

THE CLINICAL ASSOCIATIONS AND PHYSIOLOGICAL MECHANISMS LINKING
BONE AND CARDIAC HEALTH

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by
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University of Missouri-Kansas City, 2024

ABSTRACT

Osteoporosis and cardiovascular disease represent two significant clinical burdens worldwide, which have been found to be clinically intertwined. The skeleton is highly sensitive to mechanical stimuli with osteocytes/osteoblasts sensing mechanical strain and activating bone remodeling. Bone is multifaceted exhibiting endocrine/paracrine functions and connections to the central nervous system through which it modulates a number of tissues including itself. During osteoporosis, bone cellular network integrity and the anabolic response to loading is compromised. Thus, the clinical associations of cardiovascular and skeletal health may be explained by crosstalk via bone-humoral and/or bone-neural mechanisms. To investigate the links among bone and heart health clinically, the impact of osteoporosis on cardiovascular disease patient outcomes was examined utilizing data from Cerner Health Facts. Next, cellular and animal studies were undertaken to determine the effects of bone cell-secreted molecules

and *in vivo* bone mechanical loading on cardiac physiology. The Health Facts analysis revealed a significant association of history of osteoporosis with prolonged length of hospital stay, elevated NT-proBNP levels and greater mortality in patients admitted for cardiovascular disease. Men with a history of osteoporosis displayed worse overall outcomes for cardiovascular disease compared to women including remaining hospitalized for 1.02 days longer and having 2.13-fold higher mortality. *In vitro* studies showed that administration of conditioned media from bone cells exposed to fluid flow shear stress enhanced cardiomyocyte cell viability during CoCl₂ treatment (p<0.05) and hypoxia/reoxygenation (p<0.05). *In vivo*, tibia mechanical loading in adult mice acutely and transiently lowered heart rate (p<0.01) and enhanced heart rate variability (p<0.01), which was mediated by neuronal afferents in the hindlimb and downregulated sympathetic nervous system tone. This cardiac response to loading was largely diminished by middle age. Daily tibia loading over three weeks in adult mice resulted in significantly lower resting heart rate (p<0.05) and higher heart rate variability (p<0.05) compared to non-loaded mice. These findings suggest that endocrine/neural pathways associated with bone mechanical loading may regulate cardiac physiology and link bone and cardiovascular health. Investigation of bone-heart crosstalk networks could aid in identifying novel therapeutic targets to improve standard of treatment for musculoskeletal and cardiovascular diseases.

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CONTENTS

ABSTRACT..... iii

LIST OF ILLUSTRATIONS..... ix

LIST OF TABLES xi

LIST OF ABBREVIATIONS xii

ACKNOWLEDGEMENTS..... xiv

Chapter

1. INTRODUCTION..... 1

2. REVIEW OF LITERATURE..... 3

 Bone 3

 Ultrastructure 3

 Mechanical Loading..... 4

 Endocrine Functions of Bone..... 6

 Aging..... 8

 Heart..... 9

 Physiology and Pathophysiology 9

 Evidence of Bone-Heart Crosstalk..... 11

 Specific Aims and Hypotheses 13

3. METHODOLOGY 14

 Clinical Studies 14

 Retrospective Cohort Study 14

Database.....	14
Subjects.....	15
Outcome Measures.....	15
Statistical Analysis for Clinical Studies.....	16
<i>In Vitro</i> Studies	17
H9C2 Cell Culture	17
RNA Extraction and Real-Time RT-PCR.....	17
Generation of Bone Conditioned Media (CM).....	18
Cell Viability Assay.....	19
<i>Ex Vivo</i> Heart Contractility.....	20
Statistical Analysis for <i>In Vitro</i> Studies.....	21
Animal Studies.....	21
Animals.....	21
Tibia Axial Mechanical Loading.....	22
<i>In Vivo</i> Electrocardiogram (ECG)	23
Injection of Neural Inhibitors.....	24
Strain Gauging	24
Statistical Analysis for Animal Studies	25
4. RESULTS.....	26
Clinical Studies	26
<i>In Vitro</i> Studies	40
Animal Studies.....	47

5. DISCUSSION	66
Clinical Studies	67
<i>In Vitro</i> Studies	73
Animal Studies	79
Conclusion	88
REFERENCES	93
VITA.....	107

LIST OF ILLUSTRATIONS

Figure	Page
1. Longer average CVD-related LOS in those with a history of osteoporosis	30
2. History of osteoporosis in females is an independent predictor for prolonged LOS using logistic regression.....	35
3. History of osteoporosis in males is an independent predictor for prolonged LOS using logistic regression.....	36
4. Interaction effect between CVD subtype and history of osteoporosis for LOS	37
5. Increased NT-proBNP levels in CVD patients with a history of osteoporosis.....	38
6. Increased mortality during CVD-related hospital stay in males with a history of osteoporosis	39
7. Cardiac phenotype of H9C2 cells is induced during differentiation	41
8. Bone CM protects H9C2 cells against cell death during long-term CoCl ₂ treatment	42
9. Bone CM protects H9C2 cells against cell death during long-term hypoxia/reoxygenation.....	44
10. Bone CM (0.75%) does not protect against <i>ex vivo</i> cardiac contractility impairment from acute ischemia/reperfusion injury.....	46
11. Bone CM (7.5%) does not protect against <i>ex vivo</i> cardiac contractility impairment from acute ischemia/reperfusion injury.....	47
12. Heart rate decreases and HRV increases in response to acute tibia mechanical loading in adult mice	49
13. ECG intervals are not affected during and after acute tibia mechanical loading in adult mice	51

14.	Effect of mechanical loading at various magnitudes on heart rate and HRV in adult 2-6 month old mice.....	53
15.	Heart rate and HRV responses to acute tibia mechanical loading are diminished in middle age mice	55
16.	ECG intervals are not affected in response to acute tibia mechanical loading in middle age mice	56
17.	The heart rate changes in response to mechanical loading are mediated by afferent neurons in the hindlimb and reduced SNS tone	58
18.	Resting heart rate decreases and HRV increases in adult mice during three-weeks of tibia loading	60
19.	ECG intervals are altered during three-weeks of tibia loading in adult mice.....	62
20.	Heart and skeletal muscle weights are not affected with three weeks of tibia loading	63
21.	<i>Ex vivo</i> skeletal muscle contractile force is unaffected by three-weeks of tibia loading	65
22.	Clinical association of history of osteoporosis with CVD-related hospital outcomes.....	73
23.	Cardioprotective factors are released by bone cells mechanically loaded <i>in vitro</i>	79
24.	Mechanistic overview of the impact of tibia mechanical loading on cardiac function <i>in vivo</i>	87
25.	Proposed endocrine and neural based mechanisms linking bone mechanical loading and cardiovascular health	92

LIST OF TABLES

Table	Page
1. Demographic characteristics and covariates for the study population	27
2. Demographic characteristics and covariates for the study population stratified by subgroups of CVD	28
3. History of osteoporosis in females is an independent predictor for LOS (continuous) using multiple linear regression.....	32
4. History of osteoporosis in males is an independent predictor for LOS (continuous) using multiple linear regression.....	33

LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

BMI – Body mass index

BMP – Bone morphogenic protein

BNP – Brain natriuretic peptide

CC index – Charlson comorbidity index

CD – Cardiac dysrhythmia

CI – Confidence interval

CM – Cardiomyopathy (clinical study), conditioned media (*in vitro* study)

CNS – Central nervous system

CV – Coefficient of variation

CVD – Cardiovascular disease

DMEM – Dulbecco's modified Eagle medium

ECG - Electrocardiogram

EDL – Extensor digitorum longus

FFSS – Fluid flow shear stress

FGF23 – Fibroblast growth factor 23

HF – Heart failure

H/R – Hypoxia/reoxygenation

HRV – Heart rate variability

HT – Hypertension & hypertensive-related heart disease

HW/BW – Heart weight to body weight ratio

ICD9/10 – International classification of disease, 9th/10th revision

IHD – Ischemic heart disease

IP – Intraperitoneal

I/R – Ischemia/reperfusion

LOS – Length of hospital stay

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW/BW – Muscle weight to body weight ratio

NT-proBNP – Amino terminal-pro-brain natriuretic peptide

PGE₂ – Prostaglandin E₂

PNS – Parasympathetic nervous system

RMSSD – Root mean square of successive differences

RT-PCR – Reverse transcription-polymerase chain reaction

SD – Standard deviation

SDRR – Standard deviation of the RR interval

SNS – Sympathetic nervous system

SOL – Soleus

Wnt – Wntless-related integration site

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CHAPTER 1

INTRODUCTION

Our understanding of the human body has evolved over the years as we continue to discover new processes and avenues of potential treatment for disease. Organ systems have long been regarded as a collection of relatively independent units operating in isolation for a specialized task. However, it is becoming clearer that organ systems, particularly the musculoskeletal system, have a larger and more involved role in the systemic regulation of other organs that maintain the health of the individual. More recently, inter-organ communication at the endocrine/paracrine level has been identified to be important for organ system homeostasis via the regulated release of hormones and/or metabolites from source organs into the bloodstream or surrounding tissue. In particular, the musculoskeletal system is now accepted as a rich reservoir of these hormones released specifically during periods of physical activity (e.g. muscle contraction, bone loading) that “talk” to other tissues and beneficially affect systemic health ¹.

In bone, recent discoveries have highlighted direct actions of bone-secreted hormones upon many different tissues including liver, kidney, brain, and fat ². Bone has also been shown to influence skeletal muscle development and function and vice-versa. Additionally, bone is highly innervated and communicates with the brain and autonomic nervous system to regulate

its structure in response to mechanical loading, suggesting possible interplay with other organs under autonomic control. While these communication pathways may also extend to the heart, studies investigating the relationship of bone and heart health remain mostly limited to clinical associations. For instance, osteoporosis has been linked to cardiovascular disease in humans and serum concentrations of bone-derived hormones correlate with the progression and severity of cardiovascular disease ^{3, 4}. Additionally, there exists a large gap in knowledge regarding how these emerging crosstalk networks govern biological aging, contribute to the progression of disease states and influence adaptation to exercise. Indeed, this novel concept of inter-organ crosstalk represents a shift in the paradigm of knowledge of systems biology and is a promising new field of therapeutic research.

CHAPTER 2

LITERATURE REVIEW

Bone

Ultrastructure

Skeletal bone is a highly mineralized tissue consisting of collagenous networks cemented by rigid hydroxyapatite crystal which provides the structural framework of the body. Within this rough environment of bone there exists several resident bone cell types including osteoblasts, osteocytes, and osteoclasts. Osteoblasts and osteoclasts are characterized functionally as the main orchestrators of *de novo* bone formation and resorption of existing bone, respectively ⁵. However, the most abundant cell type in bone, the osteocyte (>90%), has traditionally been characterized by its physical location of being embedded deep inside of mineralized bone. This is mainly due to the historically limited insight into its true functions owing to the difficulty in accessing and studying this particular cell type. Osteocytes are derived from their precursor cells, osteoblasts, which are located mainly on the bone periosteal and endosteal surfaces. During the process toward terminal differentiation into an osteocyte, osteoblasts undergo changes to their gene expression profiles which allow them to completely embed themselves within the mineralized bone matrix ^{6,7}.

Mature osteocytes are embedded individually inside of empty cavities termed lacunae. They may appear to be solitary entities of bone. Each lacuna, however, is connected via a series of small fluid-filled canals called canaliculi. This forms a complex network by which osteocytes are linked to the bone surface and receive oxygen and nutrients from the vasculature⁸. This network is known as the lacunocanalicular system and allows the rigid bone environment to become a highly vascularized organ and is essential for osteocyte viability and normal function. Intriguingly, it has been shown through tracer studies that molecules as large as 70 kDa can readily infiltrate bone from the circulation and be transported throughout the lacunocanalicular system⁹. Osteocytes also express specialized proteins, such as connexin 43, to aid in cell to cell communication through gap junctions and hemichannels¹⁰. In addition, osteocytes form numerous cytoplasmic processes, or dendrites, that can extend through the lacunocanalicular system and contact neighboring osteocytes or the bone marrow space¹¹. This suggests a role for a cell-to-cell communication network in bone homeostasis.

