor for diseases such as atrial fibrillation, which are common in older adults (Al-Rawahi *et al.*, 2015). Calcification of the aortic valve leaflets impairs their movement, which can obstruct left ventricular outflow and promote the development of heart failure (New & Aikawa, 2011), especially in older men (Milin *et al.*, 2014). The concept that age adversely affects heart structure and predisposes elderly people to develop heart diseases has encouraged interest in the effects of age on electrical and contractile function of the heart.

1.3.4 Age-related change in heart rate and conduction

Heart rate (HR) is not affected by age in supine men and women (Lakatta & Levy, 2003). There is also no difference in resting HRs between young adult and aged rodents of both sexes (Fannin *et al.*, 2014; Medrano *et al.*, 2016; Koch *et al.*, 2013). Older adults also have an impaired ability to increase HR in response to exercise, resulting in a 30% decrease in cardiac output during exercise between the ages of 20 and 85 (Lakatta &

Levy, 2003). This impaired ability to increase HR arises, in part, because of the age-associated decline in responsiveness to sympathetic stimulation (Ferrara et al., 2014). A decline in pacemaker cell number and reduced ion channel expression in SAN cells may diminish automaticity and contribute to lower HRs in older adults (Mirza et al., 2012; Tellez et al., 2011). Age-related SAN dysfunction may help explain the development of bradyarrhythmias and symptoms that require pacemaker implantation in older adults (Mirza et al., 2012; Tellez et al., 2011). The cardiac conduction system changes characteristically with age. Age-related prolongation of the QRS, consistent with a slowing of conduction, is seen in humans and animals (Mirza et al., 2012; Bonda et al., 2015). Animal studies demonstrate that reduced cell-to-cell connections, mediated by lower expression of connexin-43, slow conduction in the ageing heart (Bonda et al., 2015). These age-dependent changes in conduction may promote dysrhythmias in older adults.

1.3.5 Age-related change in atrial remodelling

In young adults, early left ventricular filling occurs rapidly, so that very little filling is due to atrial contraction later in diastole. By contrast, slow early left ventricular filling is a characteristic feature of the aging heart. Slow left ventricular filling increases diastolic filling pressure, which results in atrial dilation and hypertrophy (Fleg & Strait, 2012). This enhances the force of atrial contraction and promotes late diastolic filling to compensate for reduced filling early in diastole (Strait & Lakatta, 2012). In consequence, the atria make a larger contribution to ventricular filling in older adults than in younger adults. Thus, attenuation of atrial contraction in diseases such as atrial fibrillation can markedly reduce diastolic volumes in older individuals (Fleg & Strait,

2012). This reduces cardiac output, which predisposes older people towards the development of heart failure.

Other studies have investigated age-dependent changes in atrial electrophysiology. Most studies have used atrial myocytes from patients with underlying cardiovascular disease and few have examined individuals older than 70-years of age (Dun & Boyden, 2009). Thus, whether age influences the electrophysiological properties of atrial myocytes from healthy, older humans is not clear. Studies in older animals have shown that right atrial myocytes are depolarized, with longer action potentials than younger animals (Dun & Boyden, 2009). These changes in action potential configuration are associated with an increase in K^+ currents, although Ca^{2+} currents in these cells decline with age (Dun & Boyden, 2009). Together, age-related changes in ion channels along with atrial fibrosis and hypertrophy provide an ideal substrate for the development of atrial fibrillation, which is common in older adults (Brandenburg et al., 2016). There is evidence for a higher incidence of atrial fibrillation in men, although women often have worse outcomes including stroke and systemic embolization (Pancholy et al., 2014). Interestingly, susceptibility to atrial fibrillation also increases with age in mice, at least in males (Guo et al., 2014). Whether age-dependent atrial remodelling differs between the sexes is not yet clear and additional studies are warranted.

1.3.6 Age-related change in systolic and diastolic function

Traditionally, measures of systolic function, including stroke volume and ejection fraction, were said to be similar in young and older adults at rest (Lakatta & Levy, 2003). More recent evidence indicates that cardiac contractility is well preserved in women, but in men actually declines after age fifty (Claessens *et al.*, 2007). Interestingly,

echocardiography studies in animals support this view. Systolic function (stroke volume, ejection fraction) declines with age in male rodents (Medrano *et al.*, 2016; Koch *et al.*, 2013; Shinmura *et al.*, 2011) but not in females (Fannin *et al.*, 2014; Dai *et al.*, 2014).

Diastolic dysfunction, characterized by problems with relaxation, is a hallmark of cardiac ageing. In young adult hearts, left ventricular filling occurs early and rapidly as consequence of ventricular relaxation (Fleg & Straight, 2012; Lakatta & Levy, 2003). By contrast, hearts from older individuals fill with blood more slowly (Fleg & Straight, 2012; Lakatta & Levy, 2003). Although the rate of left ventricular filling in the early diastolic phase declines with age by the sixth decade in both sexes (Boyd *et al.*, 2011), this decline in diastolic function begins as early as age 30 (Brenner *et al.*, 2001). This age-dependent slowing of relaxation in diastole may predispose the aging heart towards heart failure with preserved ejection fraction, commonly referred to as heart failure with preserved ejection fraction (HFpEF; Loffredo *et al.*, 2014; Kaila *et al.*, 2012). HFpEF is characterized by increased wall thickness and diastolic dysfunction with little or no reduction in ejection fraction (Dunlay & Roger, 2012; Kaila *et al.*, 2012).

The increasing prevalence and lack of treatment options for HFpEF has promoted interest in the determinants of age-related diastolic dysfunction. Ageing rodents of both sexes exhibit slowed relaxation and diastolic dysfunction (Fannin *et al.*, 2014; Medrano *et al.*, 2016; Shinmura *et al.*, 2011; Dai *et al.*, 2014 but *c.f.* Koch *et al.*, 2013). This suggests that underlying mechanisms of potential relevance to humans can be investigated in animals. Several mechanisms have been implicated. For example, increased fibrosis is thought to increase ventricular stiffness (Horn & Trafford, 2016). This reduces ventricular compliance and impairs passive filling of the left ventricle (Loffredo *et al.*,

2014). The thicker, less distensible left ventricular walls seen in older humans and animal hearts (Straight & Lakatta, 2012; Dai *et al.*, 2012; Lindsey *et al.*, 2005) also may contribute to the development of HFpEF. In addition, increased myocyte stiffness, mediated by age-dependent changes in the sarcomeric protein titin, contribute to the increase in left ventricular stiffness in HFpEF (Hamdani *et al.*, 2013). Changes in intracellular calcium homeostasis have also been implicated in the pathogenesis of HFpEF.

1.4 Mechanisms of Dysfunction in the Ageing Heart

Mechanisms of dysfunction underlie the structural and functional changes observed with cardiac ageing. Pre-clinical studies have suggested a number of potential mechanisms. Two key mechanisms being investigated are alterations in Ca^{2+} homeostasis and chronic activation of the renin-angiotensin pathway.

1.4.1 Changes in Intracellular Homeostasis with Age

Excitation-contraction coupling is the term used to describe the translation of an action potential into the mechanical contraction of the heart. When an action potential is propagated along the sarcolemma, it travels down invaginations of the membrane, known as T-tubules, and activates voltage-gated L-type Ca^{2+} -channels. This results in an influx of Ca^{2+} into the cytosol (Bers, 2001). These L-type channels are located so that they are closely associated with ryanodine (RyR) receptors present on the sarcoplasmic reticulum (SR). The Ca^{2+} interacts with RyR (Scriven *et al.*, 2000) and signals the release of Ca^{2+} stored within the SR. This process is referred to as Ca^{2+} -induced Ca^{2+} release (CICR) (Fabiato, 1985). The overall rise in intracellular free Ca^{2+} concentration results in it

binding to troponin C, a component of myofilaments, allowing for actin to associate with myosin, therefore allowing for the contraction to occur (Bers, 2002).

In order for relaxation to occur Ca^{2+} has to be removed from the cytosol. The primary method of removal is by a transporter on the SR known as sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). SERCA activity is regulated by the phosphoprotein phospholamban (PLB; Rodriguez & Kranias, 2005). In its unphosphorylated form, PLB is able to interact with SERCA, inhibiting its activity. Phosphorylating PLB prevents the inhibition of SERCA activity, increasing its ability to remove cytosolic Ca^{2+} (Bers, 2002). Ca^{2+} is also removed from the cell by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) present on the cell membrane. Although they do not contribute as significantly, a Ca^{2+} uniporter present on mitochondria and a Ca^{2+} -ATPase on the cell membrane also work to remove cytosolic Ca^{2+} (Bers, 2002). Excitation-contraction coupling is a tightly regulated process, however components of the pathway can be affected by age. In addition, there is evidence that EC-coupling is modulated by Ang II, as Ang II signalling in cardiomyocytes can activate NCX to reverse its activity, causing it to bring Ca^{2+} in to the cell (Egger *et al.*, 2010).

As described above, cardiac contraction and relaxation reflect the rise and fall of intracellular Ca^{2+} levels in individual cardiac myocytes. Age-dependent changes in the regulation of intracellular Ca^{2+} affect the ability of the heart to contract (Feridooni *et al.*, 2015). For example, smaller Ca^{2+} transients are responsible for the smaller contractions observed in ventricular myocytes from aged male rodents compared to younger animals (Howlett, 2010; Grandy & Howlett, 2006). Smaller Ca^{2+} transients are thought to arise, in part, from a reduction in peak Ca^{2+} currents leading to less Ca^{2+} influx in myocytes from aged male rodents (Feridooni *et al.*, 2015). By contrast, age has little effect on peak Ca^{2+}

transients, Ca^{2+} currents or contractions in cells from female rodents (Feridooni et al., 2015) and actually enhances these responses in cells from aged female sheep (Dibb et al., 2004). The age-dependent decrease in peak Ca^{2+} transients/contractions in myocytes from males but not females may help explain why systolic dysfunction and HF_rEF are seen more in older men than in older women (Dunlay & Roger, 2012).

Altered Ca^{2+} homeostasis also helps explain slowed relaxation and diastolic dysfunction in the ageing heart. Prolongation of relaxation in the ageing heart reflects a slower rate of decay of the Ca^{2+} transient (Feridooni *et al.*, 2015). This is clear in older male animals but less so in females, although few studies have used female animals (Feridooni *et al.*, 2015). Slower Ca^{2+} transient decay arises from reduced Ca^{2+} uptake into stores in the SR, due reduced expression and activity of SERCA (Feridooni et al., 2015). Prolonged availability of internal Ca^{2+} causes persistent activation of contractile filaments, which delays active ventricular relaxation and compromises left ventricular filling in early diastole (Dai *et al.*, 2012; Loffredo, *et al.*, 2014). There are additional, age-related changes in contractile proteins in both sexes (Feridooni *et al.*, 2015). There is a shift from the fast α -myosin heavy chain isoform to the slower β -isoform with age in hearts from both sexes, which can slow relaxation (Feridooni *et al.*, 2015). These changes help explain the increase in HF_pEF in older adults (Loffredo, *et al.*, 2014; Kaila *et al.*, 2012). Still, why HF_pEF occurs more commonly in women whereas outcomes are worse in men obliges further investigations.

1.4.2 Chronic Activation of Renin-Angiotensin Axis in Cardiovascular Aging

The renin-angiotensin system plays an important role in the regulation of blood volume and vascular resistance (Domenighetti *et al.*, 2005). Renin, a proteolytic enzyme,

is released into circulation after being secreted from juxtaglomerular cells of the kidney and acts on angiotensinogen, cleaving it to angiotensin I. Angiotensin converting enzymes, present on vascular endothelium, further cleave the peptide, resulting in the formation of angiotensin II (Ang II). Ang II levels increase when there are hypotensive conditions or reduced sodium concentrations in the blood. At the level of the kidney, Ang II signals an increase in sodium retention, increasing water reabsorption and blood volume. It also acts directly on the vasculature, causing vessel constriction, the net effect being an increase in blood pressure. Ang II exerts the majority of its effects by binding to G_q-protein coupled receptors known as angiotensin II type 1 receptors (AT₁R; Li & Zhuo, 2016; McDonough, 2010; Ma *et al.*, 2010). As would be expected, these receptors are present within the kidneys and vasculature, however they are also located throughout a number of tissues throughout the body, including heart tissue and skeletal muscle (Allen *et al.*, 1999). Intracellularly, AT₁R signalling leads to the activation of phospholipase C (PLC), which produces inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG; Mehta & Griendling, 2007). IP₃ binds to the SR, causing a Ca²⁺ channel to open, leading to an efflux of Ca²⁺ into the cytoplasm. Ca²⁺ binds to calmodulin and activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain, which enhances the actin-myosin interaction, resulting in vasoconstriction (Mehta & Griendling, 2007). In addition, DAG can also activate phosphokinase C (PKC), resulting in the phosphorylation of various targets (Mehta & Griendling, 2007).

In fibroblasts, Ang II causes an increase in collagen deposition, while in myocytes it causes an increase in growth. To investigate the effects of Ang II on the heart, Rosenkranz (2004) chronically infused Ang II in younger animals. It resulted in cardiac

hypertrophy, fibrosis and slowed relaxation, a phenotype that resembles cardiac ageing. Studies have shown that activation of AT₁R on fibroblasts stimulates fibroblast proliferation, increases collagen synthesis and augments the expression of extracellular matrix proteins (Rosenkranz 2004). Ang II is also thought to promote growth in adult cardiac myocytes by inducing the expression of growth factors such as transforming growth factor β 1 (Rosenkranz 2004). Chronic exposure to Ang II also damages the heart by increasing cellular and mitochondrial reactive oxygen species (ROS; Dai *et al.*, 2012). These similarities between the Ang II-treated heart and the ageing heart suggest that Ang II may play a role in cardiac ageing. Indeed, evidence from rodent models has shown that cardiac Ang II levels increase with age and this has been linked to hypertrophy, fibrosis and diastolic dysfunction in the ageing heart (Dai *et al.*, 2009). Interestingly, the effects of angiotensin on the ageing heart appear to differ between the sexes. For example, female mice with elevated intra-cardiac levels of Ang II exhibit age dependent contractile depression while male mice do not (Mellor *et al.*, 2014).

The process of translating an electrical impulse into a physical cardiac contraction is a tightly regulated process, however age can influence mechanisms involved in Ca²⁺ handling. Changes in intracellular Ca²⁺ homeostasis can impair the hearts ability to contract, which manifests in functional impairment (Feridooni *et al.*, 2015). Chronic Ang II exposure, which is commonly observed with increasing age, can also contribute to functional changes by signalling an increase in fibrotic deposition and inducing myocyte hypertrophy (Dai *et al.*, 2009). By targeting Ang II production, there may be the potential to reduce some of the structural and functional cardiac changes associated with ageing.

This could improve cardiovascular function in ageing and potentially improve overall health. Whether this can affect the development of frailty however, is unclear.