Mechanical Loading

Although bone cells are locked inside of the mineralized environment of bone, they are by no means passive cells. Over a century ago, Julius Wolff first recorded the observation that bone has the remarkable ability to adapt to mechanical forces by altering its overall structure and mass¹². Osteocytes and osteoblasts both play pivotal roles in the sensation of mechanical strain and transduction of this signal into a bone formation/remodeling response, termed mechanotransduction¹³⁻¹⁵. Applied load in bone signals bone formation and remodeling, or in the case of lack of load, leads to bone degradation¹³. While the exact mechanisms of

mechanosensation in osteocytes/osteoblasts remain to be fully elucidated, it is thought that loading on bone leads to deformations in the bone microenvironment and increases flow of interstitial fluid throughout the lacunocanalicular system. This translates into increased fluid flow shear stresses generated around the osteocyte cell body¹⁶. Although it is unknown which cellular structure senses loading, primary cilia¹⁷ and membrane calcium channels¹⁸ have been proposed. Following loading, a variety of changes occur inside the osteocyte including increases in intracellular calcium, nitric oxide, ATP, prostaglandin E₂ (PGE₂) and activation of the Wnt/ β -catenin pathway which further facilitates bone anabolic remodeling. PGE₂ has been found to be a particularly important signaling molecule in the bone anabolic response to mechanical loading¹⁹. PGE₂ is released in large quantities from osteocytes into the surrounding bone matrix where it facilitates the formation of new bone and signals to osteoblasts and osteoclasts at the bone surface in an autocrine/paracrine manner^{20, 21}. There is evidence that the actions of bone produced PGE₂ may also extend to surrounding tissues such as skeletal muscle. Utilizing *in vitro* models of skeletal muscle development, PGE₂ has been found to promote the formation of new muscle fibers as well as increase the proliferation of muscle precursor cells^{22, 23}.

More recently, nerve fibers within bone have also been shown to be a necessary component for the anabolic response to mechanical load. Bone is a highly innervated tissue harboring abundant nerve fibers that typically follow the blood vessels of the Haversian and Volkman's canals and further penetrate into the periosteum, mineralized cortical bone matrix and bone marrow²⁴. Upon mechanical loading, studies have shown that sensory peripheral nerve fibers are activated, due to the binding of secreted PGE₂ and neural growth factor, among

others, from osteoblasts/osteocytes and relay of this afferent signal to the ventromedial hypothalamus in the brain^{15,25}. After signaling in the brain, it has been demonstrated that nerve signals are then relayed back to the bone through a neural efferent pathway involving the autonomic nervous system, composed of the parasympathetic and sympathetic pathways, to control of bone mass²⁵. Neural components of the sympathetic nervous system (SNS) are abundantly localized within bone tissue²⁶. Activation of β -adrenergic receptors in bone tissue leads to catabolic effects^{27,28} and inhibition of SNS leads to improved bone formation^{25,28-30}. Additionally, parasympathetic nervous system (PNS) innervation is highly present in skeletal bone, and activation of acetylcholine receptors is associated with enhanced bone formation and downregulation of resorption^{31,32}. In addition to its role in bone remodeling, the autonomic nervous system works to maintain the homeostatic balance of nearly all major bodily organ systems through relative changes in SNS and PNS tone dictated by both external and internal stimuli. These autonomic reflexes may result in widescale changes affecting many organs or may induce a more targeted response. Thus, the connection of bone with autonomic nervous system tone is suggestive of possible interplay with other organ processes under autonomic control such as cardiac function.

Endocrine Functions of Bone

While the dynamic nature of bone cells has remained obscure to investigators for many decades, the characterization of bone as having an endocrine function is not an entirely novel concept. Since the early 2000's bone has been known as a master regulator of serum phosphate retention in the kidney via the actions of the bone-secreted protein, fibroblast growth factor 23

(FGF23). FGF23 induces renal excretion of phosphate by down regulating sodium-phosphate co-transporters in the proximal renal tubule and reducing gut absorption of phosphate, indirectly, through reduction of active vitamin D^{33,34}. Circulating FGF23 also signals in the parathyroid gland to inhibit parathyroid hormone production³⁵. Apart from FGF23, bone is also known as the source of numerous growth factors including transforming growth factor beta, insulin-like growth factors and bone morphogenic proteins. These factors play a role in bone remodeling³⁶ but can also lead to effects in muscle when in excess³⁷. Bone also forms an endocrine communication pathway with the brain through secretion of lipocalin 2 which transverse the blood-brain barrier and activates melanocortin 4 receptor to downregulate appetite³⁸. More recently, increased attention has been given to osteocalcin, a protein produced by osteoblasts/osteocytes which is stored within the mineralized matrix of bone and is released into the circulation in its undercarboxylated form³⁹. Osteocalcin has roles in bone remodeling and has been found to regulate metabolism in a variety of organ systems, such as stimulating insulin expression in the pancreas and increasing glucose uptake and metabolism in fat and muscle⁴⁰. The metabolic actions of osteocalcin make it an attractive candidate for treating metabolic disease. Hence, osteocalcin has been tested in metabolic disease models and has been found to be effective in preventing type 2 diabetes and obesity in mice fed a high-fat diet⁴¹. Maternal osteocalcin derived from the skeleton has been demonstrated to cross the placenta and contribute to the fetal brain development of offspring⁴². Interestingly, recent observations have found that blood levels of osteocalcin increase abruptly during endurance exercise and decline over the course of aging in both humans and rodents⁴³. Injections of mice with recombinant osteocalcin restored exercise capacity of old mice to that of young mice and prevented sarcopenia, the aging related decrease in muscle mass and strength⁴³. Osteocalcin

also appears to regulate heart rate in mice during periods of acute stress via the autonomic nervous system ⁴⁴. Therefore, there is increasing evidence of the endocrine role of bone and its implications for systemic regulation, exercise adaptation and the aging process.

Aging

The age demographics of our society are quickly shifting towards that of an older population. The U.S. Census Bureau anticipates that for the first time people aged 65 years and older will outnumber those less than 18 years old by the year 2035 ⁴⁵. As the aging process results in the functional decline of many organ systems, disease prevalence within the population and the resulting economic burden will also continue to rise in the coming decades, necessitating the development of novel treatments for common diseases. The consequences of aging-related diseases are also compounded by lack of exercise, as it has been estimated that health care costs attributed to physical inactivity alone numbered over \$50 billion internationally in 2013 ⁴⁶. Osteoporosis, characterized by the decrease in structural quality and strength of bone, represents a significant clinical problem that is experienced with aging. Osteoporosis afflicts over 200 million individuals worldwide and is associated with increased falls and fracture risk. Interestingly, osteoporosis and sarcopenia, the loss of muscle mass and strength with age, have been referred to as twin diseases as they tend to develop concomitantly in the same individual suggesting a common etiology and potential treatment strategy ⁴⁷. The burden of musculoskeletal disorders is staggering, affecting up to 1 in 2 individuals worldwide and is the leading cause of disability ⁴⁸. In addition to societal burden of musculoskeletal disorder, cardiovascular disease is a major cause of age-related mortality worldwide even

considering the progress made to cardiovascular disease treatment and prevention measures ⁴⁹. Intriguingly, osteoporosis is highly associated with the development of cardiovascular disease, even when controlling for traditional risk factors including age, body mass index, hypertension and other comorbidities ⁵⁰⁻⁵². Clearly there exists a gap in the understanding and treatment of musculoskeletal and cardiovascular diseases during aging which may be bridged by looking more closely at the endocrine/neural signaling pathways between bone and heart.

Heart

Physiology and Pathophysiology

Similar to musculoskeletal tissue, heart muscle has the ability to remodel and alter functional output in order to meet physiological demands. The adult heart consists of terminally differentiated muscle cells, called cardiomyocytes, which make up the contractile tissue of the atrial and ventricular chambers. Cardiomyocytes express contractile proteins and contract and generate force in a synchronous manner in order to pump and transport blood throughout the body. The intrinsic pace-making ability of the heart originates from a specialized unit of cells within the atria known as the sinoatrial node. Heart rate can be further modulated by the sympathetic and parasympathetic pathways of the autonomic nervous system and is also under the control of endocrine signaling of epinephrine from the adrenal glands. Pathological dysregulation of the intrinsic pace-making of the heart is manifested in the form of cardiac arrhythmias that may originate from the atrial or ventricular portions of the heart. Atrial fibrillation is the most common type of arrhythmia seen in patients and is associated

with subsequent stroke and heart failure ⁵³. Ventricular arrhythmias include premature ventricular contraction, ventricular fibrillation and ventricular tachycardia, which if left untreated may lead to sudden cardiac death ⁵⁴.

Cardiac remodeling may occur under chronic physiological or pathological stimuli that is mediated through distinct cell-signaling pathways ⁵⁵. In response to endurance exercise, the heart adapts by increasing the size of ventricular chamber muscle through growth that is eccentric, or around the outside of the heart. This process is known as physiological hypertrophy and results in increased contractile force and cardiac output ⁵⁶. Conversely, concentric pathological hypertrophy is characterized by the inward growth of heart, tissue fibrosis, and ultimately reduced cardiac output due to smaller chamber size and stiffness ⁵⁶. Concentric pathological cardiac hypertrophy can lead to heart failure with preserved ejection fraction, known to occur with aging, obesity, type 2 diabetes mellitus, and chronic kidney disease ^{55, 57, 58}. Other types of pathological structural remodeling of the ventricles may occur such as in dilated cardiomyopathy wherein ventricular dilation and systolic dysfunction are present in the absence of concentric thickening of the muscle and is a major predeterminant for heart failure with reduced ejection fraction and cardiac transplantation ⁵⁹.

Lastly, the coronary blood vessels of the heart perfuse the myocardium with oxygen rich blood necessary for the high energy demand of sustained contractile activity. In ischemic heart disease these arteries may become narrowed or occluded due to the formation of atherosclerotic plaques on the interior surfaces leading to an acute or chronic environment of low blood/oxygen supply in the myocardium contributing to cardiomyocyte death and cardiac dysfunction.

Evidence of Bone-Heart Crosstalk

Anatomically the heart is spatially separated from the skeleton but given that bone can secrete hormonal factors into the circulation and both bone and the heart share neural connections with the autonomic nervous system, the heart seems a likely target of such crosstalk mechanisms. In fact, we have already begun to uncover some of these pathways. The pathophysiology of chronic kidney disease represents a special case of a recently discovered link between dysregulated bone endocrine signaling via bone derived FGF23 and heart dysfunction. Patients with kidney disease tend to have high levels of serum FGF23 which continue to rise throughout the progression of declining renal function and are at their highest in end-stage renal disease patients receiving dialysis, which can be up to 1000-fold higher than normal individuals ⁶⁰. In human clinical studies, high levels of circulating FGF23 during chronic kidney disease have been found to be associated with cardiac hypertrophy, reduced functional output, and increased incidence of arrhythmias and sudden death ^{61, 62}. Studies in mice, including several from our laboratory, have shown that direct administration of FGF23 causes concentric cardiac hypertrophy and perturbs the electrophysiology of the heart ⁶³⁻⁶⁷. In light of these recent discoveries, novel therapies targeting the FGF23 endocrine pathway have been proposed to treat cardiac decline in kidney disease ⁶⁸.