1.5 Potential Role of ACE Inhibitors in Attenuating Frailty

It is clear that the renin-angiotensin system plays a critical role in the regulation of blood volume and the cardiovascular system. In addition, its role in the pathogenesis of a number of diseases, including hypertension, congestive heart failure and acute myocardial infarction has been well documented (Ma et al., 2010). Because of this, components of renin-angiotensin system are targets for pharmacological intervention to treat hypertension and systolic heart failure. In fact, ACE inhibitors are a class of drug that target the angiotensin converting enzyme, preventing its role in the formation of Ang II and they commonly used to improve function in cardiovascular-related diseases such as hypertension and congestive heart failure (Garg & Yusuf, 1995; Flather et al., 2000). There may, however, be potential for this drug class to target the progression of frailty. As mentioned previously, frailty may be modified, slowed or even reversed, and there is evidence that this could be achieved by targeting Ang II production. For example, a study using an AT₁R receptor knockout mouse model showed that animals missing the active Ang II receptor had prolonged lifespans, as well as smaller myocyte size and less cardiac fibrosis, compared to the control group (Benigni *et al.*, 2009). In addition, a number of studies using ACE inhibitors, in both human and animal models, showed that decreasing the production of Ang II resulted in improved skeletal muscle function (Hutcheon *et al.*, 2002; Sumukadas *et al.*, 2007). One of the studies involved the observation of elderly women (average 78.9 years of age) with hypertension. Patients who were continuously taking ACE inhibitors exhibited a slower decline of physical function and muscle

strength, even when compared to individuals on other antihypertensive drugs (Onder *et al.*, 2002). A study by Hutcheon *et al.* (2002) looked at how administration of the ACE inhibitor, perindopril, to older adults (average age 81) with left ventricular systolic dysfunction affected physical performance by measuring an individual's walking distance within a 6 minute period. Patients receiving treatment had a significantly longer walking distance compared to patients receiving placebo (Hutcheon *et al.*, 2002). To determine if this improvement would still be present in patients with no signs of heart failure, Sumukadas *et al.* (2007) investigated the effect of the same ACE inhibitor on physical function in older patients (average age 78.7) with no signs of left ventricular systolic dysfunction. After 20 weeks of perindopril, the treatment group saw a significant increase in walking distance compared to patients receiving placebo. When similar experiments were performed in rodent models, results reflected what was observed in people (Carter *et al.*, 2011; Habouzit *et al.*, 2009). When 24 mos old male rats were treated with enalapril (40 mg/kg/day), there was an attenuation of physical performance, measured using grip strength, after 3 mos of treatment (Carter *et al.*, 2011). Similarly, female rats treated with perindopril (2 mg/kg/day) had an increase in running exercise capacity compared to controls (Habouzit *et al.*, 2009). Overall, reducing AT₁R signalling, via knockout or ACE inhibitors, increases lifespan, improves physical activity and reduces signs of weakness. These effects may be beneficial in attenuating frailty, although this has not been investigated.

1.6 Objectives and Hypothesis

The specific objectives of this work were to:

- (1) Determine if ACE inhibitor treatment would attenuate frailty in middle-aged male and female mice.
- (2) To identify male-female differences in the development of frailty and cardiovascular parameters between ACE inhibitor-treated and control-treated animals.
- (3) To investigate if the attenuation of frailty occurred, at least in part, through changes in the cardiovascular system.

The hypothesis to be tested is “Administration of ACE inhibitors will attenuate frailty in middle-aged male and female mice”

Chapter 2: Methods

2.1 Animals

All experimental protocols that involved animals were done in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, Ottawa, ON: Vol 1, 2nd edition, 1993; Vol 2, 1984) and approved by the Dalhousie Committee on Laboratory Animals. Male and female C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, QC) as retired breeders and aged to approximately 9 months at which point they began the 3 month drug study. Females were housed with five animals per cage, while males were housed individually. All animals were kept in micro-isolator cages within the Carlton Care Animal Facility at Dalhousie University and maintained on a 12-hour light/dark cycle. Animals had free access to food and water.

2.2 Administration of enalapril

The ACE inhibitor study was initiated when the mice were 9 months of age. The ACE inhibitor enalapril (Cayman Chemical, Ann Arbor, MI) was incorporated into animal bacon-flavoured food tablets (Bio-Serv, Frenchtown, NJ) at a dose of 280 mg/kg. This dose was selected based on previous studies (Venegas-Pont et al., 2010; Carter et al., 2011). The control group received identical food tablets, but with the drug omitted. The food contained ground corn, dehulled soybean meal, dried beet pulp, ground oats, brewers animal fat (preserved with butylated hydroxyanisole), porcine meat meal, wheat middlings, mineral mix, vitamin mix, bacon flavour, DL-methionine, and choline chloride. At nine months of age, animals were transferred from the normal chow to either the enalapril-containing chow or control chow and were allowed to free feed for approximately three months. Food consumption was tracked by weighing the food left in

the cage and adding a known amount each week. Female mice were group housed, so average food consumption for the cage was divided by the number of animals in the cage to provide an estimate of individual consumption. Mouse drug dose was calculated by multiplying the dose of drug in the food by the animal's individual food consumption and then divided by their weight.

2.3 Quantification of Frailty

Before animals were placed on control or enalapril-containing chow, baseline frailty measurements were obtained. Once animals had started the study, frailty was examined at monthly intervals, with the final measurement occurring before animals were euthanized for isolated cardiomyocyte experiments. Frailty was assessed using a method previously developed in the Howlett laboratory (Parks et al., 2012; Whitehead et al., 2014; Feridooni et al., 2015). With this approach, frailty is measured by quantifying the accumulation of deficits. The frailty index measured a total 31 potential deficits throughout the integumentary musculoskeletal, vestibulocochlear/auditory, ocular/nasal, digestive/urogenital, and respiratory systems, as well as looking for signs of general discomfort. All deficits can be measured non-invasively through clinical examination of each animal.

Frailty assessments were conducted in a quiet room within the animal care facility. Animals were taken to the room and allowed to acclimate for 15 minutes before the assessment began. If there was no deficit present, the animal received a score of 0. If there was a mild deficit present, a score of 0.5 was given. If the deficits were severe, a score of 1 was given. Details of how the parameters were measured and how the scoring was determined are outlined in table 2.1 and 2.2. Mean body weight and body surface

Table 2.1. Mouse frailty assessment form.

Mouse #: _____ Date of Birth: _____ Sex: F M
 Body weight (g) _____ Surface body temperature (°C) _____

Mouse Frailty Assessment Form

Rating: 0 = absent 0.5 = mild 1 = severe

➤	Integument:				NOTES:
	❖ Alopecia (hair loss)	0	0.5	1	_____
	❖ Loss of fur colour	0	0.5	1	_____
	❖ Dermatitis	0	0.5	1	_____
	❖ Loss of whiskers	0	0.5	1	_____
	❖ Coat condition	0	0.5	1	_____
➤	Musculoskeletal system:				
	❖ Tumours	0	0.5	1	_____
	❖ Distended abdomen	0	0.5	1	_____
	❖ Kyphosis/hunched posture	0	0.5	1	_____
	❖ Tail stiffening	0	0.5	1	_____
	❖ Gait	0	0.5	1	_____
	❖ Tremor	0	0.5	1	_____
	❖ Forelimb grip strength	0	0.5	1	_____
	❖ Body condition score	0	0.5	1	_____
➤	Vestibulocochlear/Auditory:				
	❖ Head tilt	0	0.5	1	_____
	❖ Hearing loss	0	0.5	1	_____
➤	Ocular/Nasal:				
	❖ Cataracts	0	0.5	1	_____
	❖ Discharge/swollen/ squinting	0	0.5	1	_____
	❖ Microphthalmia	0	0.5	1	_____
	❖ Corneal opacity	0	0.5	1	_____
	❖ Vision loss	0	0.5	1	_____
	❖ Menace reflex	0	0.5	1	_____
	❖ Nasal discharge	0	0.5	1	_____
➤	Digestive/Urogenital system:				
	❖ Malocclusions	0	0.5	1	_____
	❖ Rectal prolapse	0	0.5	1	_____
	❖ Penile/Uterine prolapse	0	0.5	1	_____
	❖ Diarrhoea	0	0.5	1	_____
➤	Respiratory:				
	❖ Breathing rate/depth	0	0.5	1	_____
➤	Discomfort:				
	❖ Mouse Grimace Scale	0	0.5	1	_____
	❖ Piloerection	0	0.5	1	_____

Total Score/ Max Score:

From Whitehead et al. (2014).

Table 2.2. Clinical assessment of deficits in ageing mice to create a frailty index.

System/Parameter	Clinical assessment of deficit	Scoring
Integument		
Alopecia	Gently restrain the animal and inspect it for signs of fur loss	0 = normal fur density 0.5 = < 25% fur loss 1 = > 25% fur loss
Loss of fur color	Note any change in fur color from black to grey or brown	0 = normal color 0.5 = focal grey/brown changes 1 = grey/brown throughout body
Dermatitis	Document skin lesions	0 = absent 0.5 = focal lesions (e.g. neck, flanks, under chine) 1 = widespread/multifocal lesions
Loss of whiskers	Inspect the animal for signs of a reduction in the number of whiskers	0 = no loss 0.5 = reduced number of whiskers 1 = absence of whiskers
Coat condition	Inspect the animal for signs of poor grooming	0 = smooth, sleek, shiny coat 0.5 = coat is slightly ruffled 1 = unkempt and un-groomed, matted appearance
Physical/ Musculoskeletal		
Tumors	Observe the mice to look for symmetry. Hold the base of the tail and manually examine mice for visible or palpable tumors	0 = absent 0.5 = < 1.0 cm 1 = > 1.0 cm or multiple smaller tumors
Distended abdomen	Hold the mouse vertically by the base of the tail and tip backwards over your hand. Excess fluid visible as a bulge below the rib cage	0 = absent 0.5 = slight bulge 1 = abdomen clearly distended
Kyphosis	Inspect the mouse for curvature of the spine or hunched posture. Run your fingers down both sides of the spine to detect abnormalities	0 = absent 0.5 = mild curvature 1 = clear evidence of hunched posture
Tail stiffening	Grasp the base of the tail with one hand, and stroke the tail with a finger of the other hand. The tail should wrap freely around the finger when mouse is relaxed	0 = no stiffening 0.5 = tail responsive but does not curl 1 = tail completely unresponsive
Gait disorders	Observe the freely moving animal to detect abnormalities such as hopping, wobbling, circling, wide stance and weakness	0 = no abnormality 0.5 = abnormal gait but animal can still walk 1 = marked abnormality, impairs ability to move
Tremor	Observe the freely moving animal to detect tremor, both at rest and when the animal is trying to climb up an incline	0 = no tremor 0.5 = slight tremor 1 = marked tremor; animal cannot climb
Forelimb grip strength	Hold the mouse. Allow it grip the bars on the cage lid. Lift animal by the base of the tail and assess grip strength	0 = sustained grip 0.5 = reduction in grip strength 1 = no strength, no resistance

Body condition score	Place mouse on flat surface, hold tail base and manually assess the flesh/fat that covers the sacroiliac region (back and pubic bones)	0 = bones palpable, not prominent 0.5 = bones prominent or barely felt 1 = bones very prominent or not felt due to obesity
Vestibulocochlear/ Auditory		
Vestibular disturbance	Hold the base of the tail and lower mouse towards a flat surface. Inspect for head tilt, spinning, circling, head tuck or trunk curling	0 = absent 0.5 = mild head tilt and/or slight spin when lowered 1 = severe disequilibrium
Hearing loss	Test startle reflex. Hold a clicker ~ 10 cm from mouse, sound it 3 times and record responses	0 = always reacts (3/3 times) 0.5 = reacts 1/3 or 2/3 times 1 = unresponsive (0/3 times)
Ocular/ Nasal		
Cataracts	Visual inspection of the mouse to detect opacity in the center of the eye	0 = no cataracts 0.5 = small opaque spot 1 = clear evidence of opaque lens
Eye discharge/swelling	Visual inspection of the mouse to detect ocular discharge and swelling of the eyes	0 = normal 0.5 = slight swelling and/or secretions 1 = obvious bulging and/or secretions
Microphthalmia	Inspect eyes	0 = normal size 0.5 = one or both eyes slight small or sunken 1 = one or both eyes very small or sunken
Corneal opacity	Visual inspection of the mouse for superficial white spots and/or clouding of the cornea	0 = normal 0.5 = minimal changes in cornea 1 = marked clouding and/or spotting of cornea
Vision loss	Lower mouse towards a flat surface. Evaluate the height at which the mouse reaches towards the surface	0 = reaches >5 cm above surface 0.5 = reaches 2-5 cm above surface 1 = reaches <2 cm above surface
Menace reflex	Move an object towards the mouse's face 3 times. Record whether the mouse blinks in response	0 = always responds 0.5 = no response to 1 or 2 approaches 1 = no response to 3 approaches
Nasal discharge	Visual inspection of the mouse to detect nasal discharge	0 = no discharge 0.5 = small amount of discharge 1 = obvious discharge, both nares
Digestive/ Urogenital		
Malocclusions	Grasp the mouse by the neck scruff, invert and expose teeth. Look for uneven, overgrown teeth	0 = mandibular longer than maxillary incisors 0.5 = teeth slightly uneven 1 = teeth very uneven and overgrown
Rectal prolapse	Grasp the mouse by the base of the tail to detect signs of rectal prolapse	0 = no prolapse 0.5 = small amount of rectum visible below tail 1 = rectum clearly visible below tail
Vaginal/ uterine/ penile prolapse	Grasp the mouse by the base of the tail to detect signs of vaginal/ uterine or penile prolapse	0 = no prolapse 0.5 = small amount of prolapsed tissue visible 1 = prolapsed tissue clearly visible

Diarrhea	Grasp the mouse and invert it to check for signs of diarrhea. Also look for fecal smearing in home cage	0 = none 0.5 = some feces or bedding near rectum 1 = feces + blood and bedding near rectum, home cage smearing
Respiratory		
Breathing rate/depth	Observe the animal. Not the rate and depth of breathing as well as any gasping behavior	0 = normal 0.5 = modest change in breathing rate and/or depth 1 = marked changes in rate/depth, gasping
Discomfort		
Mouse grimace scale	Note facial signs of discomfort: 1) orbital tightening, 2) nose bulge, 3) cheek bulge, 4) ear position (drawn back) or 5) whisker change (either backward or forward)	0 = no signs present 0.5 = 1 or 2 signs present 1 = 3 or more signs present
Piloerection	Observe the animal and look for signs of piloerection, in particular on the back of the neck	0 = no piloerection 0.5 = involves fur at base of neck only 1 = widespread piloerection
Other		
Temperature	Measure surface body temperature with an infrared thermometer directed at the abdomen (average of 3 measures). Compare with reference values from sex-matched adult animals	0 = differs by <1 SD from reference value 0.25 = differs by 1 SD 0.5 = differs by 2 SD 0.75 = differs by 3 SD 1 = differs by >3 SD
Weight	Weight the mouse. Compare with reference values from sex-matched adult animals	0 = differs by <1 SD from reference value 0.25 = differs by 1 SD 0.5 = differs by 2 SD 0.75 = differs by 3 SD 1 = differs by >3 SD