There are likely more bone-heart communication pathways that affect cardiac health, yet the current evidence remains mostly limited to clinical associations. For instance, many studies have found correlations in the development of osteoporosis and cardiovascular disease. A study in over 2000 postmenopausal women found that having osteoporosis increased the likelihood of suffering a cardiovascular event by 3.9-fold ⁵². Moreover, a Third National Health

and Nutrition Examination Survey (NHANES III) based assessment of the 10-year Framingham coronary heart disease risk value found that a score of greater than 20% in individuals was associated with a 73% increased probability of having low bone-mineral density ⁴. In addition, higher coronary artery and aortic calcification was significantly associated with lower bone mineral density in the Multi-Ethnic Study of Atherosclerosis (MESA) cohort even after adjustment for lipids, inflammatory markers, and sex hormones ⁶⁹. Circulating osteocalcin is also related to atherosclerosis and cardiovascular disease outcomes. A prospective study in men aged from 51-85 years found that higher total baseline osteocalcin was associated with less all-cause mortality after a 10-year follow up ³. In Chinese individuals who received coronary angiography, decreasing osteocalcin levels were significantly associated with increasing severity of coronary vessel calcification ⁷⁰. Other potential candidate endocrine factors that may be participating in bone-heart crosstalk include Wnt3a ⁷¹, osteoprotegerin ⁷², and the negative regulators of bone formation, sclerostin ⁷³ and receptor activator of nuclear factor- κ B ligand (RANKL) ⁷⁴.

Bone has emerged as a diverse organ with recent insights showing an intimate clinical and molecular connection with the cardiovascular system, suggesting bone health and the process of bone mechanical loading are important for heart health. However, it currently remains unknown specifically how bone-derived hormones or bone-neural signaling affect heart health and function and in what way these pathways are affected across the aging process. Particularly, the physiological impact of bone mechanical loading on cardiac function is currently not well understood.

Specific Aims and Hypotheses

The overall hypothesis for these studies is that bone has a positive modulatory influence on cardiac physiology under baseline conditions and during stress via the release of bone-derived hormones and/or bone-neural signaling pathways. These pathways are altered with aging and associated with the dual clinical manifestation of bone and cardiac diseases. This overall hypothesis was tested utilizing an approach involving three specific aims:

Specific Aim 1 (Clinical study) - Determine the clinical association of history of osteoporosis and CVD-related hospital outcomes using electronic health record analysis. Hypothesis: History of osteoporosis is associated with worse hospital outcomes among CVD patients.

Specific Aim 2 (In vitro study) - Evaluate the protective effects of *in vitro* administration of bone cell-secreted factors produced within the context of bone loading, on heart cells and isolated contracting heart preparations exposed to hypoxia/reoxygenation stress. Hypothesis: Bone cell-secreted factors produced from bone loading *in vitro* are protective against the deleterious effects of hypoxia/reoxygenation on heart cells.

Specific Aim 3 (Animal study) - Determine the impact of acute and chronic bone mechanical loading on heart physiology *in vivo* in mice. Hypothesis: Acute and chronic bone mechanical loading *in vivo* alters heart functional properties.

CHAPTER 3
METHODOLOGY

Clinical Studies

Retrospective Cohort Study

Database

The population data for this study was drawn from the Cerner Health Facts database, a de-identified national warehouse which includes data from over 65 million patients and over 600 health care facilities through the year 2018. Data in Health Facts is extracted directly from the electronic medical record from hospitals in which Cerner has a data use agreement. Encounters may include pharmacy, clinical and microbiology laboratory, admission, and billing information from affiliated patient care locations. All admissions, medication orders and dispensing, laboratory orders and specimens are date and time stamped, providing a temporal relationship between treatment patterns and clinical information. Cerner Corporation has established Health Insurance Portability and Accountability Act-compliant operating policies to establish de-identification for Health Facts. This study was declared exempt by the University of Missouri Institutional Review Board (IRB) (Project# 2099694).

Subjects

The cohort selected for the study consisted of both males and females aged 50-70 years admitted to the hospital for cardiovascular disease (CVD) within the categories of heart failure, cardiomyopathy, cardiac dysrhythmias, ischemic heart disease, and hypertension/hypertensive heart disease between the dates of January 2015 and November 2018. All patient encounters included for CVD during this timeframe were index encounters, indicating there was no prior admittance history for the CVD condition for the given patient. Of this cohort of CVD patients, those with a previous diagnosis of osteoporosis occurring prior to admittance for index CVD were categorized as those with a history of osteoporosis while CVD patients with no history of osteoporosis were considered the control group. ICD9/10 diagnosis codes for osteoporosis and CVD types were utilized to build the study cohorts. The demographic characteristics of age, sex, and race/ethnicity along with the health measures of obesity (BMI > 30 kg/m²), smoking status, and Charlson comorbidity index (CC index) were included in the dataset for use as covariates and for subgroup analyses. CC index was calculated using the R package “comorbidity”⁷⁵ for comorbidities occurring within ± 30 days of index encounter of the cohort population. The updated Charlson comorbidity scoring system was used to calculate the total weighted scores for each patient^{76,77}. Subjects were further excluded from the dataset if they had any missing data for any of the parameters.

Outcome Measures

The primary outcomes assessed for the study cohort was the length of hospital stay (LOS) for CVD, calculated as a continuous variable from the difference in days from the point of admittance until discharge or mortality. In addition, prolonged LOS, defined categorically

as a LOS > 75th percentile for specific CVD subtype, was evaluated. Subjects were excluded from the dataset if LOS values were ≤ 0 . The secondary outcomes for the study included laboratory values of cardiac disease diagnostic markers, brain natriuretic peptide (BNP) and amino-terminal proBNP (NT-proBNP), and the documented incidence of mortality for patients during their index hospital stay for CVD. For all outcome measures, female and male subject groups were analyzed separately for determination of sex specific effects.

Statistical Analysis for Clinical Studies

Demographic characteristics and other covariates were compared among groups using Chi-square analyses or Mann-Whitney U tests with Bonferroni correction. LOS data followed a non-normal distribution and was positively skewed. Therefore, preliminary analysis comparing mean LOS data among groups was performed using Kruskal-Wallis tests with pairwise comparisons. Next, LOS data was \log_{10} transformed and multiple linear regression models were constructed to determine the predictive ability of history of osteoporosis for continuous LOS. Binary logistic regression models were used to assess prolonged LOS (categorical). Statistical models met the assumptions of linearity, independence of residuals, multicollinearity and homoscedasticity. Different regression models were created for data stratified by sex and CVD type and adjusted for the covariates of age, race/ethnicity, obesity, smoking status and CC index. All variables were entered into the models using simultaneous entry. Effect sizes for linear regression models were calculated as Cohen's F^2 . Laboratory values of serum biomarkers for cardiac disease, BNP and NT-proBNP, followed a non-normal distribution and were \log_{10} transformed and analyzed using one-way ANOVA with Bonferroni post hoc analyses. Other categorical outcome data including prevalence of history of

osteoporosis among CVD types and mortality was compared among groups using Chi-square analyses with Bonferroni correction. Data is presented as mean \pm 95% confidence interval (CI) for continuous variables and as percentages for categorical variables. A p-value of less than 0.05 was set as the threshold for significance. SPSS v. 28 (IBM, Armonk, NY) was used to perform all statistical tests and modeling for the clinical study.

In Vitro Studies

H9C2 Cell Culture

H9C2 cardiac cells (ATCC, Manassas, VA), originally derived from female embryonic rat cardiac tissue, were maintained in a cell incubator at 37°C and in a 5% CO₂ atmosphere. H9C2 were cultured from passage number 2-10 as proliferative cells using growth media (DMEM + 10% fetal bovine serum + 1% penicillin/streptomycin). To induce differentiation, cells were seeded at a density of 7800 cells/cm² in plastic culture dishes in growth media and after 24 hours media was switched to differentiation media (DMEM + 1% fetal bovine serum + 1% penicillin/streptomycin + 1 μ M all-trans retinoic acid). Cells were then cultured in differentiation media for six to seven days after which they were considered fully differentiated.

RNA Extraction and Real-Time PCR

H9C2 cells were cultured as non-differentiated and fully differentiated cells in six-well plates (n=1 per group). Total RNA was extracted from trypsinized cells pooled from three wells using a Total RNA Mini Kit (IBI Scientific, Dubuque, IA) following the manufacturer's protocols. Quality and yield of isolated RNA samples were assessed using a Biophotometer

(Eppendorf, Enfield, CT) and stored at -20°C. Real-time RT-PCR was performed using a one-step SYBR green quantitative PCR kit (Bio-Rad, Hercules, California). Rat specific primers for cardiac markers were obtained from Sigma (St. Louis, MO) including beta myosin heavy chain (*β-mhc*, *Myh7*; Fw: GATATAGAGACCTTCTGG, Rev: TTCAGAGTCATCTATTGAGGC), alpha myosin heavy chain (*α-mhc*, *Myh6*; Fw: ATCCTCATCACTGGAGAATC, Rev: TAGGATTGTCCTTCTTGCTAC), atrial natriuretic peptide (*Anp*, *Nppa*; Fw: GAGAGAAACCAGAGAGTGAG, Rev: GCAGGTTCTTGAAATCCATC) and brain natriuretic peptide (*Bnp*, *Nppb*; Fw: CAATCCACGATGCAGAAG, Rev: AAAGCAGCTTGA ACTATGTG). Primers for housekeeping gene beta-actin (*Actb*; Ref Seq# NM_031144(1)) were obtained from IDT (Coralville, Iowa), and was used as the reference for gene expression calculation as gene expression levels did not change between treatments. Real-time RT-PCR was performed using a Corbett Rotor-Gene 6000 thermocycler (Qiagen) and Roto-Gene Q Series software (Qiagen). RT-PCR reactions (10 μl) using 10 ng of input RNA were performed in triplicate with cycling conditions: 50°C for 10 minutes, 95°C for 1 minute, followed by 40 cycles of 95°C, 58°C, 72°C for 10, 15, and 10 seconds respectively. A melt curve was performed from 60°C to 99°C rising by 0.5°C every 5 seconds. The cycle threshold (Ct) values were calculated and converted to $2^{-\Delta Ct}$ and averaged between replicates for each gene.

Generation of Bone Conditioned Media (CM)

MLO-Y4 osteocyte-like cells originally derived from murine female long bones were seeded onto positively charged collagen coated glass slides at 5600 cells/cm² and loaded into a Flexcell® Streamer® Shear Stress Device (Flexcell International, Burlington, NC). MLO-

Y4 cell media consisted of α MEM + 2.5% calf serum + 2.5% fetal bovine serum + 1% penicillin/streptomycin. Fluid flow shear stress (FFSS) was applied at 2 dynes/cm² for 2 hours using 350 ml media, which was subsequently collected. Static media was generated from MLO-Y4 cells cultured in the same shear stress device for a similar amount of time but without applied shear stress. MLO-Y4 cell media that was not exposed to cells was used as the blank control.

Cell Viability Assays

H9C2 cells were plated in 96-well plates at 7800 cells/cm² (2500 cells/well) and induced to fully differentiate. Cells were then pretreated with concentrations of 0.075 %, 0.75% and 7.5 % FFSS CM, static CM or blank media for 24 hours. To induce cell death, cells were next challenged with CoCl₂ (hypoxia mimetic) (n=3 independent experiments) for 24 hours or placed in a hypoxia chamber (1% O₂, 5% CO₂, 94% N₂) for 24 hours followed by reoxygenation for 1 hour (H/R) (n=6 independent experiments). H9C2 cells without pretreatment with bone CM were also included to serve as internal controls for interpreting cell viability changes. All treatment groups consisted of n=5 replicate wells. Cell viability was determined using MTT assay. MTT reagent was added to each well at 0.5 mg/ml in differentiation media and incubated at 37°C for two hours. The MTT reagent was then removed and 100 μ l DMSO was added to solubilize formazan crystals and plates were placed on a rocker for 30 minutes. Absorbance was then read (570 nm, 690 nm reference) on a Victor Nivo microplate reader. For analysis of replicate experiments, data was averaged for all wells in each treatment group and normalized to untreated cells challenged with CoCl₂ or hypoxia/reoxygenation.