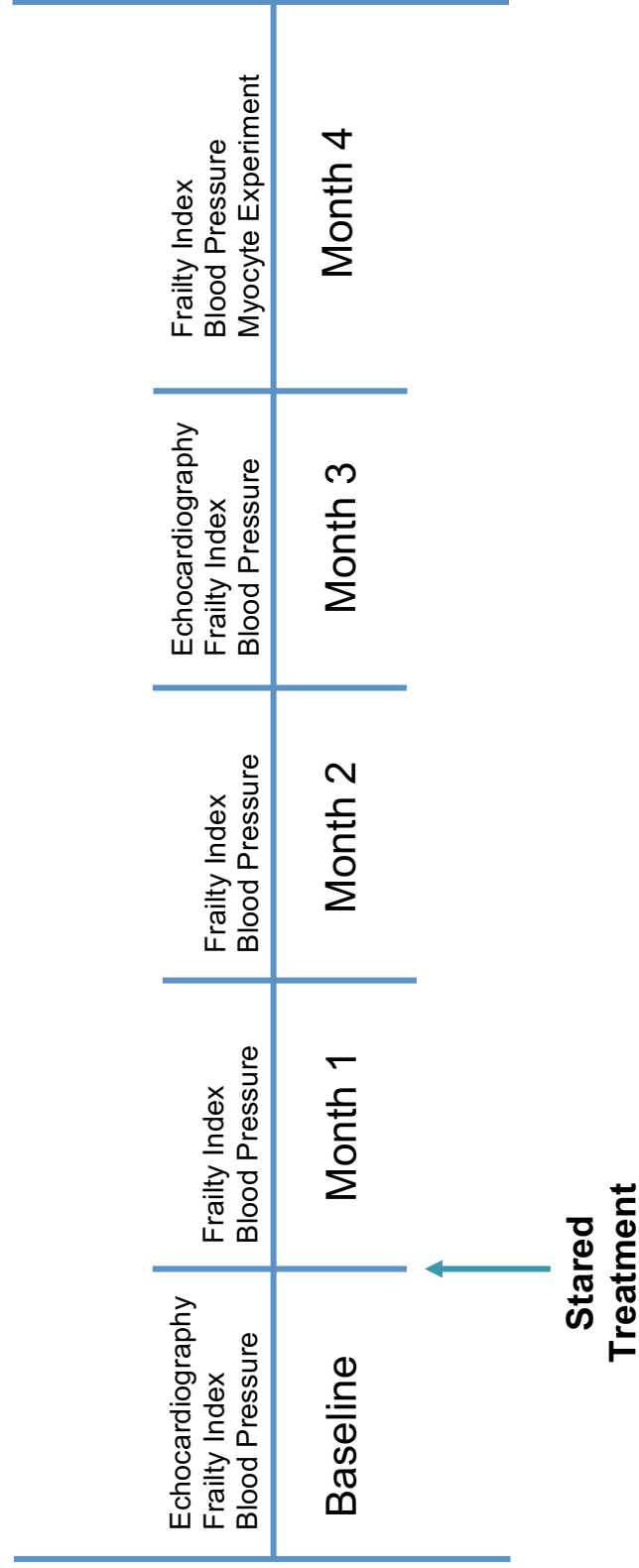
From Whitehead et al. (2014).

temperature were also measured and deviation from average values measured in baseline counted towards a deficit. To measure hearing loss, a clicker similar to that used in dog training was used. Animals were then weighed and body surface temperature was recorded using an infrared temperature probe (La Crosse Technology, La Crosse, WI) directed at the lower abdomen. Three measurements of body surface temperature were obtained and averaged. Body surface temperature and weight were measured as deficits based on their deviation from average values calculated in the baseline frailty index. A value that was +/- 1 SD from the mean received a score of 0.25. Values +/- 2 SD received a score of 0.5, +/- 3 SD received a score of 0.75 and any values that fell outside 3 SD of the reference value were given a score of 1. Once all parameters were evaluated, the deficit scores were added and divided by 31, the total number of parameters. This provided an FI value of between 0 and 1.

2.4 Measuring Blood Pressure

Blood pressure was measured using a tail cuff plethysmography machine (IITC Life Science, Inc., Woodland Hills, CA). Measurements were performed at baseline and then at monthly intervals after animals were started on enalapril or control chow (Refer to Fig. 2.1). Before blood pressure was measured, animals were obtained from the animal care facility and allowed to acclimate in a quiet location for 15 minutes. Animals were then placed in a clear restrainer to limit movement, with a dark cone placed in front of the animal during measurements to limit sight. The animal was then placed in a heated compartment and a tail cuff attached to a sensor was then placed at the base of the tail. The lights were turned off and the animal was given 10 minutes of acclimation within the restrainer. The tail cuff was then inflated to occlude blood flow to the tail and deflated,

Figure 2.1. General timeline of experimental interventions.



allowing blood flow to return. A light sensor present within the tail cuff detected systolic blood pressure, diastolic blood pressure and heart rate. Five consecutive measurements were made and this was repeated 6 times in a day, two days in a row. The systolic and diastolic blood pressure values obtained for each mouse were then averaged.

2.5 Measuring *in vivo* heart function

Two-dimensional echocardiography was performed on all animals in this study at baseline and at the end of the third month to assess changes in *in vivo* heart function. A Vivid 7 imaging system (GE Medical Systems, Horten, Norway) was used to perform the experiments. Animals were placed in an empty cage and 3% isoflurane in oxygen (1 L/min) was applied using a cone until the animal lost consciousness. The animal was then placed in the supine position on a heating pad (~37°C) under a heat lamp. Isoflurane was then reduced to 1% and maintained at this level for the rest of the procedure. Depilatory cream was used to remove hair on the animal's chest to reduce interference in image production. Electrocardiography electrodes were inserted subcutaneously at the upper left and right limbs and by the tail to obtain electrocardiography measurements. Heart rate was calculated from the resulting ECG. A temperature probe was placed rectally to measure internal temperature during the procedure. Ultrasound transmission gel was applied to a high-resolution linear transducer before placing it on the animal's chest to maximize image quality. Two-dimensional M-Mode images of the heart in short axis were used to collect physical measurements of the left ventricle in systole and diastole. These measurements included interventricular septum thickness (IVS), left ventricular internal diameter (LVID), and left ventricular posterior wall thickness (LVPW). These values were used to calculate ejection fraction (EF; the fraction of blood pumped out of

the heart during a heartbeat), and fractional shortening (FS; the degree of shortening of the left ventricle between diastole and systole). Pulse wave Doppler was performed in apical view to measure the velocity of the blood entering the left ventricle through the mitral valve. The E wave represents passive filling, through ventricular relaxation, and the A wave reflects active filling, by atrial contraction, of the ventricle (Ashley & Niebauer, 2004). Calculating an E/A ratio from these values provided a measure of diastolic function. After the experiment was complete, the mice were placed on a heating pad for a minimum of 1 hour to allow the animals to recover from the anesthetic. Once proper motor function had fully returned, the animals were returned to their home cages. Animals were then given a minimum of a week to recover before being euthanized for isolated cardiomyocyte experiments.

2.6 Ventricular Myocyte Isolation

Ventricular myocytes were isolated via enzymatic digestion of the heart to evaluate cellular contractile function and intracellular calcium homeostasis. A mouse was placed in a clean cage in animal care and brought to the lab. Food and water were provided and the animal was placed in a quiet corner for 30 minutes to acclimate. Sodium pentobarbital (220 mg/kg) and heparin (3000 U/kg) were co-administered through intraperitoneal injection to anesthetize the mouse and prevent blood clotting, respectively. Once the mouse lost consciousness it was weighed and placed in the supine position on the surgical table.

To confirm anaesthesia, pedal withdrawal and corneal reflexes were tested. Forelimbs were secured to the surgical surface to maintain the animal's position during surgery. A sternal incision was then made to expose the ribs. A lateral cut was made on either side of

the ribs and they were lifted towards the head to expose the heart. The aorta was severed, a cannula inserted and secured with a suture. The heart was then removed from the body. *Ex-vivo* retrograde perfusion of the heart occurred at 2.2 ml/min (5 min) with oxygenated Ca^{2+} -containing buffer solution heated to 37°C. The solution contained (mM) 105 NaCl, 5 KCl, 25 HEPES, 0.33 NaH_2PO_4 , 1 MgCl_2 , 20 glucose, 3 Na-pyruvate, 1 lactic acid and 0.4 CaCl_2 . The solution was brought to a pH of 7.4 with NaOH. Once this was complete, the heart was perfused with oxygenated solution, as described above but with the CaCl_2 omitted, for 7 minutes. Finally, the heart was perfused with the Ca-free buffer supplemented with 50 μM CaCl_2 , collagenase type I (8.0 mg/30 mL; Worthington, Lakewood, NJ), dispase II (3.0 mg/30 mL; Roche Diagnostics, Laval, QC) and trypsin (0.5 mg/30 mL; Sigma Aldrich, Oakville, ON) for 8-10 minutes. All solutions were bubbled with 100% O_2 (Praxair, Dartmouth, NS) and delivered to the heart via a perfusion apparatus powered by a peristaltic pump (Masterflex L/S Easy-Load II; Cole-Parmer, Montreal, QC). A bubble trap was present to prevent air bubbles from reaching the heart.

Following enzymatic digestion the ventricles were isolated from the atria, minced into smaller segments and placed in a high potassium buffer comprised of (mM): 45 KCl, 3 MgSO_4 , 30 KH_2PO_4 , 50 L-glutamic acid, 20 taurine, 0.5 EGTA, 10 HEPES, and 10 glucose. The solution was brought to a pH of 7.4 using KOH. The tissue was rinsed with this solution three times to ensure that no enzyme-containing solution was present. The tissue was then stored in a small beaker containing the high potassium solution. To release individual myocytes, the beaker was gently swirled. To remove any large tissue fragments, the cells were filtered through a 225 μm polyethylene mesh filter.

2.7 Field Stimulation: Intracellular Ca^{2+} and contraction measurements

Isolated cardiomyocytes were field stimulated to measure characteristics of cell contractions and underlying Ca^{2+} transients. Myocytes were incubated with fura-2AM (2.5 μM) in the dark for 20 minutes in a plexiglass chamber. The chamber had a glass bottom and it was mounted to the stage of an inverted microscope (Nikon Eclipse TE200; Nikon Canada, Mississauga, ON). The microscope was contained within a Faraday cage covered in black vinyl to limit light entering and interfering with the fluorescence recordings. A custom-made air table supported the microscope to reduce vibration. After the incubation was complete, myocytes were superfused at 3 ml/min with a buffer solution that contained (mM): 135 NaCl, 10 glucose, 10 HEPES, 4 KCl, 1 MgCl_2 , 4 CaCl_2 , and brought to a pH of 7.4 with NaOH. The buffer was maintained at 37°C via a circulating water bath (Polystat, Model # 12112-10, Cole Parmer, Vernon Hills, IL).

Quiescent myocytes with clear striations were located and two platinum electrodes were placed on either side of the cell within the plexiglass chamber. Bipolar pulses were generated by a stimulation unit (Model #SIU-102; Warner Instruments, Hamden, CT) and myocytes were stimulated at 2 Hz, with pacing controlled by pClamp 8.2 software (Molecular Devices, Sunnyvale, CA). The simultaneous measurement of fluorescence and cell contraction was made possible by dividing light between a photomultiplier tube (Photon Technologies International (PTI), Birmingham, NJ) and a video camera (Philips, Markham, Ontario) via a dichroic cube (Chroma Technology Corp., Rockingham, VT). To obtain fluorescence measurements, a photomultiplier aperture was closed to fit the precise size of the cell. A fluorescence system excited cells with light at alternating wavelengths of 340 nm and 380 nm. Fluorescence emitted at 510

nm was collected for both wavelengths at a rate of 200 samples/sec and was recorded for a total of 20 seconds. At the end of each experiment a background recording was taken for each individual cell in an area near the cell, but free of other myocytes or cell fragments. Simultaneously, cell shortening was detected using a video edge detector (Model #105; Crescent Electronics, Sandy, UT) at a rate of 120 samples/second. Cell length and width were also measured in quiescent cells. Cell contractions were recorded with pClamp 8.2 software.

2.8 Chemicals

Fura-2 AM was obtained from Invitrogen (Burlington, ON). Stock preparations were made by dissolving 50 µg of fura in anhydrous DMSO. DMSO had a final concentration of 0.2%. The stock was stored at -5°C until needed. Enalapril was purchased from Cayman Chemical (Ann Arbor, MI) and incorporated into food pellets provided by Bio-serv (Frenchtown, NJ) at 280 mg/kg. Food was stored in a dark, dry place at room temperature. All chemicals used to make buffer solutions were purchased from Sigma Aldrich (Oakville, ON).

2.9 Statistics

Sigma Plot 12.0 (Systat Software, Inc., Point Richmond, CA) was used to create all graphs and for statistical analysis. To evaluate if frailty development was influenced by enalapril treatment and if there were any sex differences, 2-way repeated measures (RM) ANOVA was performed. Systolic and diastolic blood pressure were analyzed using the blood pressure monitor software (IITC Life Science, Inc., Woodland Hills, CA) and 2-way RM ANOVA was performed. Echocardiography was analyzed using Vivid 7 imaging systems (GE Medical Systems, Horten, Norway). Contraction and field

stimulation data was analyzed using Clampfit 8.2 (Molecular Devices, Sunnyvale, CA). Contractions were quantified by measuring the difference between cell lengths at rest and at peak contraction. To obtain the Ca^{2+} concentrations, Felix software (PTI) was used to subtract background fluorescence from each wavelength. The resulting values were converted to an emission ration (340/380) and then to Ca^{2+} concentrations. Ca^{2+} transients were measured as the difference between diastolic and systolic Ca^{2+} . Differences between groups were detected by performing a 2-way ANOVA. Multiple comparisons were performed using a Holm-Sidak post-hoc test. Data are presented as mean +/- SEM and differences were reported as significant if $p < 0.05$.

Chapter 3: Results

3.1 Weight and enalapril dose

Baseline characteristic of animals used in this study are summarized in Table 3.1 (mean \pm SEM). To determine if weight varied across female control, female enalapril-treated, male control and male enalapril-treated groups, weights were recorded at various time points throughout the study and this is illustrated in Figure 3.1A. Female enalapril-treated mice were significantly heavier at the start of the study when compared to control. However, this did not remain the case throughout the rest of the study, with weights no longer being different after 11 days of treatment. This remained so throughout the study, with the exception of weights measured on day 68. Male control and enalapril-treated animal weights were not different at the start of the study and remained that way throughout. When weights from female and male control animals were compared, no differences were detected. However, enalapril-treated male mice started at a higher weight compared to the enalapril-treated females, and remained higher until day 33, where they were no longer significant different. Mean (\pm SEM) values of animal weights are summarised in table 3.2.

In order to determine the average enalapril dose consumed, food consumption was tracked throughout the course of the study and dose calculated from those values. This is shown in Figure 3.1B. After 11 days, male and female mice were receiving similar enalapril doses. Amounts received remained similar, with the exception of one measurement, until day 50. At this point males were receiving a higher dose of enalapril.

Table 3.1. Baseline characteristics of animals.

Parameter	Female Control	Female Enalapril	Male Control	Male Enalapril
Age (mos)	8.88 ± 0.22	9.57 ± 0.01	9.68 ± 0.04	9.65 ± 0.04
Body Weight (g)	33.2 ± 0.9	35.8 ± 0.8	36.7 ± 2.3	40.3 ± 1.0
Initial FI	0.15 ± 0.01	0.13 ± 0.01	0.17 ± 0.02	0.19 ± 0.01

Values are mean ± SEM.

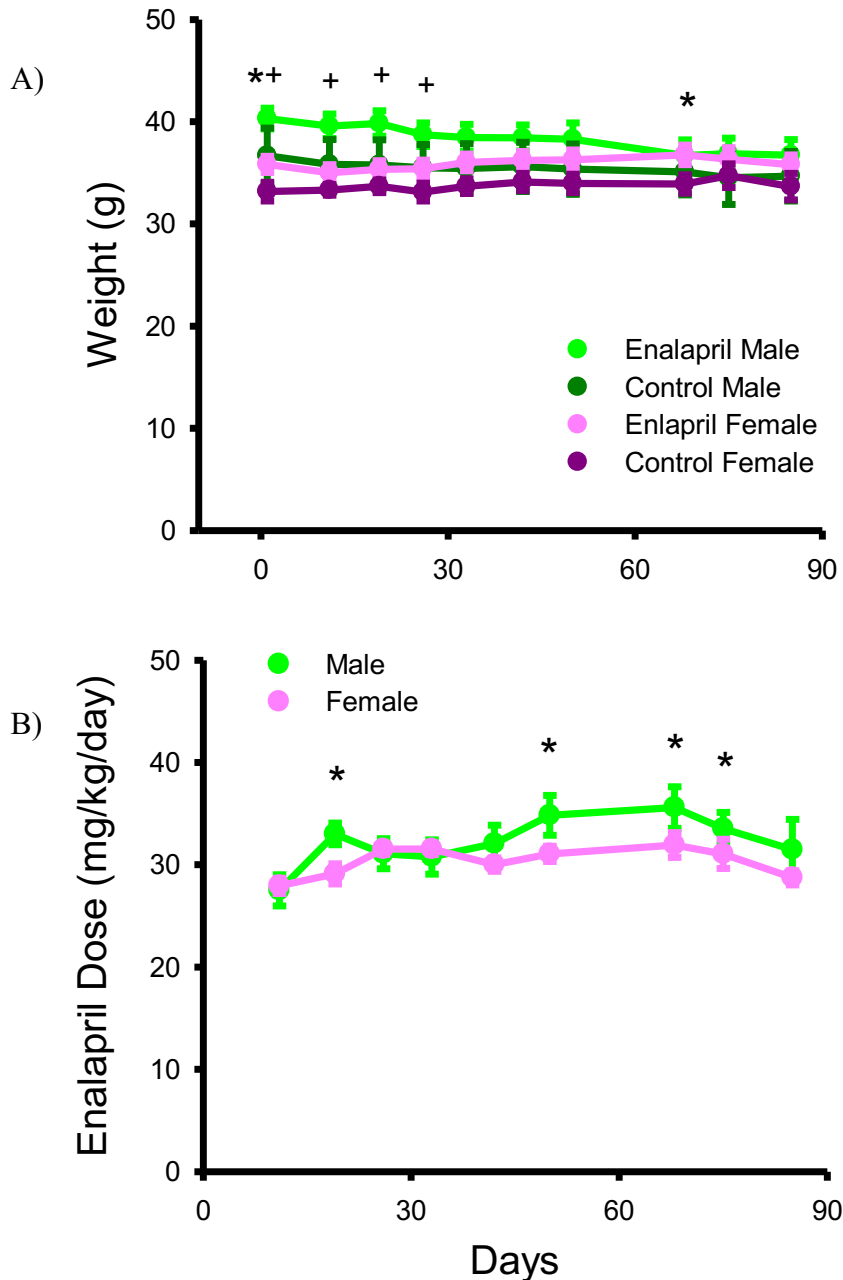


Figure 3.1. Weight was similar between male and female enalapril-treated and control mice, with a few exceptions. Male and female mice started out consuming similar amounts of enalapril, however males were receiving a higher dose after day 50. Female enalapril-treated mice were similar weights throughout the study, with a few exceptions. Males enalapril-treated animals started at a higher weight compared to females, however weights were no longer different at day 33 (A; significant differences are * female enalapril vs. control; + enalapril females vs. males). Enalapril consumption (B) was similar at the beginning of the study, but after 50 days of treatment males were receiving a higher dose. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.3. Average enalapril dose (mg/kg/day) in male and female mice over the 3 mos

Days	Female Enalapril (n=10)	Male Enalapril (n=6)	Dose difference p<0.05
11	27.9 ± 0.7	27.5 ± 1.5	NS
19	29.1 ± 1.0	33.0 ± 1.1	Yes
26	31.5 ± 0.5	31.1 ± 1.5	NS
33	31.5 ± 0.5	30.8 ± 1.7	NS
42	30.0 ± 0.7	32.1 ± 1.8	NS
50	31.1 ± 0.8	34.8 ± 1.9	Yes
68	31.1 ± 1.4	35.6 ± 2.0	Yes
75	28.7 ± 0.7	33.5 ± 1.6	Yes
85	30.2 ± 1.4	31.5 ± 2.9	NS

Values are mean ± SEM. 2-way RM ANOVA. NS=Not Significant.

Table 3.2. Average weights (g) of male and female enalapril-treated and control mice over 3 mos.

Days	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
1	33.2 ± 0.9	35.8 ± 0.8	36.7 ± 2.7	40.3 ± 1.0	Yes	NS	NS	Yes
11	33.3 ± 0.5	35.0 ± 0.8	35.8 ± 2.4	39.6 ± 1.2	NS	NS	NS	Yes
19	33.7 ± 0.6	35.3 ± 0.8	35.8 ± 2.5	39.8 ± 1.2	NS	NS	NS	Yes
26	33.1 ± 0.9	35.4 ± 0.9	35.5 ± 2.3	38.7 ± 1.2	NS	NS	NS	Yes
33	33.7 ± 0.7	36.0 ± 0.8	35.4 ± 2.4	38.4 ± 1.3	NS	NS	NS	NS
42	34.1 ± 0.9	36.2 ± 0.9	35.6 ± 2.4	38.4 ± 1.3	NS	NS	NS	NS
50	34.0 ± 0.9	36.3 ± 1.0	35.4 ± 2.5	38.3 ± 1.6	NS	NS	NS	NS
68	33.9 ± 0.9	36.8 ± 1.0	35.1 ± 2.3	36.7 ± 1.5	Yes	NS	NS	NS
75	34.7 ± 1.2	36.3 ± 1.1	34.5 ± 2.6	36.9 ± 1.5	NS	NS	NS	NS
85	33.7 ± 1.3	35.8 ± 1.0	34.7 ± 2.4	36.7 ± 1.5	NS	NS	NS	NS

Values are mean ± SEM. 2-way RM ANOVA. NS=Not Significant.

Males continued to receive a significantly higher dose than females, with exception of the last measurement. These data show that the males received either the same or a higher dose of enalapril throughout the study compared to females. Table 3.3 summarizes these mean (\pm SEM) values.

3.2 Impact of enalapril administration on development of frailty

The next series of experiments was designed to determine if ACE inhibitor treatment would attenuate frailty in middle-aged male and female mice. Figure 3.2A illustrates FI scores for female control and enalapril-treated mice at baseline and monthly for four months of treatment. FI scores were similar in control and enalapril-treated mice for the first 3 months. However, after 3 months of treatment, FI scores were significantly higher in control female animals when compared to enalapril-treated animals. Figure 3.2B compares FI scores from male mice treated with enalapril with scores from animals that received control food. By contrast, FI scores were similar in control and treated male mice until the third month, when FI scores were significantly lower in controls than in enalapril-treated male mice. At four months however, FI scores were no longer significantly different in males.

FI scores in control male and female mice were also compared directly (Figure 3.2C). There was an interesting sex difference in FI scores. Males started with significantly higher FI scores than females. After one month, scores were no longer different, and remained so throughout the experiment. At the third and fourth month, control FI scores in females appeared higher than males, although this was not statistically significant. When FI scores from male and female animals treated with

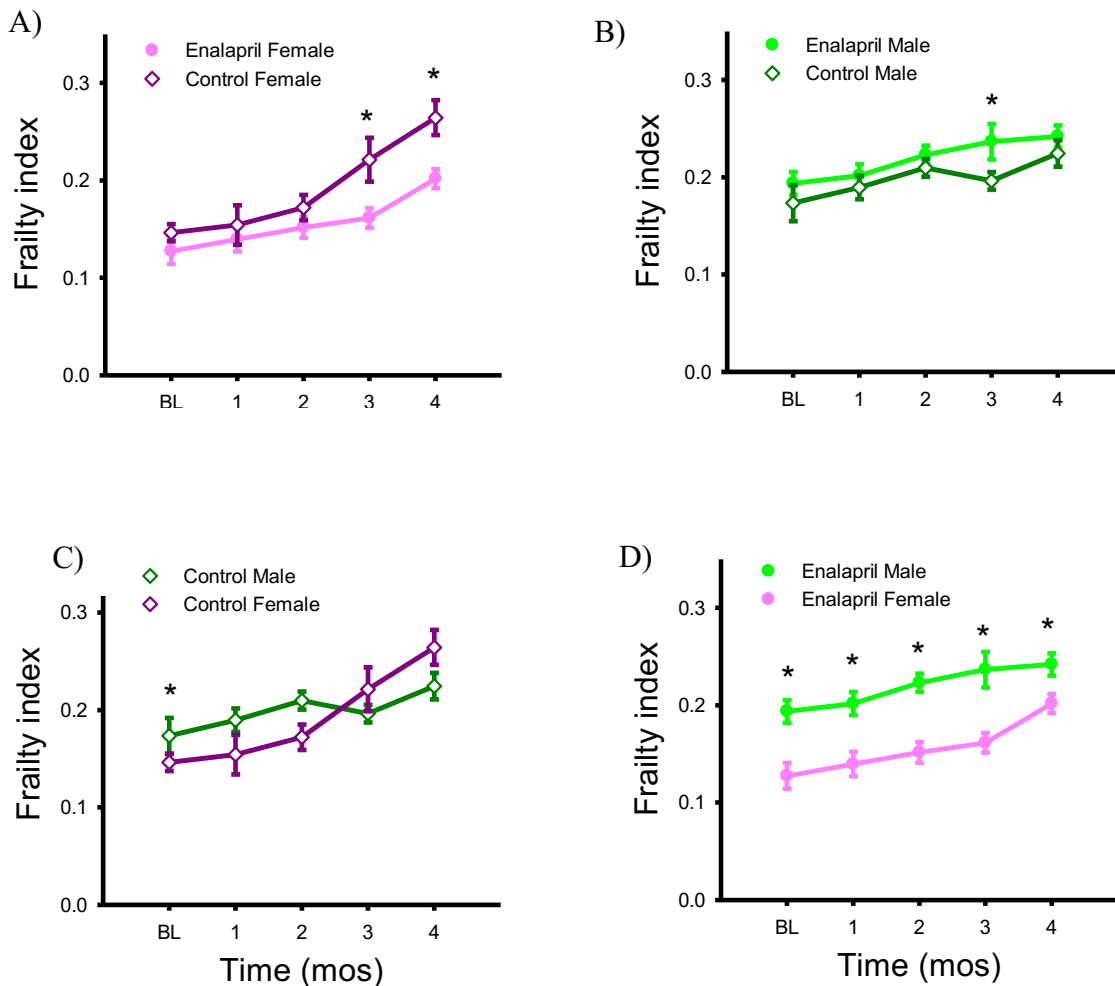


Figure 3.2. Enalapril treatment attenuated frailty in females, but not in males. (A) In females, frailty scores were higher in control mice compared to enalapril-treated mice after 3 months of treatment. (B) In males, frailty scores were not significantly different between enalapril-treated and control mice, except at 3 mos where control animals had lower scores than enalapril-treated animals. At month 4 however, frailty scores were not different between the two groups. (C) Male mice in control groups started with a higher frailty score at the beginning of the study compared to females, but were not different throughout the rest of the study. (D) Within the enalapril-treated group, males had higher FI scores than females. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.4. Frailty Index scores measured at baseline and monthly intervals.

	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
Baseline	0.15 ± 0.01	0.13 ± 0.01	0.17 ± 0.02	0.19 ± 0.01	NS	NS	Yes	Yes
Mos 1	0.15 ± 0.02	0.14 ± 0.01	0.19 ± 0.01	0.20 ± 0.01	NS	NS	NS	Yes
Mos 2	0.17 ± 0.01	0.15 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	NS	NS	NS	Yes
Mos 3	0.22 ± 0.02	0.16 ± 0.01	0.20 ± 0.01	0.24 ± 0.02	Yes	Yes	NS	Yes
Mos 4	0.26 ± 0.02	0.20 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	Yes	NS	NS	Yes

Values are mean ± SEM. 2-way RM ANOVA. NS=Not Significant.

enalapril were compared (Figure 3.2D), scores were significantly higher in males than in females at baseline and throughout the experiment. Table 3.4 summarizes mean FI values (\pm SEM) from female and male control and enalapril-treated animals.

3.3 Impact of enalapril on blood pressure

To investigate whether enalapril treatment affected blood pressure, a tail cuff blood pressure machine was used to measure diastolic and systolic blood pressure at baseline, and then monthly for the next three months. Figure 3.3A and 3.3B show mean (\pm SEM) systolic and diastolic blood pressures in female and male control and enalapril-treated mice, respectively. In both sexes, systolic and diastolic blood pressure did not change over the course of drug treatment. Blood pressure also was unaffected by enalapril treatment. These mean (\pm SEM) values are summarized in Table 3.5.

3.4 Impact of enalapril on *in vivo* cardiac function

To determine whether enalapril treatment affected *in vivo* cardiac morphology and function, two-dimensional guided M-mode and pulse wave Doppler echocardiography measurements were evaluated. Initially, changes in cardiac morphology were evaluated by measuring M-mode images of the left ventricle. Figure 3.4A shows mean (\pm SEM) interventricular septum (IVS) diameters during diastole for control and drug-treated mice. Enalapril had no effect on IVS thickness during diastole in any group and there were no differences between the sexes. Figure 3.4B shows IVS thickness during systole in males and females. Although drug treatment did not affect this parameter, male control animals did have a thicker IVS during systole compared to female control mice. Figures 3.4C,

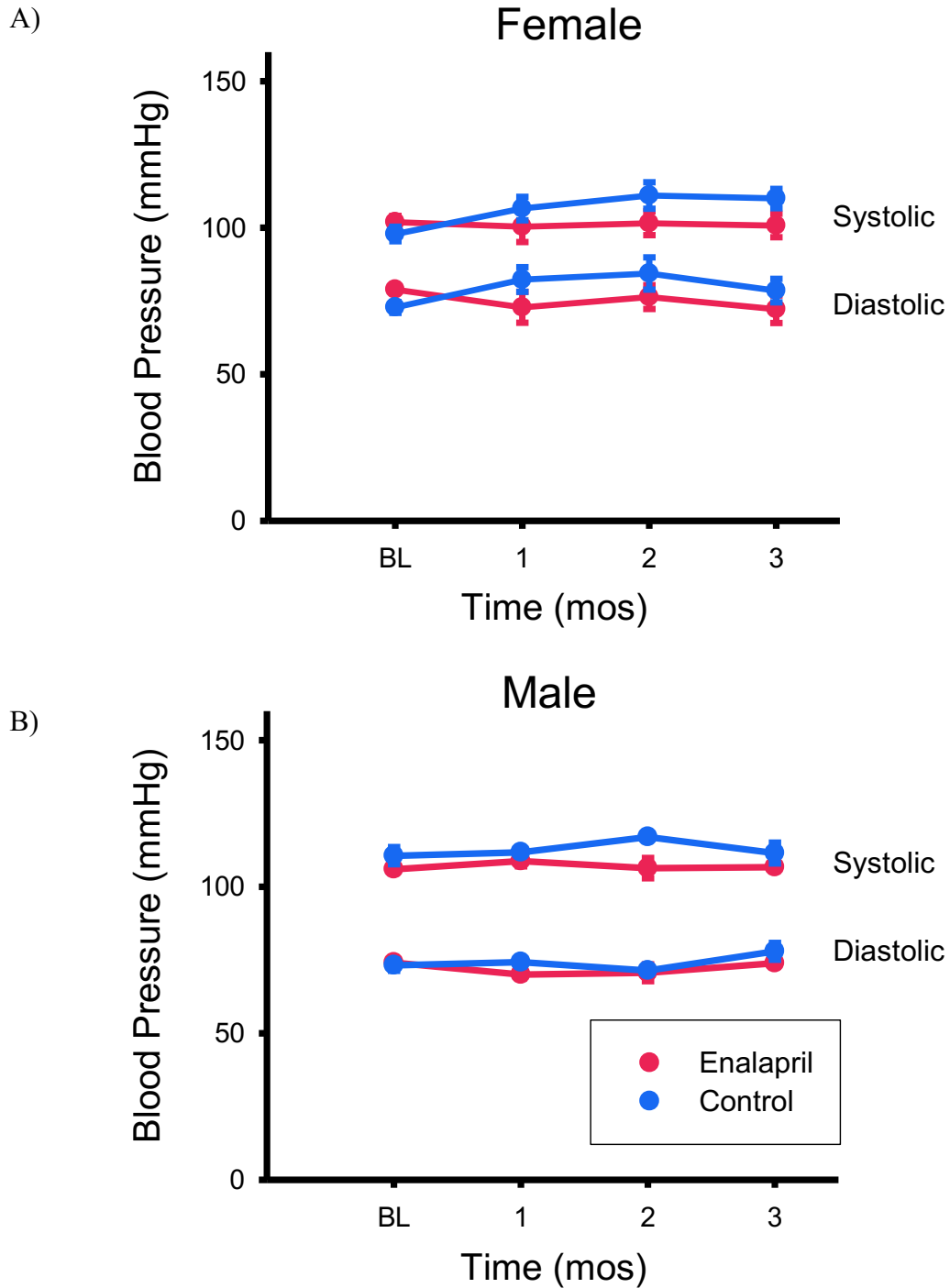


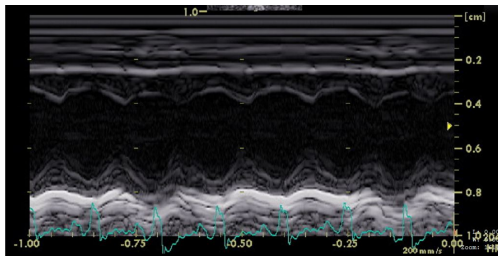
Figure 3.3. Blood pressure did not change with the administration of enalapril. Systolic and diastolic blood pressures were not significantly different between enalapril-treated and control animals in female (A) or male (B) mice. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.5. Systolic and diastolic blood pressure measured at baseline and monthly intervals during drug treatment.