Ex Vivo Heart Contractility

All animal procedures were approved by the University of Missouri-Kansas City Institutional Animal Care and Use Committee (UMKC-IACUC) (Protocol #42048). Hearts were dissected from two to three month old CD-1 male mice (Envigo, Indianapolis, IN) under 3% isoflurane anesthesia (1.5 L/min) as previously described from our laboratory⁶⁶. Isolated hearts were placed in a dish containing ice-cold Krebs-Henseleit solution (NaCl 118.5 mM, CaCl₂ 1.8 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 25.0 mM, glucose 10 mM and pH 7.4) bubbled with 95%/5% oxygen/carbon dioxide to arrest the heart. Fat and excess connective tissues were then removed. A small cannula was then inserted into the aorta and secured using 4-0 silk suture and the heart was Langendorff-perfused to begin intrinsic contractile rhythm and function. Hearts were next attached to a force transducer via small metal clips and submerged in 25-ml of glass tissue chamber with oxygenated buffer heated to 37° C. Tension was applied until the maximal contractile force was obtained from the beating heart samples. Hearts were then allowed a baseline of one hour to allow for stabilization of contractile function. Hearts were pretreated with 0.75% or 7.5% FFSS CM, static CM or blank control media by administration to the contractile chamber 30 minutes prior to ischemia (0.75% CM: n=6 per group; 7.5% CM: n=5 FFSS, n=6 static, n=5 blank). To induce ischemia/reperfusion injury (I/R), perfusion and oxygen supply to the heart were stopped for a period of 30 minutes, followed by restoration of perfusion and oxygen for a period of one hour, as described previously⁷⁸. Changes in contractility parameters during the reperfusion period including systolic and diastolic force, rates of force development and relaxation, and heart rate were determined by averaging over 30 seconds of contractility data at various timepoints

during reperfusion and were expressed as a percentage of baseline values or absolute change from baseline values in the case of diastolic force.

Statistical Analysis for *In Vitro* Studies

Statistical analysis for *in vitro* studies was performed with GraphPad Prism V.10 (GraphPad, La Jolla, CA). Shapiro-Wilks tests or QQ-plots were used to assess the normality of the data and of the residuals, respectively. Outliers were identified by Grubb's test and removed from the dataset prior to statistical analyses. Comparisons of the changes in cell viability in MTT assay experiments were made using a one-way ANOVA with Bonferroni post hoc analysis. Comparisons in *ex vivo* heart contractility parameters were made using a Mixed Model ANOVA. In all cases, $p < 0.05$ was established a priori as the threshold for significance. All data are presented as mean \pm standard deviation.

Animal Studies

Animals

All animal procedures were approved by the University of Missouri - Kansas City Institutional Animal Care and Use Committee (UMKC-IACUC) (Protocol #42048). Male and Female CD-1 background TOPGAL mice aged to 5-6 months, and wild type CD-1 male mice ages 2-6 months (adult) and 11 months (middle age) (Envigo, Indianapolis, IN) were utilized for all mechanical loading studies. Mice were housed in a temperature-controlled facility with a 12 hour light-dark cycle and *ad libitum* access to food and water.

Tibia Axial Mechanical Loading

Mice were maintained on isoflurane anesthesia (3%, 1.5 L/min) via a Fortec vaporizer and secured in a precision bone loading apparatus (Bose, Electroforce 3220, MA, USA). Tibia compressive loading was performed as previously described^{79, 80} with some modifications. Briefly, the right hindlimb of the mouse was positioned for axial loading of the tibia with the heel of the paw secured and the knee in contact with the loading cell. A pre-load of 0.5 N was applied to the loading cell to maintain the hindlimb in position prior to implementation of the loading protocol. For the acute loading protocol in 5-6 month old TOPGAL mice, the tibia was cyclically loaded with 9 N of force at a frequency of 2 Hz for 300 cycles lasting two and a half minutes (n=7 male control, n=7 male loaded; n=6 female control, n=7 female loaded). Loading at 9 N was chosen as it generates sufficient strain to induce a bone anabolic response in the tibia. The acute loading protocol (2 Hz, 300 cycles lasting 2.5 min) was also performed in 2-6 month old CD-1 male mice with 0 N (control n=15), 1 N (n=8), 2 N (n=8), 3N (n=12) or 9 N (n=13) of force. Additionally, the acute loading protocol (2 Hz, 300 cycles lasting 2.5 min) was performed in 11 month old CD-1 male mice with a force of 3 N followed by 9 N after a 10 minute rest period in the same animal (n=6 control, n=7 loaded). Of these 11 month old mice, a subset were monitored for an additional 15 minutes after the loading session (n=4 control, n=5 loaded) and three loaded mice died before completing the experiment. For all experiments, control mice were anesthetized and right hindlimb secured in the loading apparatus under 0.5 N preload for the same amount of time as loaded mice but did not receive the dynamic compressive loading.

For the three-week loading procedure, 6-month old male CD-1 mice were loaded three times per week (Monday-Wednesday-Friday) over a three week period. Right hindlimb tibias

were mechanically loaded using 100 cycles of 9 N loading force at a frequency of 2hz. Control mice were anesthetized and right hindlimb secured in the loading apparatus under 0.5 N preload for the same amount of time as loaded mice over the three-week period but did not receive the dynamic compressive loading.

In Vivo Electrocardiogram (ECG)

ECG was recorded during the acute loading experiments in anesthetized mice using 29-gauge needle electrodes (ADInstruments) inserted subcutaneously in each of the limbs to obtain a six-lead ECG. Body temperature was maintained at physiological temperature using a water-jacketed heating pad and monitored via a rectal probe. ECG signals and body temperature were recorded using a PowerLab system with LabChart 8 software (ADInstruments). An ECG baseline was established for 5-8 minutes prior to loading, during the loading process, and during a 15-minute period subsequent to loading. Analysis of heart rate was performed by averaging over an interval of five seconds of data collection during baseline, during loading at the lowest heart rate, and at specific time intervals after the loading procedure. Heart variability (HRV) and lead II ECG signal intervals were analyzed by averaging over an interval of 30 seconds of data collection during baseline, during loading at the lowest heart rate, and at specific time intervals after the loading procedure. All data were normalized to baseline values and reported as fold change from baseline.

In vivo conscious electrocardiogram (ECG) was measured before the study and after each week of tibia loading throughout the three-week regimen using a non-invasive ECGenie apparatus (Mouse Specifics, Boston, MA). Mice were first placed on a platform outside the apparatus and allowed a five minute acclimation period. Next, mice were allowed to enter the

ECG measurement compartment where ECG signals were recorded via the paws by electrodes in the floor. ECG signals were collected over a 20 minute time period and analyzed using Emouse software (MouseSpecifics).

Injection of Neural Inhibitors

Lidocaine HCl monohydrate (2.5mg/kg) (Thermo Scientific™, Waltham, MA) was injected subcutaneously around the tibia receiving loading five minutes prior to loading in 2-6 month old male CD-1 mice (n=7 control, n=8 treated) in order to effectively block nerve activity in the hindlimb⁸¹. Atropine sulfate monohydrate (2mg/kg) (TCI America™, Portland, OR)^{82, 83} (n=8 control, n=11 treated) or propranolol HCl (10mg/kg) (Thermo Scientific™, Waltham, MA)⁸⁴ (n=7 control, n=8 treated) were administered by intraperitoneal (IP) injection in mice ~10-15 minutes prior to loading in order to block parasympathetic muscarinic and sympathetic autonomic nervous system activity, respectively. All drugs were dissolved in phosphate buffered saline (PBS) for injection delivery. Control mice were injected with similar volumes of vehicle solution (PBS) prior to undergoing tibia mechanical loading.

Strain Gauging

Experimental strains were obtained from isolated right limb tibias of 6 month old male CD-1 mice (n=3). Muscle and soft tissues were removed from the tibia using micro-dissection tools and the bone surface was prepared with 320-grit sandpaper and cleaned with acetone using a cotton applicator. A strain gauge (EA-06-015DJ-120/LE Vishay Precision Group, Malvern, PA) was then attached to the mid shaft region of the medial surface of the tibia using adhesive and electrical wires were carefully soldered to the strain gauge and multimeter was

used to check the resistance. These wires were then soldered to the wires coming from the StrainSmart Data Acquisition system (System 7000 Vishay Precision Group, Malvern, PA) for strain recording. The tibia sample was placed in the loading fixture and mechanical loading was applied at 3 N, 5 N, 7 N and 9 N of load (2 Hz, 15 cycles) and the resultant strains were recorded. Average peak strains were plotted for each loading force.

Statistical Analysis for Animal Studies

Statistical analysis for animal studies was performed with GraphPad Prism V.10 (GraphPad, La Jolla, CA). Animal data was assessed for normality using the Shapiro-Wilks test and QQ-plots. Outliers in the data were identified using Grubb's test and were removed prior to performing statistical analyses. Comparisons in the changes of heart rate, HRV and ECG parameters over time during the acute and three-week bone loading experiments were made using a Mixed Model ANOVA with Bonferroni post hoc analysis. One-way ANOVA with Bonferroni multiple comparisons post hoc analysis was utilized to compare heart rate and HRV changes in response to the different magnitudes of load. For the neural inhibitor injection studies, a two-tailed t-test was used to compare the vehicle and inhibitor-injected groups. Heart weight after the three-week loading study was compared using a two-tailed t-test, and skeletal muscle size and contractility was compared using one-way ANOVA. In all cases, $p < 0.05$ was established a priori as the threshold for significance. All data are presented as mean \pm standard deviation.

CHAPTER 4

RESULTS

Clinical Studies

Retrospective Cohort Study

The clinical sample size of patients examined in the dataset for this study totaled 2,078,104 individuals who were admitted for CVD as an index encounter over the approximately four-year study period of January 2015 to November 2018. Males comprised 1,044,154 (50.2%) and females 1,033,950 (49.8%) of the study population. Of the total patients, there were 59,393 who were found to have a prior history of osteoporosis as seen in Table 1. Among the demographic characteristics and covariables analyzed in all CVD patients, those with history of osteoporosis were significantly older in age, predominantly female, more likely Caucasian in race, less likely to be obese, and had a higher CC index compared to those without a documented history of osteoporosis (control group) (Table 1).

Table 1.--Demographic characteristics and covariates for the study population

Covariates		All CVD	
		Control (n=2,018,711)	Osteoporosis (n=59,393)
Age, yrs (SD)		60.4 (6.0)	63.6 (5.1)†
CC Index (SD)		0.72 (1.52)	0.96 (1.69)†
Sex, n (%)	Male	1,035,756 (51.3)	8398 (14.1)†
	Female	982,955 (48.7)	50,995 (85.9)
Race, n (%)	White	1,489,089 (73.8)	47,678 (80.3)†
	Non-White	529,622 (26.2)	11,715 (19.7)
Obesity, n (%)	No	1,799,451 (89.1)	54,110 (91.1)†
	Yes	219,260 (10.9)	5283 (8.9)
Smoking, n (%)	No	1,925,405 (95.4)	56,566 (95.2)
	Yes	93,306 (4.6)	2827 (4.8)

†Significant statistical difference ($p < 0.001$) between control and osteoporosis groups. Chi-square test or Mann-Whitney U test with Bonferroni correction.