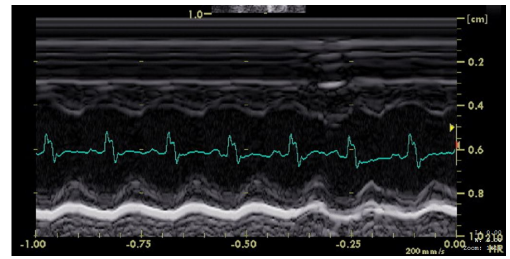
	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
Systolic Blood Pressure (mmHg)								
Baseline	97.9 ± 2.5	101.9 ± 2.2	110.6 ± 2.9	106.0 ± 1.2	NS	NS	NS	NS
Mos 1	106.5 ± 4.0	100.4 ± 5.2	111.8 ± 1.0	108.8 ± 1.8	NS	NS	NS	NS
Mos 2	111.0 ± 4.5	101.5 ± 4.1	111.9 ± 1.6	106.3 ± 3.5	NS	NS	NS	NS
Mos 3	110.0 ± 3.3	100.8 ± 4.0	111.5 ± 3.6	106.7 ± 1.2	NS	NS	NS	NS
Diastolic Blood Pressure (mmHg)								
Baseline	72.8 ± 1.9	76.7 ± 2.3	73.2 ± 1.9	74.0 ± 1.3	NS	NS	NS	NS
Mos 1	82.3 ± 4.2	72.8 ± 5.3	74.3 ± 1.4	70.1 ± 1.3	NS	NS	NS	NS
Mos 2	84.4 ± 5.5	76.3 ± 4.1	71.4 ± 1.1	70.7 ± 2.9	NS	NS	NS	NS
Mos 3	78.5 ± 4.0	72.2 ± 4.7	78.0 ± 2.9	73.9 ± 1.2	NS	NS	NS	NS

Values are mean ± SEM. 2-way RM ANOVA. NS=Not Significant.

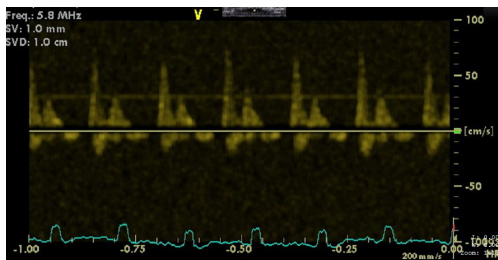
A)



B)



C)



D)

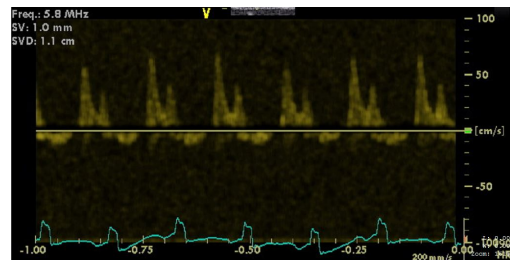


Figure 3.4. Representative M-mode and Doppler images of in vivo cardiac function. A) M-mode image from a male control mouse. B) M-mode image from a male-enalapril treated mouse. C) Pulse wave Doppler image from male control mouse. D) Pulse wave image from male enalapril-treated mouse. Images were similar in female mice.

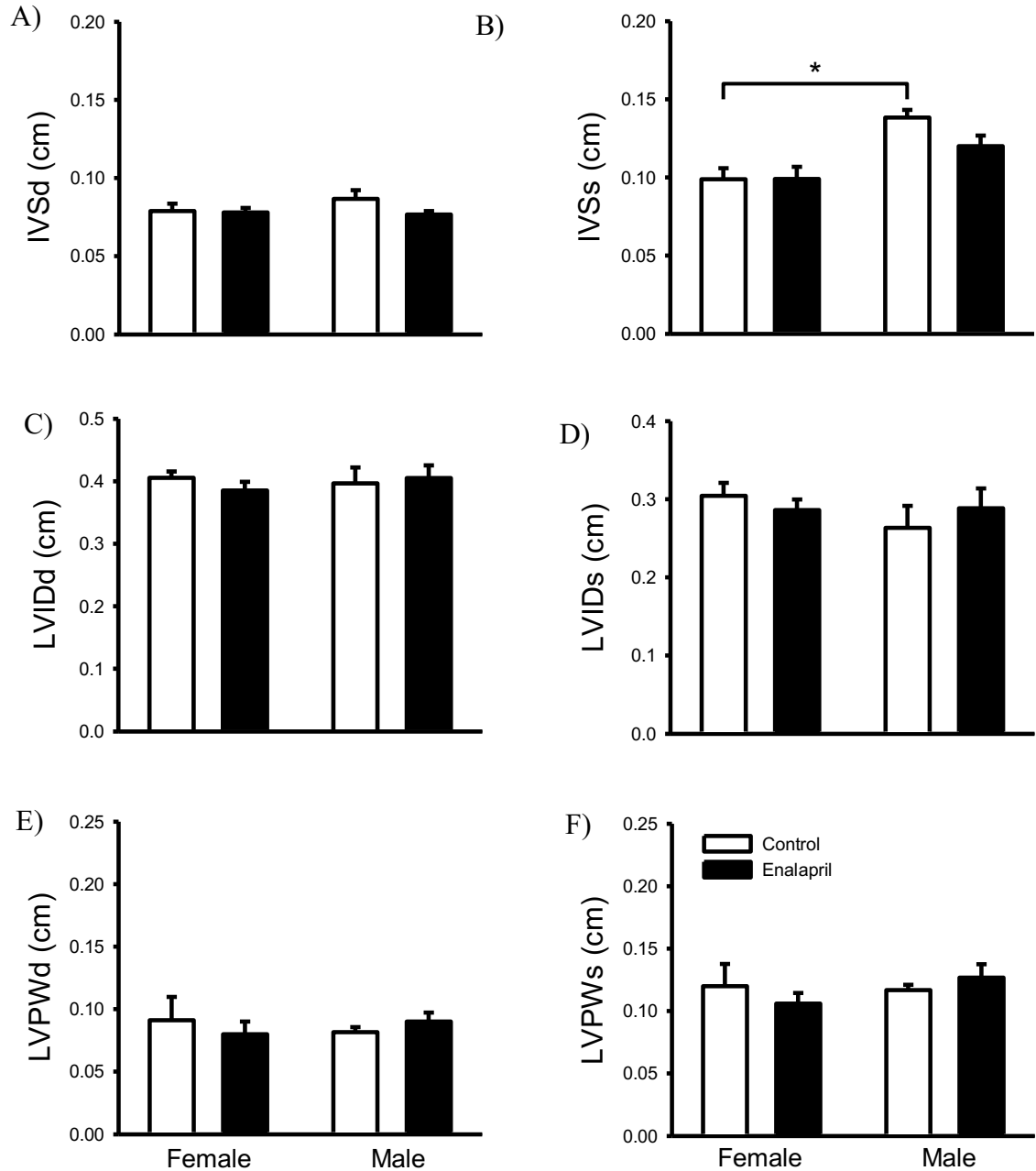


Figure 3.5. Enalapril had no effect on physical parameters of the left ventricle in males or females. Sex did not influence these parameters either, with the exception of interventricular septal thickness in systole. The interventricular septal thickness (IVS) during diastole (A) and systole (B) were not altered by drug treatment, however IVSs in control males was thicker in male controls than female controls. Left ventricular internal diameter (LVID) and left ventricular posterior wall (LVPW) measurements in diastole and systole (C-F) were unaffected by drug and sex. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

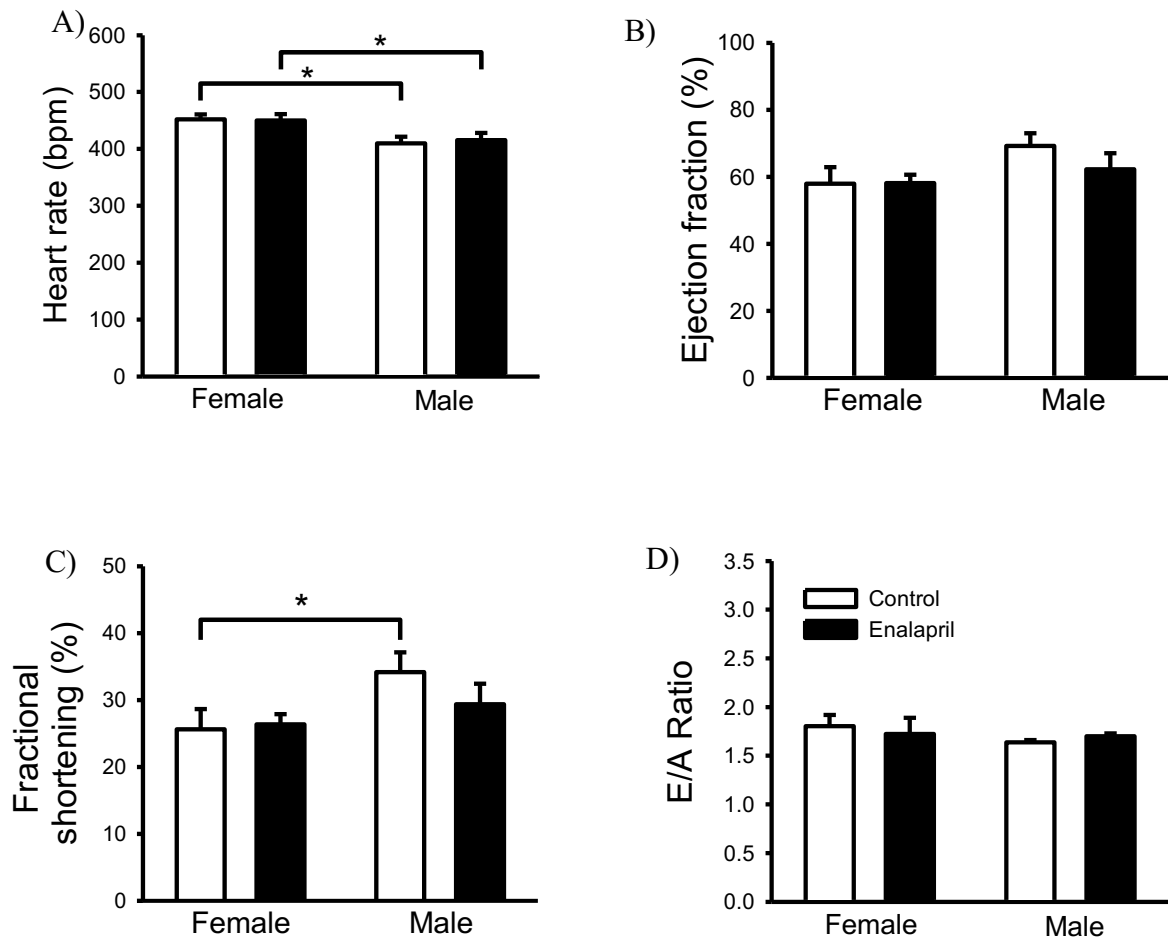


Figure 3.6. Heart rate was reduced in male mice compared to females. In vivo systolic and diastolic function were unaffected by enalapril in both male and female animals, although some baseline sex differences were observed. Heart rate (A) was lower in male mice when compared to females. Ejection fraction (B) was not effected by either sex or drug treatment, however fractional shortening (C) was enhanced in male control animals when compared to females. Diastolic function (E/A ratio) was not affected by sex or drug treatment (D). Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.6. Morphological and Functional cardiac parameters measured via echocardiography.

Parameter	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
Cardiac Morphology								
IVSd (mm)	0.79 ± 0.05	0.78 ± 0.03	0.87 ± 0.06	0.77 ± 0.02	NS	NS	NS	NS
IVSs (mm)	0.99 ± 0.07	0.99 ± 0.08	1.38 ± 0.05	1.20 ± 0.07	NS	NS	Yes	NS
LVIDd (mm)	4.06 ± 0.10	3.85 ± 0.14	3.97 ± 0.25	4.05 ± 0.20	NS	NS	NS	NS
LVIDs (mm)	3.04 ± 0.17	2.86 ± 0.14	2.63 ± 0.28	2.88 ± 0.26	NS	NS	NS	NS
LVPWd (mm)	0.91 ± 0.19	0.80 ± 0.10	0.82 ± 0.04	0.90 ± 0.07	NS	NS	NS	NS
LVPWs (mm)	1.20 ± 0.18	1.06 ± 0.08	1.17 ± 0.04	1.27 ± 0.11	NS	NS	NS	NS
Cardiac Function								
HR (bpm)	452.1 ± 8.3	449.9 ± 11.0	409.9 ± 11.2	415.2 ± 12.5	NS	NS	Yes	Yes
EF (%)	58.0 ± 4.9	58.1 ± 2.5	69.2 ± 3.8	62.2 ± 4.8	NS	NS	NS	NS
FS (%)	25.6 ± 3.0	26.4 ± 1.5	34.2 ± 2.9	29.4 ± 3.1	NS	NS	Yes	NS
E/A	1.80 ± 0.11	1.72 ± 0.16	1.64 ± 0.02	1.70 ± 0.03	NS	NS	NS	NS

Values are mean ± SEM. 2-way ANOVA. NS=Not Significant.

3.4D, 3.4E and 3.4F illustrate left ventricular internal diameter (LVID) and left ventricular posterior wall thickness (LVPW) during diastole and systole. Neither enalapril treatment nor sex affected LVID and LVPW.

To evaluate the impact of enalapril treatment on cardiac function, M-mode images were used to calculate systolic function parameters (fractional shortening and ejection fraction) and pulse wave Doppler was used to evaluate diastolic function (E/A ratio). Figure 3.5A shows that enalapril-treatment in both males and females had no effect on heart rate. Interestingly, male mice had a significantly lower heart rate when compared to age-matched females regardless of whether they were on treatment. Ejection fraction (Fig 3.5B) was not affected by enalapril in male and female animals, and ejection fraction did not differ between the sexes. Fractional shortening (Fig 3.5C) was unaffected by drug treatment in males and females, although it was higher in male control animals compared to female counterparts. Diastolic function, assessed by measuring the velocity of blood entering the left ventricle through the mitral valve and expressed as E/A ratio (Fig 3.5D), was not affected by either drug treatment or sex. Table 3.6 summarizes mean (\pm SEM) values obtained with echocardiography.