After stratifying the patient dataset by subgroups of CVD including ischemic heart disease (IHD), heart failure (HF), cardiac dysrhythmias (CD), cardiomyopathy (CM), and hypertension/hypertension related heart disease (HT), demographic and covariate characteristics were found to follow similar trends among groups (Table 2). Different CVD subgroups also had statistically significant differences in the prevalence of history of osteoporosis. Heart failure patients had the highest prevalence of history of osteoporosis (3.4%), followed by those with cardiac dysrhythmia (2.9%) and hypertension/hypertension related heart disease (2.9%), and was lowest in those with ischemic heart disease (2.4%) and cardiomyopathy (2.4%) (Table 2).

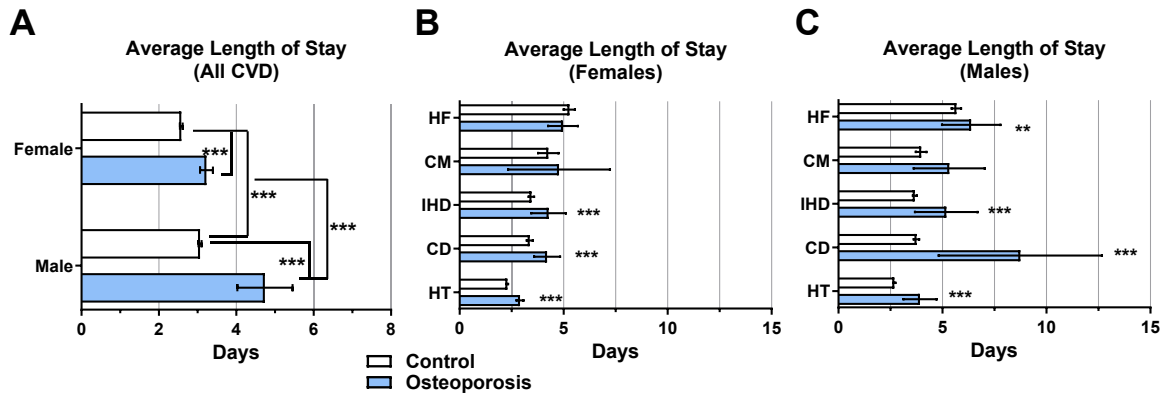
Table 2.--Demographic characteristics and covariates for the study population stratified by subgroups of CVD

Covariates	Ischemic Heart Disease (IHD)		Heart Failure (HF)		Cardiac Dysrhythmias (CD)		Cardiomyopathy (CM)		Hypertension & Hypertensive Heart Disease (HT)	
	Control	Osteoporosis	Control	Osteoporosis	Control	Osteoporosis	Control	Osteoporosis	Control	Osteoporosis
	(n=263,275)	(n=6514) ^a	(n=62,865)	(n=2188) ^b	(n=161,405)	(n=4807) ^c	(n=20,934)	(n=504) ^a	(n=1,510,232)	(n=45,380) ^c
Age, yrs (SD)	61.4 (5.9)	63.7 (5.0)†	61.0 (5.9)	63.5 (5.2)†	61.3 (6.0)	63.9 (5.0)†	60.3 (6.0)	63.0 (5.2)†	60.1 (6.0)	63.6 (5.1)†
CC Index (SD)	0.88 (1.60)	1.26 (1.83)†	3.22 (1.78)	3.59 (1.88)†	0.87 (1.79)	1.18 (2.00)†	2.65 (1.62)	3.07 (1.96)†	0.54 (1.34)	0.74 (1.48)†
Gender, n (%)										
Male	173,438 (65.9)	1365 (21.0)	34,495 (54.9)	408 (18.6)	91,518 (56.7)	828 (17.2)	12,838 (61.3)	100 (19.8)	723,467 (47.9)	5697 (12.6)
Female	89,837 (34.1)	5149 (79.0)†	28,370 (45.1)	1780 (81.4)†	69,887 (43.3)	3979 (82.8)†	8096 (38.7)	404 (80.2)†	786,765 (52.1)	39,683 (87.4)†
Race, n (%)										
White	211,321 (80.3)	5559 (85.3)	42,819 (68.1)	1744 (79.7)	133,046 (82.4)	4171 (86.8)	14,910 (71.2)	407 (80.8)	1,086,993 (72.0)	35,797 (78.9)
Non-White	51,954 (19.7)	955 (14.7)†	20,046 (31.9)	444 (20.3)†	28,359 (17.6)	636 (13.2)†	6024 (28.8)	97 (19.2)†	423,239 (28.0)	9583 (21.1)†
Obesity, n (%)										
No	233,499 (88.7)	5881 (90.3)	50,644 (80.6)	1794 (82.0)	145,596 (90.2)	4449 (92.6)	18,346 (87.6)	451 (89.5)	1,351,366 (89.4)	41,535 (91.5)
Yes	29,776 (11.3)	633 (9.7)†	12,221 (19.4)	394 (18.0)	15,809 (9.8)	358 (7.4)†	2588 (12.4)	53 (10.5)	158,866 (10.6)	3845 (8.5)†
Smoking, n (%)										
No	245,647 (93.3)	6009 (92.3)	58,478 (93.0)	2008 (91.8)	154,446 (95.7)	4522 (94.1)	19,916 (95.1)	469 (93.1)	1,446,918 (95.8)	45,558 (96.0)
Yes	17,628 (6.7)	505 (7.7)†	4387 (7.0)	180 (8.2)	6959 (4.3)	285 (5.9)†	1018 (4.9)	35 (6.9)	63,314 (4.2)	1822 (4.0)

†Significant statistical difference (p<0.001) between control and osteoporosis groups. Different letters (a, b, c) denote significant statistical difference in osteoporosis prevalence between CVD groups. Chi-square test or Mann-Whitney U test with Bonferroni correction.

To determine the impact of history of osteoporosis on hospital-related outcomes of CVD, length of hospital stay (LOS) was first examined, which represents the total number of days a patient remains hospitalized from admittance to discharge and is impacted by sociodemographic as well as disease-related factors. Figure 1 displays the average LOS values among male and female patients when considering all cause CVD. Although females exhibited the majority of osteoporosis diagnoses compared to males (5.2% vs. 0.8% prevalence), the percent increase in average LOS for all-cause CVD in female patients with history of osteoporosis was +25%, increasing from 2.58 days to 3.23 days, compared to +55% in males with history of osteoporosis which rose from 3.06 days to 4.74 days (Figure 1A). When stratified by CVD type, the effect of history of osteoporosis in females was statistically significant for IHD ($p<0.001$), HT ($p<0.001$), and CD ($p<0.001$), but not for HF or CM (Figure 1B), while males had significantly longer LOS across all CVD subtypes ($p<0.01$) except for CM (Figure 1C). Interestingly, CD showed the highest change in average LOS in males with a history of osteoporosis (+233%).

Figure 1.--Longer average CVD-related LOS in those with a history of osteoporosis



Average LOS in females and males for all cause CVD admissions (A) and after stratification for different CVD subtypes (B, C). HF=heart failure, CM=cardiomyopathy, IHD=ischemic heart disease, CD=cardiac dysrhythmia, HT=hypertension/hypertension related heart disease. ***p<0.001, **p<0.01, *p<0.05, Kruskal-Wallis Test with pairwise comparisons. Mean \pm 95% CI.

To determine if history of osteoporosis can predict LOS in CVD patients, multiple linear regression and binary logistic regression models adjusted for traditional risk factors were constructed. Covariables included in the models along with history of osteoporosis were age, race, obesity, smoking status, and Charlson comorbidity index (CC index). The CC index is of particular importance in order to control for the bias introduced by the overall burden of disease by providing a calculation of individual weighted scores for patients based on comorbidity number, type and severity⁸⁵. Of all these factors included, obesity exhibited the greatest predictive power for LOS in the multiple linear regression models ($\beta=0.319$ for all cause CVD, $p<0.001$). Importantly, history of osteoporosis was found to be a significant independent predictor of all cause CVD-related LOS (log transformed) when analyzed as a continuous variable adjusted with covariates using multiple linear regression, for both females ($\beta=0.060$, $p<0.001$) (Table 3) and males ($\beta=0.071$, $p<0.001$) (Table 4). Among CVD subtypes, females showed a significant relationship of history of osteoporosis with IHD, CD, and HT but not HF

or CM in the covariate adjusted models (Table 3). In males, the β -coefficients for history of osteoporosis were higher than females, reflecting the longer LOS for males, and were significant among all CVD subtypes except HF (Table 4). However, females displayed up to a 4.8-fold greater effect size (Cohen's F^2) for history of osteoporosis in the adjusted linear regression models for all-cause CVD, IHD, CD and HT than males (Table 3 and Table 4).

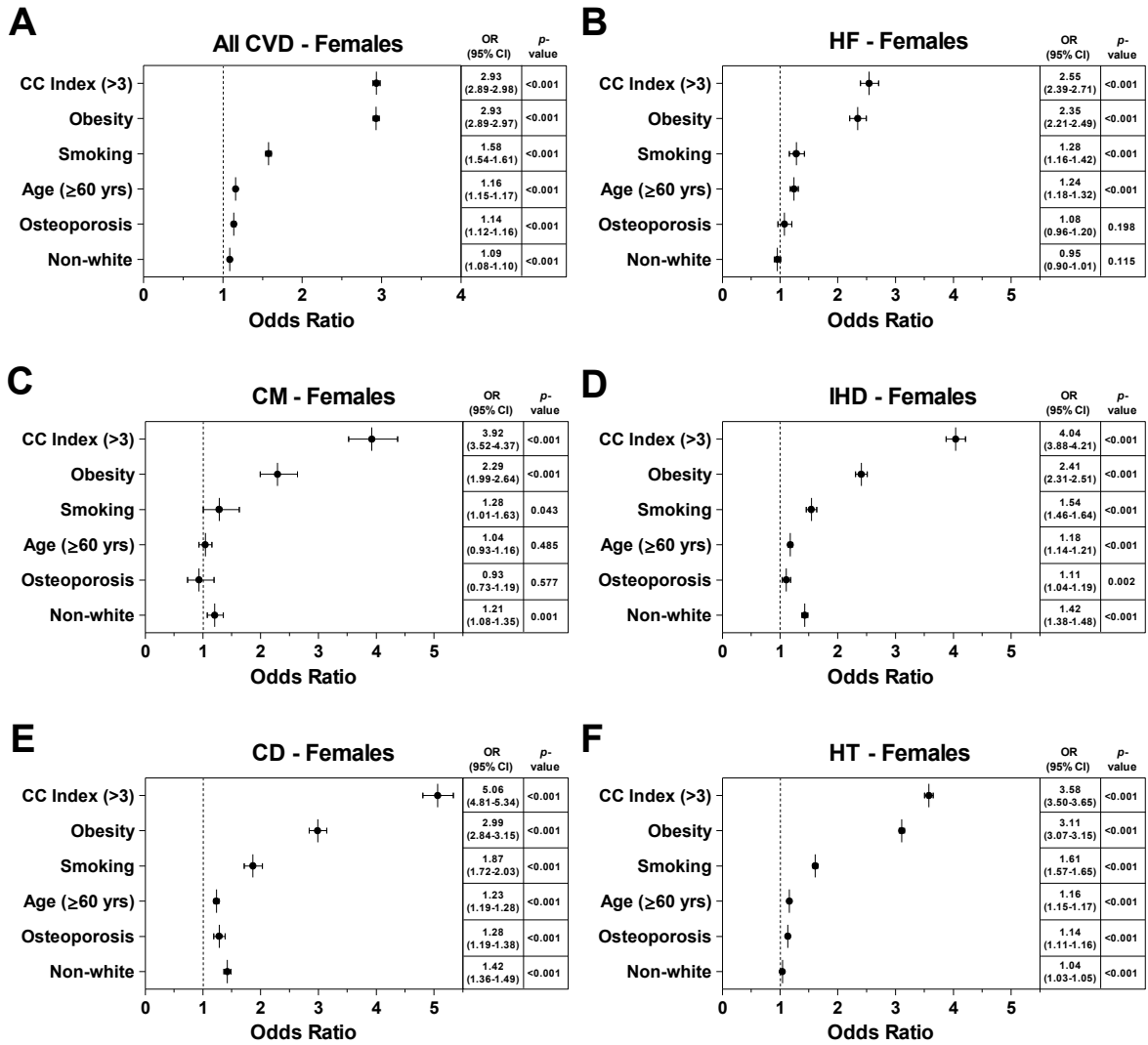
Table 3.--History of osteoporosis in females is an independent predictor for LOS (continuous) using multiple linear regression