3.6 Impact of enalapril on ventricular myocyte contractions and Ca²⁺ transients

To investigate the influence of enalapril on myocyte morphology, contractile function and underlying Ca²⁺ transients, cardiomyocytes were obtained from control and enalapril-treated animals and after 3-4 months of treatment. Figure 3.6 shows that enalapril treatment and sex had no effect on myocyte length (A), width (B) or surface area (C) as values were similar across all groups. Male and female animals were used to assess sex differences. Figure 3.7 illustrates representative examples of contractions

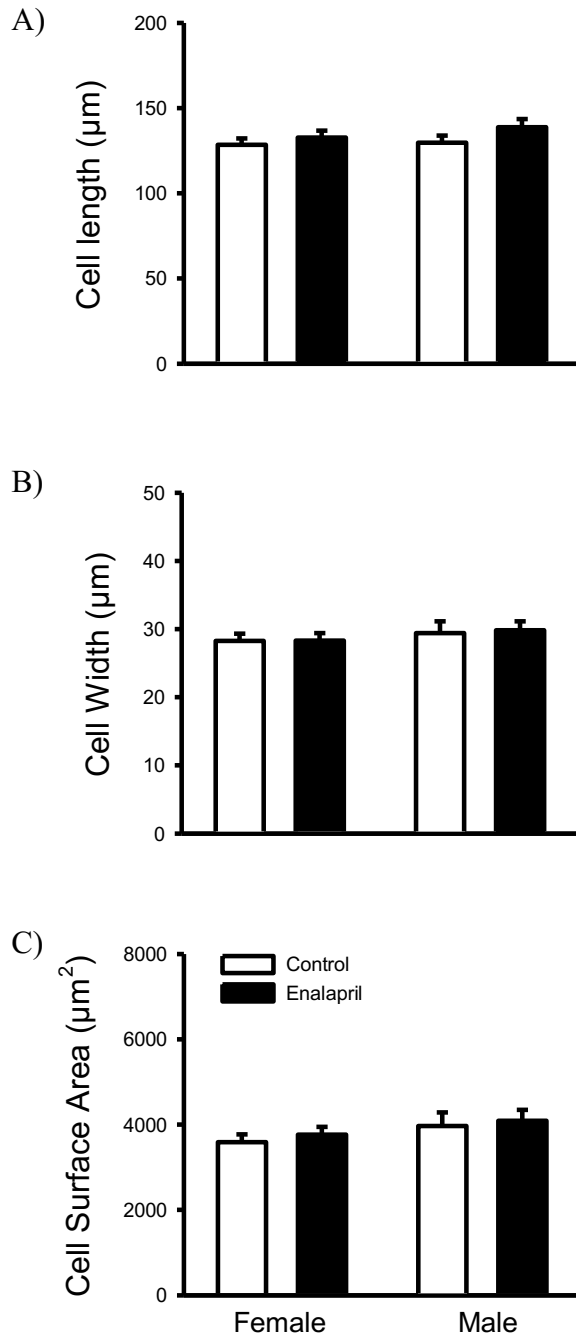


Figure 3.7 Cell length, width and surface area were not affected by either drug treatment or sex. Resting length (A), width (B) and surface area (C) of ventricular myocytes were similar in control and enalapril-treated male and female mice. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

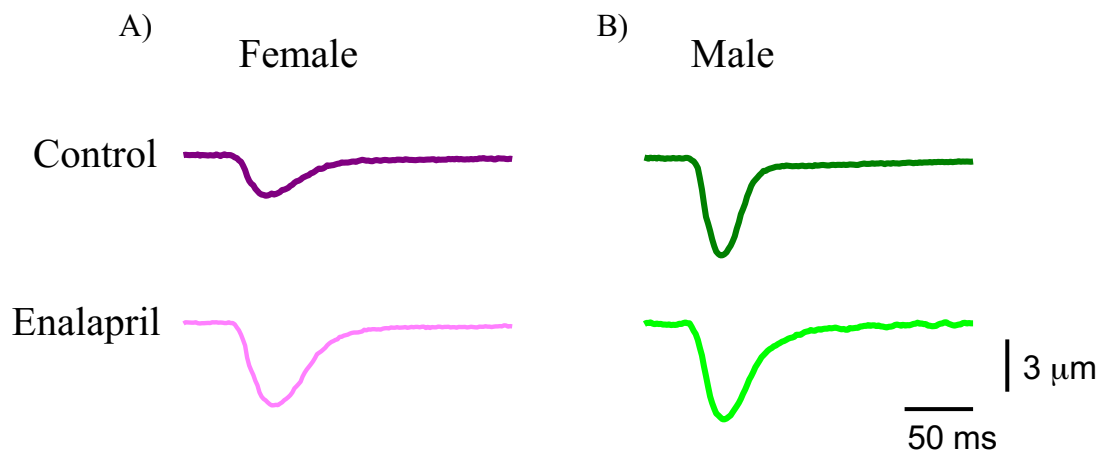


Figure 3.8. Representative traces of ventricular myocyte contractions and from female and male control and enalapril-treated animals. Enalapril-treated female mice had significantly larger contractions compared to controls (A). Male animals were unaffected by enalapril-treatment as contraction size was similar (B). Control female animals also had a significantly smaller contraction size compared to males.

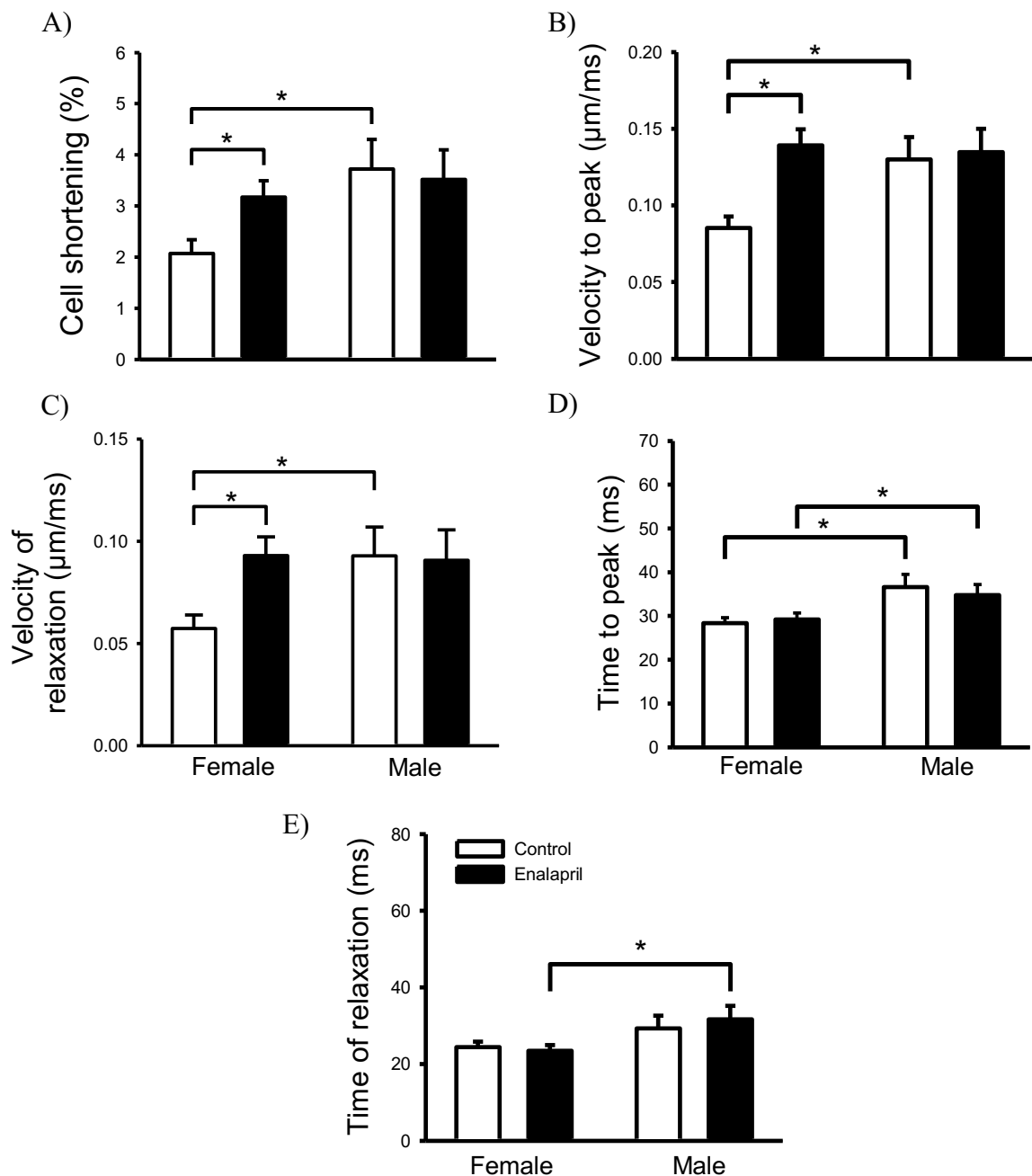


Figure 3.9. Enalapril-treatment enhanced myocyte contractile function in females, but not males and male mice had larger, faster contractions compared to female controls. Myocytes from females treated with enalapril had an increased % cell shortening (A), and velocity of contraction (B) and relaxation (C) compared to control. Males were unaffected by treatment, but had increased % cell shortening, velocity to peak, velocity of relaxation, time to peak (D) and timing of relaxation (E) compared to females. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.7. Contractile properties measured in isolated ventricular myocytes.

Parameter	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
Cell Shortening (%)	2.07 ± 0.27	3.17 ± 0.32	3.72 ± 0.58	3.52 ± 0.58	Yes	NS	Yes	NS
Velocity to Peak (µm/ms)	0.05 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	Yes	NS	Yes	NS
Velocity of Relaxation (µm/ms)	0.06 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.02	Yes	NS	Yes	NS
Time to Peak (ms)	28.36 ± 1.22	29.18 ± 1.48	36.62 ± 2.88	34.79 ± 2.42	NS	NS	Yes	Yes
Time of Relaxation (ms)	24.45 ± 1.40	23.47 ± 1.47	29.29 ± 3.31	31.64 ± 3.53	NS	NS	NS	Yes
Cell Length (µm)	128.42 ± 3.76	132.66 ± 4.17	129.56 ± 4.29	138.76 ± 4.82	NS	NS	NS	NS
Cell Width (µm)	28.26 ± 1.03	28.32 ± 1.07	29.39 ± 1.75	29.81 ± 1.33	NS	NS	NS	NS
Cell Surface Area (µm ²)	3589.0 ± 180.6	3760.4 ± 184.9	3968.4 ± 318.1	4087.0 ± 256.5	NS	NS	NS	NS

Values are mean ± SEM. 2-way ANOVA. NS=Not Significant. 'n' represents number of mice with 2-3 cells obtained from each mouse.

obtained from myocytes paced at 2 Hz in control mice of both sexes. These examples indicate that contractions appeared to be larger in the enalapril-treated female cell when compared to control. By contrast, this was not seen in males. However, the control response was larger in males compared to females.

To determine whether enalapril treatment affected contractile function in isolated myocytes, mean cell shortening, as well as velocity and timing of contraction were measured. Contraction was expressed as a percentage of resting cell length to compensate for variation in cell size. Fig 3.8A shows that, in females, enalapril treatment increased mean (\pm SEM) cell shortening compared to control. In contrast, male myocytes were not affected by drug treatment. Interestingly, sex differences were also apparent. Control males had significantly larger contractions than control females. The velocity of contraction is illustrated in Fig 3.8B and 3.8C. In females, enalapril increased the velocity to peak (B) as well as the velocity of relaxation (C). By contrast, there was no difference in the speed of contraction between control and enalapril-treated cell for the males. There also were sex differences in contraction velocity, with faster contractions in control males compared to control females. The timing of contraction was not affected by enalapril in either sex (Fig 3.8D and 3.8E). Time to peak was longer in both groups of males when compared to females (Fig 3.8D), while time to relaxation was longer only in enalapril-treated males when compared to females (Fig 3.8E). Again, both control and enalapril-treated males had significantly higher values when compared to females. Overall, enalapril-treatment did not affect contractile properties of myocytes in males, although in females it caused larger and faster contractions. Contractions were also larger and faster in males when compared to females.

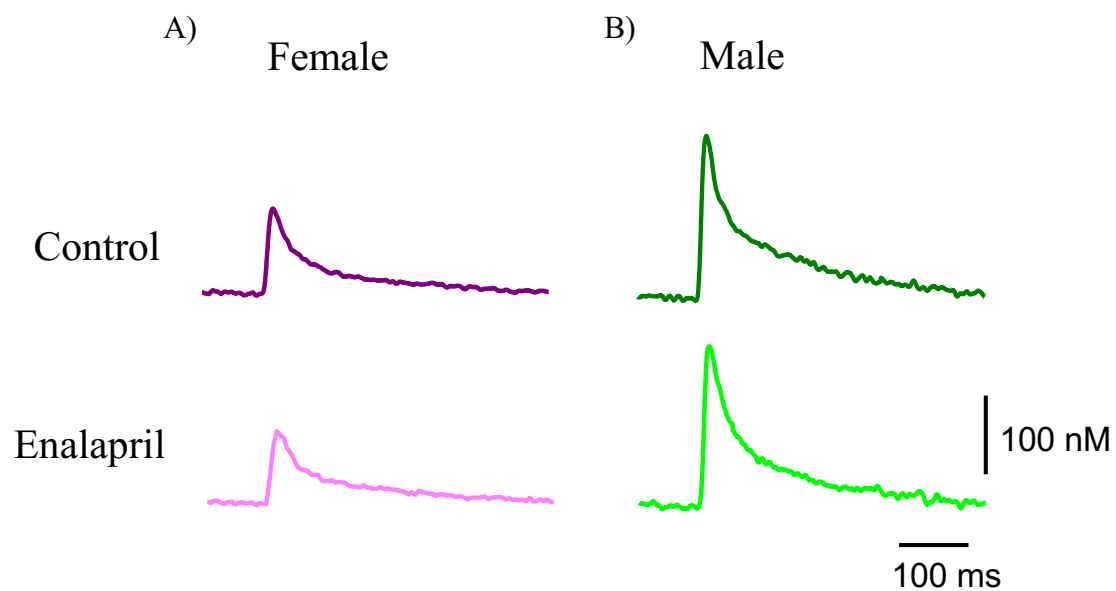


Figure 3.10. Representative traces of Ca^{2+} transients in male and female enalapril-treated and control cardiomyocytes. Enalapril-treated and control female mice had similar amplitudes of Ca^{2+} transients (A). Males also had similar Ca^{2+} amplitudes in enalapril-treated and control mice (B). Male Ca^{2+} transients had a higher amplitude compared females.

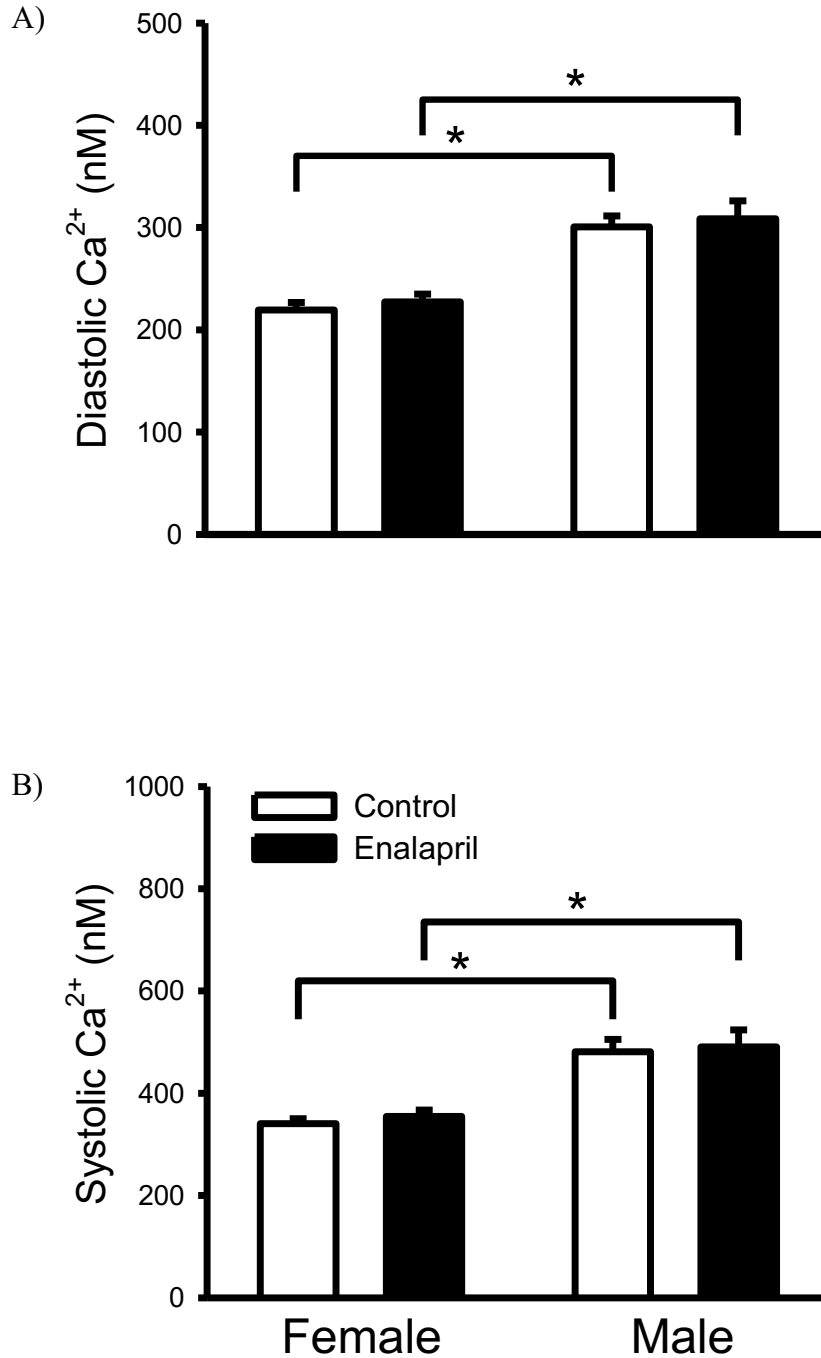


Figure 3.11. Enalapril had no effect on diastolic or systolic intracellular Ca^{2+} in isolated ventricular myocytes, but males had higher intracellular calcium levels compared to females. Diastolic (A) and systolic (B) Ca^{2+} were significantly lower in females compared to males. Control and enalapril-treated mice of both sexes had similar diastolic and systolic Ca^{2+} . Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

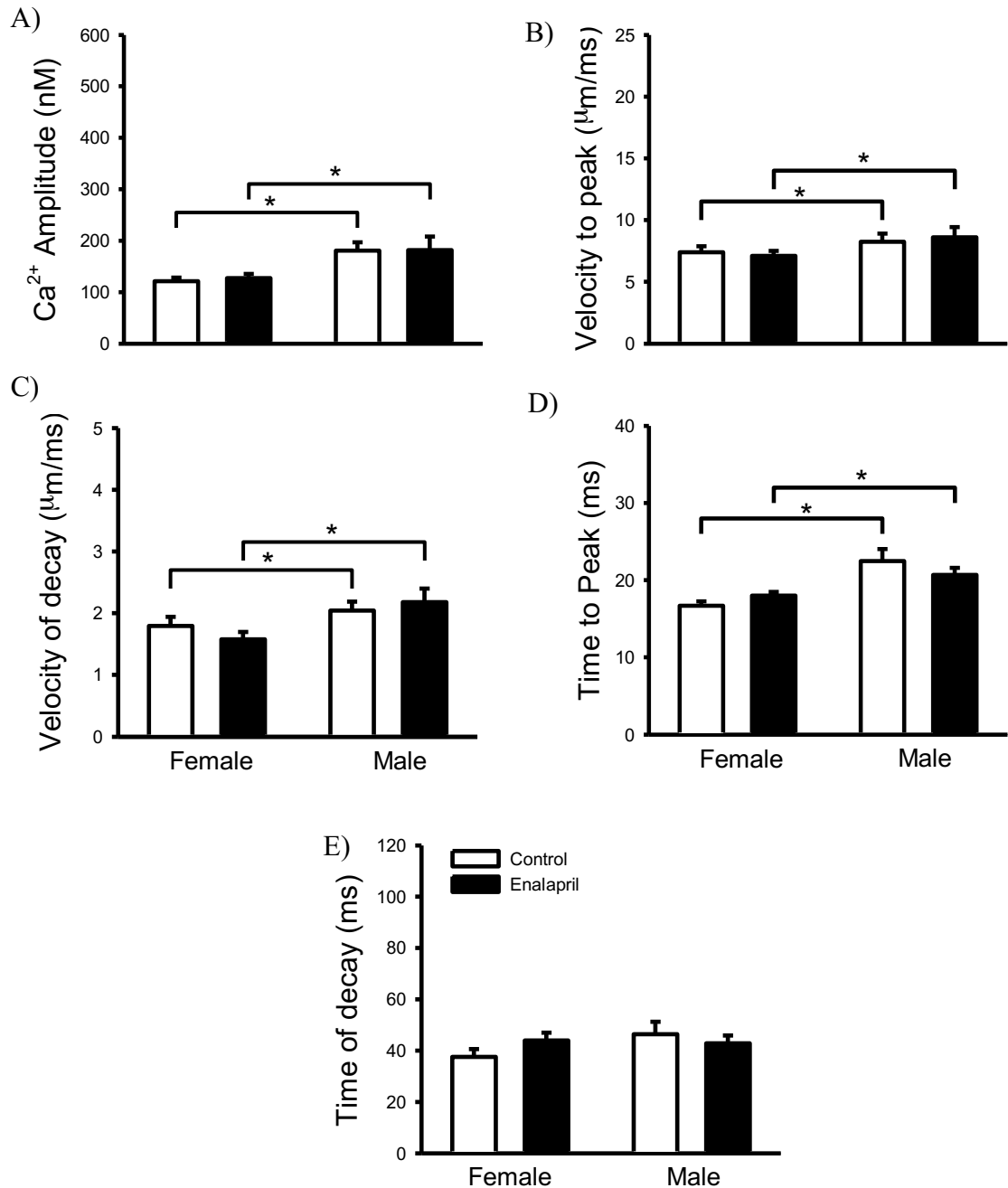


Figure 3.12. Enalapril had no effect on Ca²⁺ handling in cells from male and female mice, although male animals had increased Ca²⁺ transient amplitude, velocity and time to peak. Enalapril-treatment did not affect Ca²⁺ transient amplitude (A), velocity to peak (B), velocity of decay (C) time to peak (D) and time of decay (E) in female or male animals. However, males had increased amplitude, velocity to peak, velocity of decay and timing of contraction when compared to female control and enalapril groups. There were no sex differences in time of decay. Female enalapril-treated n=10; female control n=9; male enalapril-treated n=6; male control n=6.

Table 3.8: Intracellular Ca²⁺ transient parameters measured from isolated ventricular myocytes.

Parameter	Female Control (n=9)	Female Enalapril (n=10)	Male Control (n=6)	Male Enalapril (n=6)	Female Control vs. Enalapril (p<0.05)	Male Control vs. Enalapril (p<0.05)	Control Female vs. Male (p<0.05)	Enalapril Female vs. Male (p<0.05)
Diastolic Ca²⁺ (nM)	219.4 ± 7.4	227.3 ± 7.6	300.7 ± 10.6	308.6 ± 17.3	NS	NS	Yes	Yes
Systolic Ca²⁺ (nM)	340.4 ± 9.7	354.5 ± 12.8	481.3 ± 23.8	490.5 ± 33.5	NS	NS	Yes	Yes
Ca²⁺ Amplitude (nM)	121.0 ± 7.1	127.2 ± 8.1	180.6 ± 16.1	181.8 ± 26.4	NS	NS	Yes	Yes
Velocity to Peak (µm/ms)	7.39 ± 0.48	7.09 ± 0.42	8.24 ± 0.67	8.61 ± 0.81	NS	NS	Yes	Yes
Velocity of Relaxation (µm/ms)	1.79 ± 0.15	1.58 ± 0.12	2.04 ± 0.14	2.18 ± 0.22	NS	NS	Yes	Yes
Time to Peak (ms)	16.67 ± 0.57	18.02 ± 0.45	22.47 ± 1.57	20.69 ± 0.91	NS	NS	Yes	Yes
Time of Relaxation (ms)	37.6 ± 3.0	44.0 ± 3.0	46.4 ± 4.9	42.9 ± 3.0	NS	NS	NS	NS

Values are mean ± SEM. 2-way ANOVA. NS=Not Significant. 'n' represents number of mice with 2-3 cells obtained from each mouse.

To determine whether enalapril influenced intracellular Ca^{2+} handling, Ca^{2+} transients were measured simultaneously with contraction in cells paced at 2 Hz. Figure 3.9 illustrates representative recordings of Ca^{2+} transients from myocytes from control mice and enalapril-treated mice of both sexes. These examples suggest that intracellular Ca^{2+} was not affected by enalapril, but was influenced by sex, with males having a larger Ca^{2+} transient amplitude and faster velocity. Mean data are shown in Figure 10 and 11. Parameters measured included diastolic and systolic Ca^{2+} (Fig 3.10A and 3.10B), Ca^{2+} transient amplitude, velocity to peak, velocity of decay, time to peak and time to decay of the Ca^{2+} transient (Fig 3.11A-E).

Although enalapril treatment affected myocyte contractile properties in females, the underlying Ca^{2+} transients were unaffected by drug treatment (Fig 3.11A-E). Male control and enalapril-treated animals also exhibited no change in intracellular Ca^{2+} handling (Fig 3.11A-E). There were however sex differences in Ca^{2+} homeostasis. Both control and enalapril-treated mice had higher levels of systolic and diastolic Ca^{2+} (Fig. 3.10A and 3.10 B) as well as larger, faster Ca^{2+} transients (Fig. 3.11A-C). Interestingly, the time to peak was higher in male cardiomyocytes when compared to females (Fig 3.11D), but there was no difference in the time of decay (Fig 3.11E). These mean values (\pm SEM) are presented in Table 3.8.

Chapter 4: Discussion

4.1 Overview of Key Findings

The first objective of this study was to determine if ACE inhibitor treatment would attenuate frailty in middle-aged male and female mice. FI scores from male and female mice on enalapril or control food were assessed at baseline, then again at monthly intervals. In females, the mean FI score from enalapril-treated mice was significantly lower when measured at the 3rd and 4th mos compared to controls. In males however, the mean FI scores from enalapril-treated mice were not lower than control values at any point throughout the study. In fact, at the 3rd mos, FI scores from control mice were significantly higher than enalapril-treated mice, although the scores were no longer different at 4 mos. These findings suggest that ACE inhibitor treatment in middle-aged mice attenuates frailty in females, but not males.

An additional objective of this study was to investigate if the attenuation of frailty occurs, at least in part, through changes in the cardiovascular system. In males, no attenuation of frailty was observed with ACE inhibitor treatment. In addition, there were no changes in blood pressure, *in vivo* cardiac structure or function, myocyte contraction or Ca²⁺ transients between control and enalapril-treated males. By contrast, in females, where ACE inhibitor treatment attenuated frailty, there were a number of differences in cardiomyocyte contraction between control and enalapril-treated mice. Although no changes in blood pressure and *in vivo* cardiac function were observed, cardiomyocytes from enalapril-treated females had larger and faster contractions when compared to controls. Interestingly, this change in contractile function was not reflected as changes in Ca²⁺ handling. The amplitude and velocity of Ca²⁺ transients was similar in control and

enalapril-treated animals. These results suggest that attenuation of frailty observed in female mice, may be mediated, in part, by enhanced contractile function at the level of the cardiomyocyte.

4.2 Enalapril attenuates frailty in middle-aged mice

The concept of frailty, which is a state of increased vulnerability to adverse health outcomes for people of the same age, was developed to explain the heterogeneity in clinical outcomes for older patients (Rockwood *et al.*, 1994). Frailty is a major health care problem, as frail individuals have higher mortality and use more health care services than fit people (Rockwood *et al.*, 2012). Little is known about interventions that may work to reduce or slow the onset of frailty. Studies done in older men and women show that ACE inhibitor treatment improves muscle function, or at the very least, prevents the decline in physical ability that is associated with age (Hutcheon *et al.*, 2002; Sumukadas *et al.*, 2007; Onder *et al.* 2002). It is important to note that although, some of these studies included male and female patients, sex differences in the patient's response to the treatment were not discussed (Hutcheon *et al.*, 2002; Sumukadas *et al.* 2007). In addition, the study by Onder *et al.* (2002) only included females. Similar results have been observed in a rodent model, where aged rats treated with an ACE inhibitor exhibited improved physical performance (Carter *et al.*, 2011; Habouzit *et al.*, 2009). However, neither of these studies used both sexes, with Carter *et al.* (2011) using male rats, and Habouzit *et al.* (2009) using female rats. Other studies have shown that knocking out the AT₁R prolonged the lifespan of male mice (Benigni *et al.*, 2009). As physical ability is an important component of frailty, this evidence suggests that chronic administration of ACE inhibitors may attenuate frailty. While it is important for pre-clinical and clinical

studies to include and discuss male and female responses to interventions, it is clear that most previous studies lack sex comparisons. The present study investigated ACE inhibitor treatment in both sexes to assess how the effects of the intervention differed between males and females.

With an established simple, non-invasive method of measuring frailty in a mouse model only being recently developed (Whitehead *et al.*, 2014; Feridooni *et al.*, 2015), very few studies have investigated how interventions could impact the development of frailty. Kane *et al.* (2015) were one of the first to look at how an intervention would affect an animal's frailty by investigating how caloric restriction and resveratrol treatment altered the onset of frailty in aged male and female mice. Caloric restriction began at 6 mos of age with animals remaining on the diet for the rest of the study. On the other hand, resveratrol treatment began at 18 mos of age, and continued for 6 mos. Interestingly, both interventions were associated with lower FI scores in males but not females (Kane *et al.*, 2015). Another study that utilized the mouse clinical FI investigated whether polypharmacy, which is common in older adults, altered frailty development over a 2 week period (Huizer-Pajkos *et al.*, 2015). Although no change in frailty was observed, the treatment period was extremely short and may not have been long enough for a change in frailty to develop. These preliminary studies provide evidence that frailty can be used as an outcome measure in interventional studies.