Predictors	All CVD			HF			CM			IHD			CD			HT		
	R	β	F ²	R	β	F ²	R	β	F ²	R	β	F ²	R	β	F ²	R	β	F ²
	0.308	0.060	0.00048	0.358	0.010	0.528	0.303	-0.013	0.657	0.299	0.017	0.037	0.355	0.070	0.00063	0.271	0.069	0.00063
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.010	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	0.006	0.006	0.00326	0.083	0.083	0.04799	0.076	<0.001	0.05233	0.086	<0.001	0.05383	0.099	<0.001	0.0743	0.085	<0.001	0.03584
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.102	<0.001	0.00628	0.070	<0.001	0.00231	0.086	<0.001	0.00271	-0.018	<0.001	0.00020
	0.319	0.319	0.03012	0.400	0.400	0.06221	0.275	<0.001	0.02494	0.251	<0.001	0.02058	0.356	<0.001	0.02976	0.317	<0.001	0.03049
	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.107	<0.001	0.0013	0.084	<0.001	0.00116	0.137	<0.001	0.00168	0.062	<0.001	0.00040
	0.081	0.081	0.00068	0.133	0.133	0.00231	0.107	<0.001	0.0013	0.084	<0.001	0.00116	0.137	<0.001	0.00168	0.062	<0.001	0.00040

HF=heart failure, CM=cardiomyopathy, IHD=ischemic heart disease, CD=cardiac dysrhythmia, HT=hypertension/hypertension related heart disease.

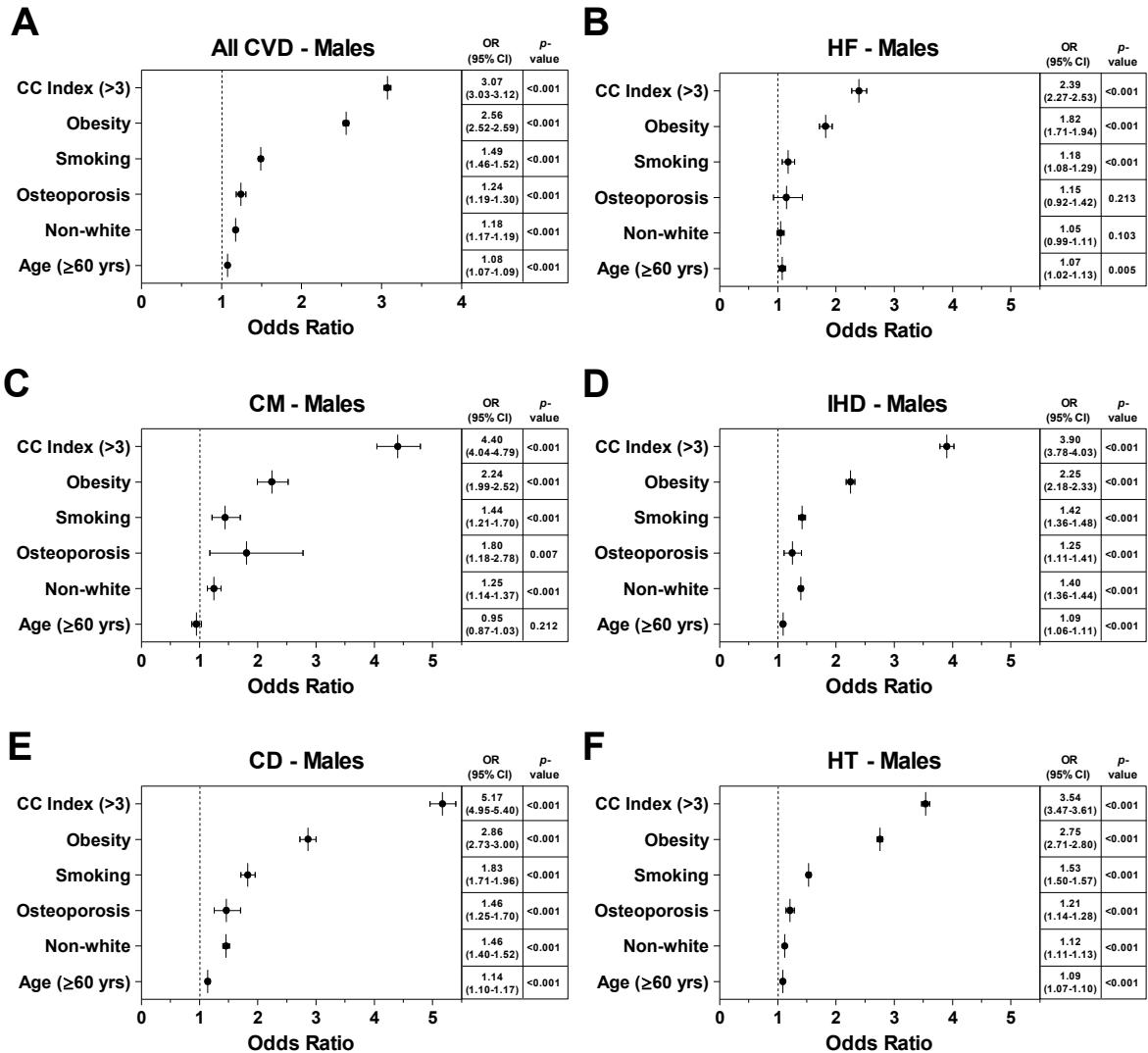
Risk of prolonged LOS (>75th percentile) was next examined through logistic regression analysis. History of osteoporosis significantly and independently increased the odds of experiencing a prolonged LOS in female and male all cause CVD patients by 1.14 times (Figure 2A) and 1.24 times (Figure 3A), respectively, even after adjusting for covariables. Similar to the linear regression results, history of osteoporosis in males showed a greater number of significant associations and higher odds of prolonged LOS across different CVD subtypes than did females (Figures 2 and 3).

Figure 2.--History of osteoporosis in females is an independent predictor for prolonged LOS using logistic regression



Female odds ratios for prolonged LOS in all cause CVD (A), heart failure (B), cardiomyopathy (C), ischemic heart disease, (D), cardiac dysrhythmias (E), and hypertension/hypertension related heart disease (F).

Figure 3.--History of osteoporosis in males is an independent predictor for prolonged LOS using logistic regression

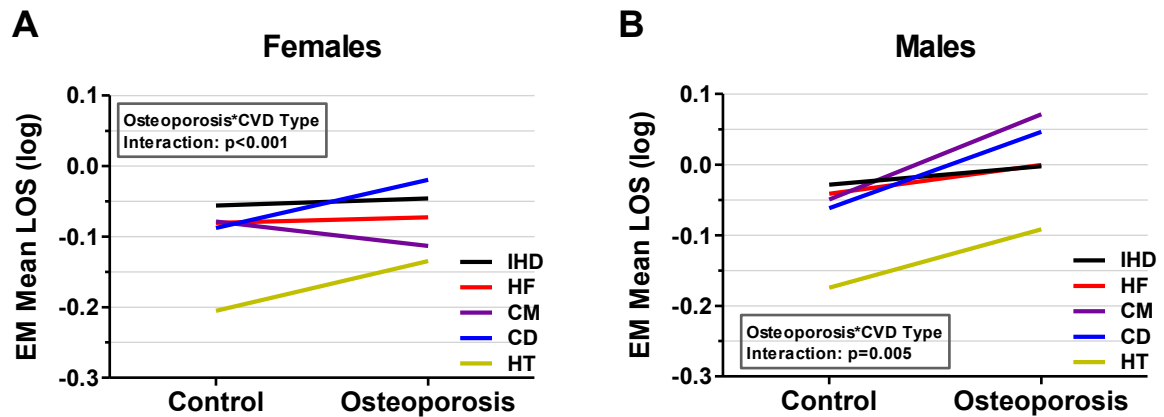


Male odds ratios for prolonged LOS in all cause CVD (A), heart failure (B), cardiomyopathy (C), ischemic heart disease, (D), cardiac dysrhythmias (E), and hypertension/hypertension related heart disease (F).

The results of the regression analysis suggest a differential effect of history of osteoporosis among the various CVD subtypes examined. Testing for an interaction among history of osteoporosis and different subtypes of CVD utilizing a generalized linear model with

interaction terms revealed and significant interaction effect with history of osteoporosis in both females and males for continuous LOS (Figure 4). This indicates that effect of history of osteoporosis on LOS is dependent upon CVD subtype.

Figure 4.--Interaction effect between CVD subtype and history of osteoporosis for LOS

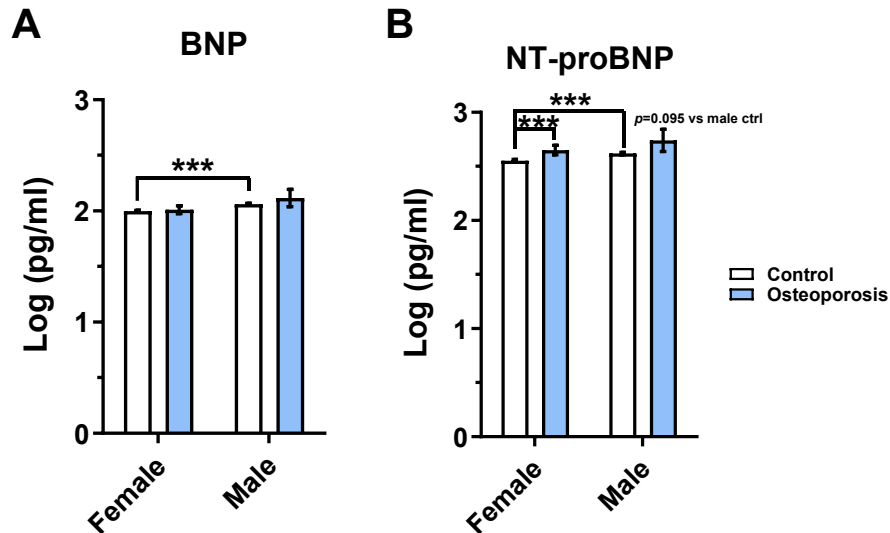


Estimated marginal means of log transformed LOS in female (A) and male (B) CVD patients across the various CVD subtypes. HF=heart failure, CM=cardiomyopathy, IHD=ischemic heart disease, CD=cardiac dysrhythmia, HT=hypertension/hypertension related heart disease. Generalized linear model univariate analysis.

The clinical manifestations of prolonged LOS for CVD-related hospital admittance in those with a history of osteoporosis may be directly related to changes in cardiac health. Therefore, levels of cardiac disease diagnostic biomarkers including brain natriuretic peptide (BNP) and amino-terminal proBNP (NT-proBNP) were compared to determine if the setting of osteoporosis exacerbates cardiac disease and stress. BNP and NT-proBNP are fragments derived from the same pro-peptide, however NT-proBNP levels are more stable over time and have shown greater sensitivity in prediction of CVD-related outcomes⁸⁶. With respect to BNP, history of osteoporosis did not significantly impact serum levels in CVD patients (Figure 5A).