A major objective of the present study was to determine whether ACE inhibitor treatment could attenuate frailty in middle-aged mice, as well as to identify sex differences in frailty development. The results of this study showed that, in middle-aged females but not males, chronic administration of the ACE inhibitor enalapril attenuated

the development of frailty. Although this was not the expected outcome based on the previous studies using ACE inhibitor treatment in humans and rodents (Onder *et al.*, 2002; Hutcheon *et al.*, 2002; Habouzit *et al.*, 2009; Sumukadas *et al.*, 2007 ; Carter *et al.*, 2011), this could be partially attributable to the age of animals used in the present study. Previous work showing that ACE inhibitor treatment improved physical function used older patients with an average age above 70 years of age (Onder *et al.*, 2002; Hutcheon *et al.*, Sumukadas *et al.*, 2007) and aged rodents of 24 mos of age (Habouzit *et al.*, 2009; Carter *et al.*, 2011). By contrast, the present study used middle-aged mice that were approximately 12 mos at the end of the study. Older animals and people would be expected to have accumulated more deficits and therefore present with higher FI scores. If ACE inhibitor treatment had been extended for a longer time, it is possible attenuation of frailty might have occurred in the enalapril-treated male mice. In addition, a previous interventional study using resveratrol in 18 mos old male and female mice noted a difference in frailty after 6 mos of treatment, but only in males (Kane *et al.*, 2015). This suggests that a longer treatment period may be needed for ACE inhibitors to have noticeable effects in males.

In addition to the age of the animals used in the study, the lack of effect of enalapril treatment in male mice could be due to sex differences in responses to the drug. Information on sex differences in drug responses however is limited. A review by Seeland & Regitz-Zagrosek (2012) noted that a number of multicentre studies have reported that ACE inhibitor treatment is associated with a smaller reduction in mortality in woman than in men. On the other hand, these studies contained a small percentage of women compared to men and, because of that, were not powered to detect sex

differences. Other studies that did include a larger sample of women did not include detailed data on sex (Seeland & Regitz-Zagrosek 2012), making it difficult to evaluate sex differences in responses to ACE inhibitors. These conflicting data emphasize the importance of using both male and female subjects in both clinical and experimental studies. The results of the present study are compatible with the idea that there are male-female differences in responsiveness to ACE inhibitors and further studies are warranted.

In addition to studying the effect of enalapril on frailty development, male-female differences in the development of frailty were also observed. Previous studies that used the clinical frailty index to quantify frailty in male and female mice have reported no sex differences in frailty values of animals of the same age, although very few male animals were investigated (Whitehead *et al.*, 2014). Parks *et al.* (2012) found similar results with a different FI tool, with frailty values in males and females being similar, at least in a small sample. In this study, with a larger group of animals, males had higher baseline FI scores than females. Although control male FI scores were not different from female scores throughout the rest of the study, enalapril-treated males started with and continued to have higher FI scores compared to females. This differs from reports of sex differences in the clinical literature, where women have higher frailty scores than men (Puts *et al.*, 2005). In this study, the sample size for males was smaller than the females, which could potentially account for the results. Clearly, additional studies exploring sex differences in frailty in pre-clinical models should be conducted.

4.3 Effects of enalapril on blood pressure

ACE inhibitors are drugs that are classically used to reduce blood pressure in hypertensive patients through decreasing blood volume and reducing vasoconstriction.

This study aimed to assess if attenuation of frailty was accompanied by changes in changes in blood pressure. Because of this, it was important to track blood pressure change to see if frailty attenuation was partially due to a reduction in blood pressure. Results showed that ACE inhibitor treatment had no effect on diastolic or systolic blood pressure. Although ACE inhibitors play an important role in reducing blood pressure in hypertensive individuals (Garg & Yusuf, 1995; Flather *et al.*, 2000), this study used animals that were normotensive. Thus, the lack of effect on blood pressure was not surprising. This study is novel in its reporting of blood pressure in normotensive mice receiving ACE inhibitor treatment.

4.4 Effects of enalapril on *in vivo* cardiac function

As an individual ages, the heart undergoes a number of physical and functional changes (Keller & Howlett, 2016). These changes accumulate over time and many of them can be observed at middle age. Some of the changes observed include thickening of the left ventricular walls (Gerstenblith *et al.*, 1977), reduced systolic function (Claessens *et al.*, 2007) and slowed relaxation (Lakatta & Levy, 2003). In fact, Brenner *et al.* (2001) suggest that changes in diastolic function can be observed as early as 30 years of age. These changes in heart structure and function are also reflected at the cellular level. Indeed, age is associated with an increase in myocyte hypertrophy and fibrosis (Olivetti *et al.*, 1991; Olivetti *et al.*, 1995). One mechanism thought to contribute to both hypertrophy and fibrosis is an increased concentration of circulating Ang II (Dai *et al.*, 2009). Ang II can act directly on myocytes and fibroblasts through AT₁R receptors present on these cells and chronic AT₁R activation can result in excessive myocyte growth and collagen deposition (Rozenkranz 2004). It is important to note that many of

the changes observed in humans are mimicked in rodent models (Lindsey *et al.*, 2005; Dai *et al.*, 2012; Lau *et al.*, 2013; Feridooni *et al.*, 2015), making them an appropriate model for investigating changes in cardiac structure and function.

To identify whether enalapril affected adverse cardiac remodelling and improved cardiac function, *in vivo* cardiac morphology and function were assessed with echocardiography after treatment with enalapril. Results showed that enalapril had no effect on cardiac morphology in males or females. It also had no effect on functional parameters such as systolic and diastolic function in either sex. Thus, enalapril treatment did not affect cardiac structure or function, measured via echocardiography. There were, however, sex differences. Untreated males had a thicker interventricular septum compared to females. This may be a result of the relatively small male sample size; a larger number of experiments could clarify this.

In the present study, heart rate differed between males and females. This agrees with previous reports that heart rate differs between the sexes, with studies reporting that women have a higher resting heart rate compared to men (Taneja *et al.*, 2001). The present study also observed higher fractional shortening and, although not significant, increased ejection fraction in males compared to females. Previous studies evaluating cardiac function in age-matched 18 week old male and female rats report smaller ejection fraction and less fractional shortening in females when measured in a working heart model (Parks *et al.*, 2013). In contrast, one previous study that measured fractional shortening and ejection fraction using echocardiography reported no sex differences in mice (Stypmann *et al.*, 2006). These results however, could be misleading if the Stypmann *et al.* (2006) used a large dose of anesthetic. Anesthetic used during

echocardiography depresses heart rate (Parks *et al.*, 2013) which could potentially mask any sex differences present.

4.5 Changes in ventricular myocyte contractile function in enalapril-treated and control animals

Although enalapril treatment was not associated with changes in *in vivo* measurements, there could still be changes in myocyte structure and function. Although there were no differences in the structure of isolated myocytes between control and enalapril-treated animals, enalapril treatment did result in significant changes in cellular function, at least in females. This study reports the novel observation that contractions in female enalapril-treated animals were significantly larger and faster than controls. By contrast, there was no difference in contractile function between control and enalapril-treated males. The fact that frailty was attenuated in females, but not males, and that changes in contractile function were only observed in females, suggests that attenuation of frailty was due, at least in part, to improved cardiomyocyte function. Although frailty attenuation in males was not observed in this study, were the study to have a longer treatment period, or be repeated in animals of a more advanced in age, a difference may emerge.

The fact that ACE inhibitor treatment had no effect on myocyte morphology, but enhanced contractile function, suggests that ACE inhibitors may attenuate frailty through improved myocyte function. As mentioned, Ang II can lead to hypertrophy through excessive growth (Rozenkranz 2004), but chronic exposure to Ang II can also damage the heart by increasing cellular and mitochondrial ROS (Dai *et al.*, 2012). Reducing Ang II formation may reduce formation of ROS within myocytes, which could potentially

improve myocyte contractile function in female enalapril-treated mice. In addition, the effect of Ang II on the ageing heart appear to differ between the sexes. For example, female mice with elevated intra-cardiac levels of Ang II exhibit age dependent contractile depression while male mice do not (Mellor et al., 2014). This may explain why female controls had smaller and slower contractions compared to males and why ACE inhibitors were effective in improving contractions in males but not females.

The amplitudes of cardiac contractions are dependent both on myofilaments and the magnitude of the rise in intracellular Ca^{2+} . Because of this relationship, it was expected that Ca^{2+} transients would be larger in cells from female enalapril-treated animals. However, this was not the case, as the improved myocyte contractile function was not associated with changes in Ca^{2+} transients. This suggests that the improvement in myocyte contractile function was mediated through another mechanism, most likely changes in the sensitivity of the myofilaments to Ca^{2+} . Myofilament sensitivity can be influenced by phosphorylation, as PKC mediated phosphorylation of troponin is associated with a reduction in myofilament contractility (Belin *et al.*, 2007). Seeing as AT_1R signalling can activate PKC, the reduction in Ang II in enalapril-treated mice could reduce PKC mediated phosphorylation of myofilaments, increasing their Ca^{2+} sensitivity.

As expected, there were also no differences in Ca^{2+} transients between enalapril-treated and control male cells. Interestingly, the study by Mellor et al. (2014) that observed age-dependent contractile depression in cardiomyocytes from aged females with elevated Ang II, noted that there were changes in Ca^{2+} handling, but myofilament Ca^{2+} sensitivity was maintained (Mellor *et al.*, 2014). These observations suggest that further studies are needed on the effects of ACE inhibitor treatment on cardiomyocyte

function in males and females to further investigate the mechanisms behind these sex differences.

Some notable sex differences were observed in both myocyte contractile function and intracellular Ca^{2+} handling. Males had larger and faster contractions compared to females, and this was reflected in the Ca^{2+} transients. Males also had higher intracellular diastolic and systolic Ca^{2+} concentrations compared to females, as well as higher amplitudes and velocities of Ca^{2+} transients. This generally reflects what is found in the literature (Parks *et al.*, 2013). Although some studies using field stimulation found no sex differences in peak contraction in rat myocytes, these experiments were performed at room temperature instead of the physiologically relevant temperature of 37°C and were paced at frequencies well below the animal's physiological heart rate (Parks *et al.*, 2013). When other experiments used more physiologically relevant stimulation frequencies and temperature, female rats exhibited significantly smaller and slower contractions compared to age-matched males (Parks *et al.*, 2013). This sex-difference in contractile function is associated with differences in the size of the underlying Ca^{2+} transients. A number of previous studies using field stimulation or voltage clamp techniques reported that cardiomyocytes obtained from male rats had larger Ca^{2+} amplitudes compared to females (Parks *et al.*, 2013). Diastolic Ca^{2+} levels have also been reported to be lower in female rats than males (Parks *et al.*, 2013). Lastly, the decay of Ca^{2+} transients in female rats is significantly slower when compared to males (Parks *et al.*, 2013). This had been suggested to be due to a difference in the rate of Ca^{2+} reuptake into the SR, with slower reuptake in female cells when compared to males (Parks *et al.*, 2013). The fact that the results from the present study reflect the established male-female differences in myocyte

contractile function and Ca^{2+} transients attests to the quality and validity of the techniques used here.

4.6 Limitations

To investigate if the attenuation of frailty was associated with structural and functional changes in the heart, echocardiography was used to assess *in vivo* function, and field stimulation to measure myocyte function. This study observed a number of differences in myocyte contractile function in female enalapril-treated animals that did not translate into changes in *in vivo* function. In addition to evaluating the effect of enalapril treatment on cardiac function, male-female differences in cardiac structure and function were also measured with these techniques. Previous studies in rats observed sex differences in cardiac functional parameters when using a working heart model, with females exhibiting a smaller ejection fraction and fractional shortening (Parks *et al.*, 2013). The present study reflected similar findings, with fractional shortening being significantly lower in females compared to males. However, ejection fraction was not significantly between the sexes. Echocardiography in mice requires the use of anesthetic, which causes a decrease in heart rate. This could potentially mask differences in cardiac function that may be present between sexes and within treatment groups. In addition, echocardiography on a mouse requires a great deal of precision due to the small size of their heart. Small movements of the transducer could potentially affect structural and functional measurements.

Although echocardiography is an important tool for measuring structural features of the heart, to study heart function in the absence of anesthetic, whole hearts could be isolated from male and female enalapril-treated and control mice and studies using the

Langendorff-perfused heart preparation. This method involves retrograde perfusion of the heart through the aorta, allowing heart to be studied without the influence of other organ systems (Bell *et al.*, 2011). Through the insertion of a balloon into the left ventricle, myocardial contractility and left ventricular function can be assessed. The advantage of this method is that the myocardium is intact and myocytes are still subjected to a hemodynamic load, something that is lost when evaluating myocyte function in isolated cardiomyocytes.

Lastly, there was a difference in sample size and housing conditions between males and females. This was due to time constraints and the length of the drug treatment. Increasing the sample size could clarify whether some of the trends observed in the present study, such as the increase in ejection fraction in males, are significant or if this was due to the variance caused by the small sample. Males were also housed singly, compared to females who were housed in groups. This was due to the tendency of male mice to fight when housed in groups. As no sex differences in frailty in mice have been recorded (Whitehead *et al.*, 2014), this variation in living environment could contribute to the male-female differences observed in this study. Clearly, additional studies of sex differences in frailty are warranted.

4.7 Summary

The present study demonstrated that, in middle-aged female mice, enalapril treatment attenuated frailty. This attenuation was not associated with changes in blood pressure or *in vivo* cardiac structure and function. At the cellular level, enalapril-treatment was associated with changes in myocyte contractile function, but no changes in Ca²⁺ transient properties were observed. Frailty attenuation was not observed in males, and there were

no differences in blood pressure, *in vivo* and myocyte contractile function and Ca^{2+} handling. This study demonstrated that the FI can successfully be used in interventional studies. Within this study, a number of sex differences in frailty development and cardiovascular parameters were observed. With frailty, males started with a higher FI score at 9 mos of age compared to females, and in enalapril-treated animals, scores remained higher throughout the study. Fractional shortening was larger in male controls when compared to females. On a cellular level, isolated cardiomyocytes from males had larger and faster contractions, with parallel changes in Ca^{2+} transients. These results reflect sex-differences that have been seen in previous rodent studies. The results of this study emphasize the importance of using both male and female animals in pre-clinical studies.

4.8 Future Work

This work was the first study to investigate how ACE inhibitor treatment affected frailty in middle-aged mice. The results suggest that, in females, treatment with ACE inhibitors may attenuate frailty, and this may be mediated, in part, by preventing some of the age-associated changes in cardiomyocytes. Previous studies in human and animal models show that frailty is correlated with age (Parks *et al.*, 2012; Whitehead *et al.*, 2014), meaning, as an individual ages, so does their likelihood to accumulate deficits. Because of this, it would be interesting to investigate if a longer treatment period would continue to attenuate frailty in females, and if attenuation of frailty occurs in older males as they begin to accumulate more deficits. This intervention could also be used in a much older cohort to see if ACE inhibitors attenuate, or even reverse, frailty in animals with higher initial FI scores.

This study identified changes in cardiomyocyte function in females treated with enalapril, but this was not associated with changes in intracellular Ca^{2+} handling. This suggests that enalapril may desensitize the myofilament to Ca^{2+} . To identify if this is the case, some hearts from control and enalapril-treated animals could be isolated and used for myofilament Ca^{2+} sensitivity studies. Although field stimulation experiments allowed for overall intracellular Ca^{2+} to be measured, potential changes in the L-type Ca^{2+} current could not be identified, as only a small amount of extracellular Ca^{2+} contributes to the overall intracellular Ca^{2+} transient. To identify if enalapril administration influences the Ca^{2+} current patch clamp experiments could be performed.

Although this study identified some potential mechanisms for frailty attenuation, it would be interesting to explore additional mechanisms that could be involved. Frailty is thought to be associated with an increase in inflammation (Afilalo *et al.*, 2014), and Ang II has pro-inflammatory actions (Phillips & Kagiya, 2002). Administering ACE inhibitors and decreasing the amount of Ang II production could potentially result in lower levels of pro-inflammatory cytokines in enalapril-treated animals. This could be investigated by obtaining a blood sample when animals are euthanized and testing them for concentrations of inflammatory cytokines. Still, the novel observations reported here suggest that the mouse FI is a powerful new tool that can be used to investigate potential beneficial effects of drugs in the setting of frailty.

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