A significant sex difference was observed among control patients without a history of osteoporosis with males showing significantly elevated BNP compared to females (Figure 5A). A similar sex difference was detected among control patients for NT-proBNP levels (Figure 5B). Interestingly, in contrast to BNP, NT-proBNP was found to be statistically significantly increased in female CVD patients with a history of osteoporosis compared to control patients (Figure 5B). Males CVD patients also exhibited a trend for higher levels of NT-proBNP in those with a history of osteoporosis ($p=0.095$), however this was not statistically significant (Figure 5B). Pearson correlation of NT-proBNP with hospital LOS data among all cause CVD patients showed a significant moderate positive correlation ($R=0.371$, $p<0.001$) suggesting that those with history of osteoporosis may remain hospitalized for longer periods due to more severe cardiovascular pathology.

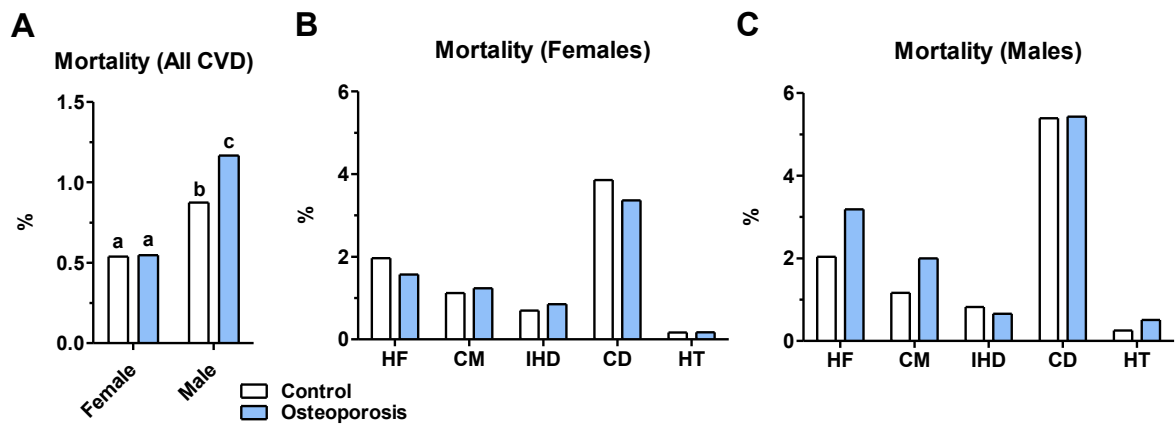
Figure 5.--Increased NT-proBNP levels in CVD patients with a history of osteoporosis



Clinical serum biomarkers of heart disease, BNP (A) and NT-proBNP (B) were examined in female and male all cause CVD patients. *** $p<0.001$, One-way ANOVA with Games-Howell post hoc analysis. Mean \pm 95% CI.

The prolongation of hospital LOS and increased CVD biomarker levels observed in CVD patients with a history of osteoporosis suggested a worse overall prognosis for the health of this population. Therefore, the incidence of mortality during CVD-related index hospital stay was next investigated. With regard to the control group without a history of osteoporosis, sex differences were found with male CVD patients having significantly elevated mortality incidence compared to females for all cause CVD (Figure 6A). History of osteoporosis did not significantly impact mortality in females; however, in males there was a 34% increase in mortality for all cause CVD in those with a history of osteoporosis (Figure 6A). In line with these findings, when mortality was examined across different CVD subtypes, males with a history of osteoporosis showed an overall higher incidence, which was not observed in the female groups (Figure 6B-C).

Figure 6.--Increased mortality during CVD-related hospital stay in males with a history of osteoporosis



Incidence of mortality in both female and male patients during CVD-related index hospital stay for all cause CVD (A), and according to CVD subtype in females (B) and males (C).

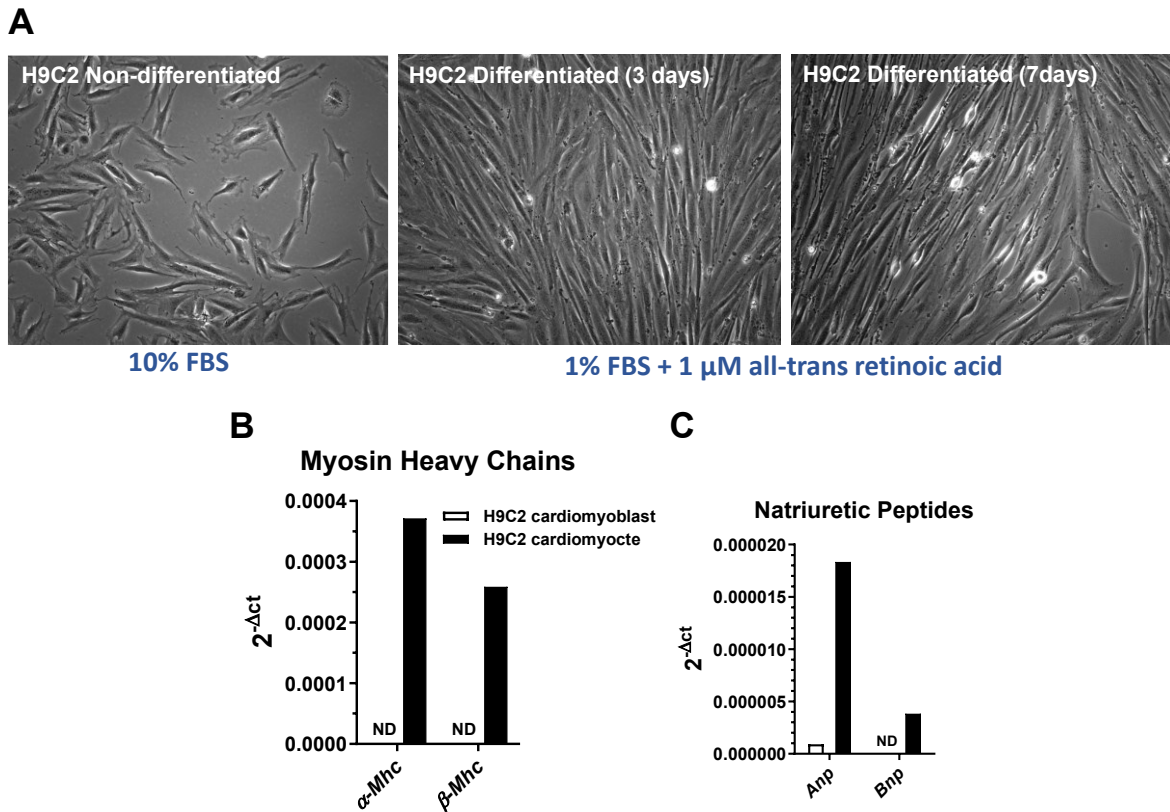
HF=heart failure, CM=cardiomyopathy, IHD=ischemic heart disease, CD=cardiac dysrhythmia, HT=hypertension/hypertension related heart disease. Groups with different letters (a, b, c) are statistically significantly different from each other, Chi-square analysis with Bonferroni correction.

***In Vitro* Studies**

Cardiomyocyte viability

Bone is an endocrine organ capable of secreting humoral factors, which have been found to regulate distant organ systems. Toward this notion, an *in vitro* model of the endocrine functions of mechanically loaded bone was utilized to explore a potential beneficial crosstalk link with the heart. The H9C2 cell line is a commonly used cardiomyocyte model existing as proliferating cardiomyoblasts which can be differentiated to exhibit a cardiomyocyte phenotype using low serum and supplementation with all-trans retinoic acid. Micrographs taken before and after H9C2 differentiation show changes to cell morphology (more spindle-like shape) and cell fusion resulting in numerous bi-nucleate and multi-nucleated cells like that of cardiomyocytes *in vivo* (Figure 7A). To determine how differentiation affects the mRNA expression of cardiac cell markers, real-time RT-PCR was undertaken in non-differentiated and differentiated H9C2 cells. Among these genes, myosin heavy chain isoforms *Myh6* (α -myosin heavy chain) and *Myh7* (β -myosin heavy chain), and natriuretic peptides *Nppa* (*Anp*) and *Nppb* (*Bnp*) were found to be at very low to undetectable levels in non-differentiated cells, but their expression was induced in response to differentiation (Figure 7B-C). These cardiac-specific markers are typically highly expressed in adult cardiomyocytes. Based on these results, seven days differentiated H9C2 cells were utilized for further studies.

Figure 7.--Cardiac phenotype of H9C2 cells is induced during differentiation

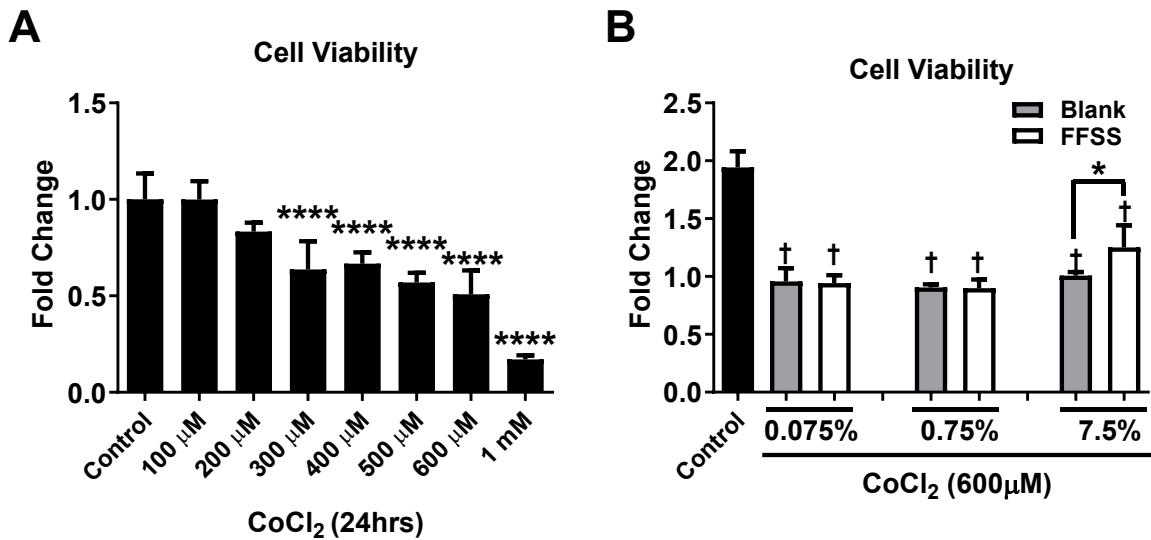


A) Phase contrast micrograph (10X) of (Left) non-differentiated H9C2 cells cultured in media supplemented with 10% fetal bovine serum (FBS), (Middle) H9C2 cells cultured in media supplemented with 1% FBS + 1 μ M all trans retinoic acid after 3 days, and (Right) after 7 days. Real-time RT-PCR analysis was performed in non-differentiated cells and 7 days differentiated cells for (B) cardiac contractile genes α -mhc and β -mhc, and (C) natriuretic peptides, *Anp* and *Bnp*. ND = not detectable. N=1 per group.

Bone cell conditioned media (CM) containing factors secreted from osteocytes during fluid flow shear stress (FFSS), which mimics bone mechanical loading *in vitro*, was utilized to characterize protective effects in heart cells under long-term hypoxia-related stress. Fully differentiated H9C2 cells were first exposed to increasing concentrations of CoCl₂, a chemical hypoxia-mimetic that induces hypoxia-related cell signaling by stabilizing hypoxia-inducible factors 1 alpha and 2 alpha (HIF-1 α and HIF-2 α) under normal oxygen conditions⁸⁷. CoCl₂

treatment resulted in a dose-dependent decrease in cell viability as measured by MTT assay (Figure 8A). To determine if bone-secreted factors promote the survival of cardiomyocytes during long-term CoCl_2 treatment, differentiated H9C2 cells were pre-treated with 0.075% - 7.5% of FFSS CM or control (blank) media before challenge with $600\mu\text{M}$ CoCl_2 . Pre-treatment with 7.5% FFSS CM statistically significantly enhanced cell viability compared to blank media after 24 hours of exposure to CoCl_2 (Figure 8B).

Figure 8.--Bone CM protects H9C2 cells against cell death during long-term CoCl_2 treatment

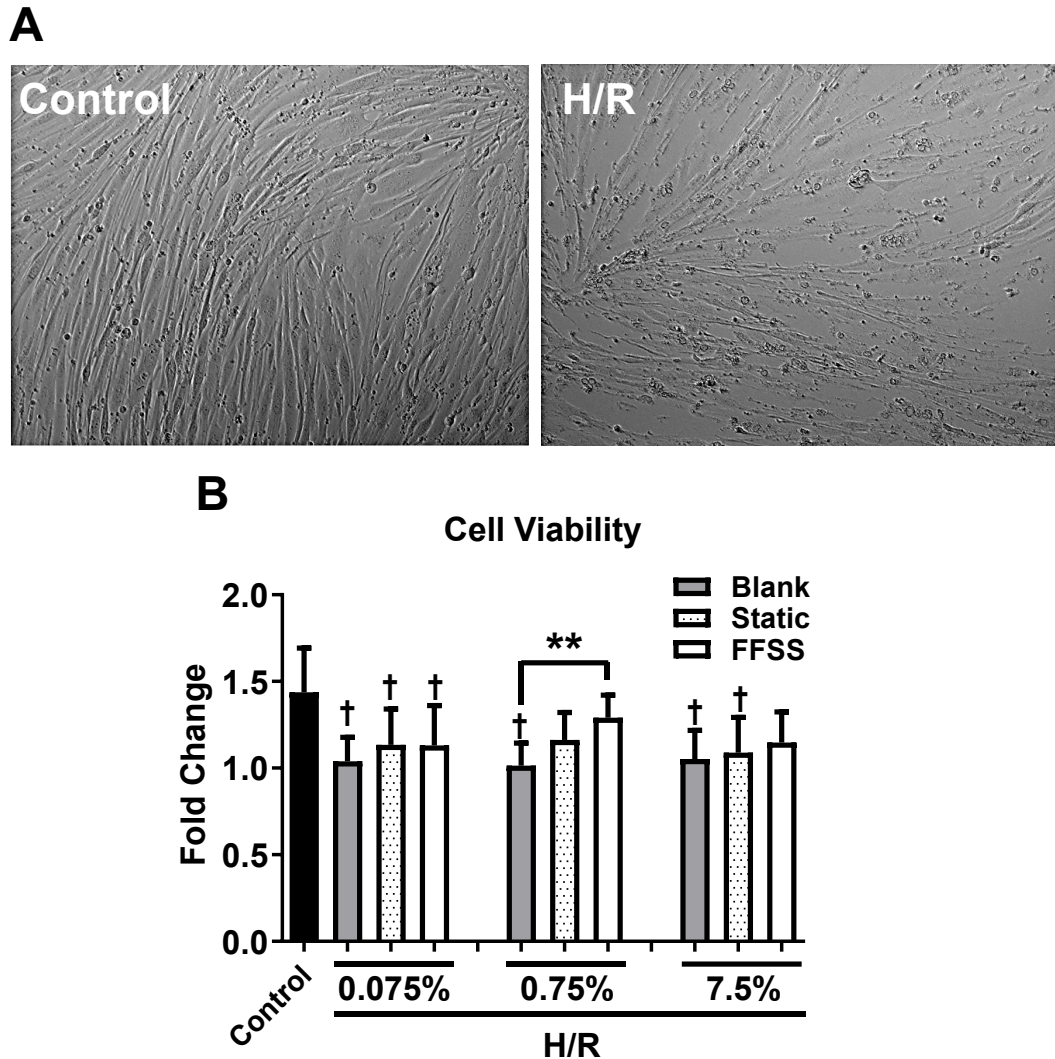


A) Dose response for hypoxia mimetic CoCl_2 -induced cell death in H9C2 cardiomyocytes treated for 24hrs as measured by MTT assay. **** $p < 0.0001$ vs. control, one-way ANOVA with Bonferroni post hoc analysis. B) Average cell viability as measured by MTT assay in differentiated H9C2 cells pre-treated with 0.075% - 7.5% FFSS CM or blank media for 24 hours followed by challenge with $600\mu\text{M}$ CoCl_2 for 24 hours. * $p < 0.05$ vs blank, † $p < 0.05$ vs control, one-way ANOVA with Bonferroni post hoc analysis, $n=3$ independent experiments. Mean \pm SD.

In light of these findings with CoCl_2 , studies were conducted using a hypoxia chamber to explore the protective effects of bone CM on cardiomyocytes under low atmospheric oxygen

(1%) followed by reoxygenation as would be experienced during prolonged myocardial I/R. In atmospheric air, oxygen partial pressure is 159 mmHg at sea level, while *in vivo*, normal arterial oxygen partial pressure is between 75-100 mmHg⁸⁸. Thus, the 1% oxygen level utilized for this study would translate to 1.59 mmHg. For these experiments, bone CM from osteocytes not exposed to FFSS (static) was also incorporated to compare to the specific influence of bone loading on protection of cardiomyocytes against hypoxia. As seen in Figure 9A, the hypoxia/reoxygenation (H/R) protocol resulted in marked alterations to the integrity of H9C2 cell cultures including lower cell density and a fragmented appearance. When cell viability after H/R was compared to cells that received normal oxygen levels (control), all treatment groups showed significantly reduced cell viability except for 0.75% static CM, 0.75% FFSS CM, and 7.5% FFSS CM, signifying a potential protective effect from these treatments against H/R (Figure 9B). However, only administration of 0.75% FFSS CM 24 hours prior to H/R showed significant protection against cell death compared to blank media (Figure 9B). Static media from bone cells did not show a significant effect on cell viability over blank media suggesting that bone loading facilitates greater release of protective factors.

Figure 9.--Bone CM protects H9C2 cells against cell death during long-term hypoxia/reoxygenation



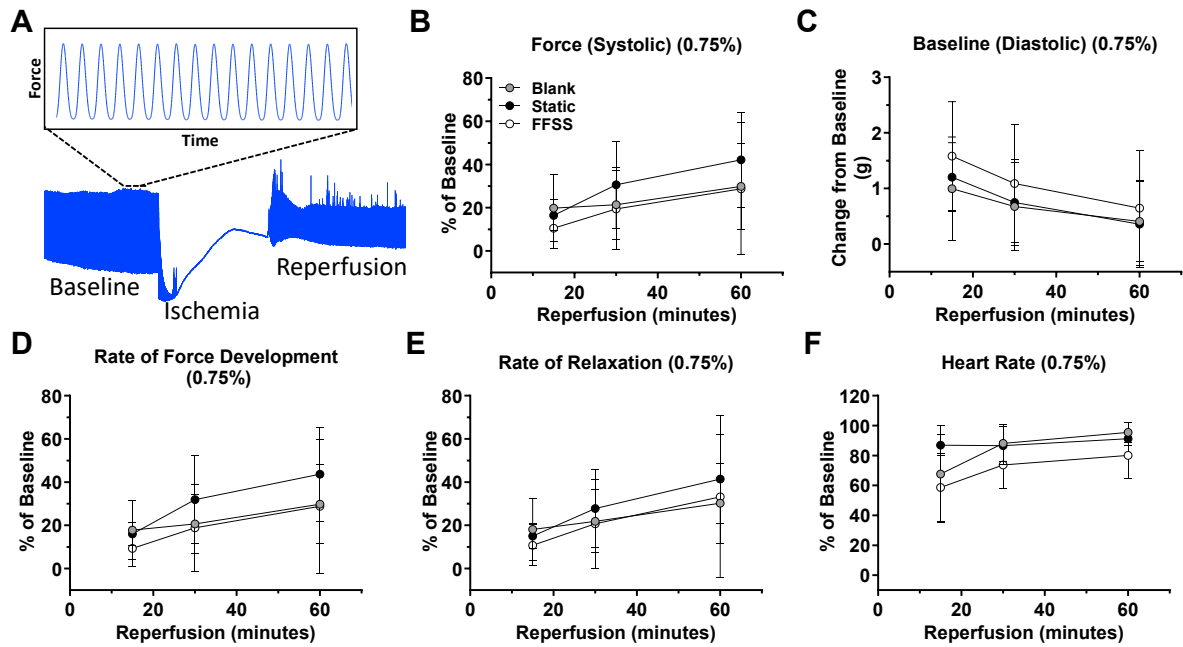
A) Representative phase contrast images (10X) of differentiated H9C2 cells cultured under normal oxygen levels (Left) and after the hypoxia/reoxygenation (H/R) protocol (Right). B) Average cell viability as measured by MTT assay in differentiated H9C2 cells pretreated with 0.075%-7.5% bone CM for 24hrs followed by H/R. ** $p < 0.01$ vs. blank media, † $p < 0.05$ vs. control, one-way ANOVA with Bonferroni post hoc analysis, $n = 6$ independent experiments. Mean \pm SD.

Whole heart contractility

At the level of the whole heart, acute ischemia results in cardiac functional decline due to the impairment of numerous cardiomyocyte processes and their contribution to contractile

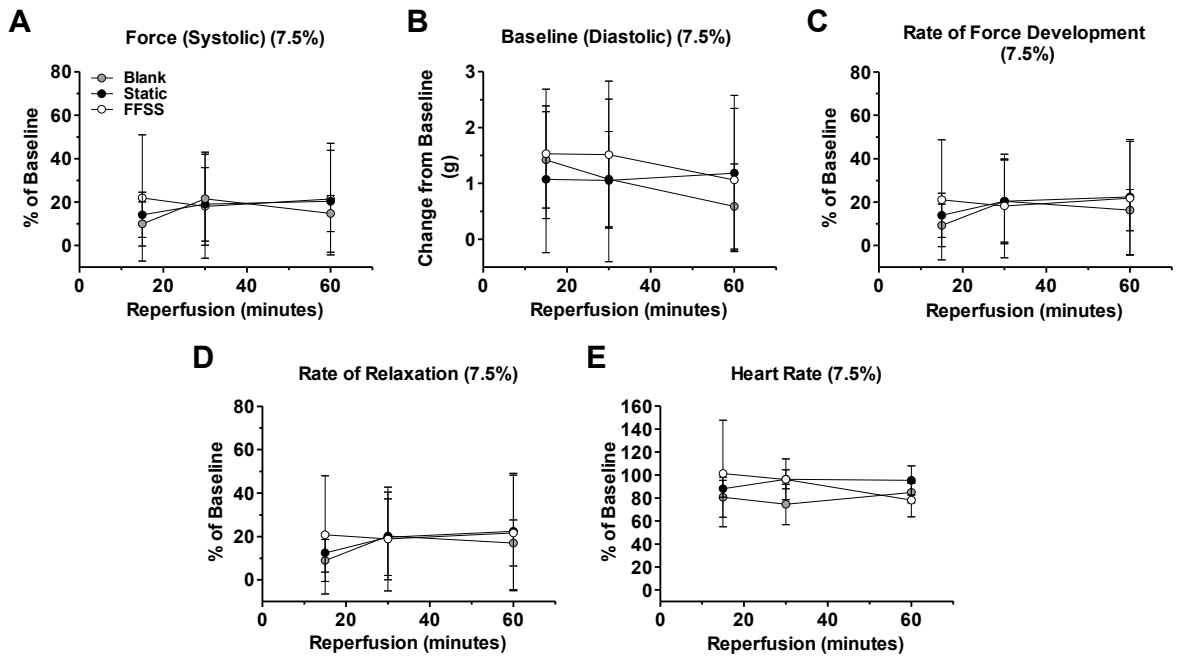
performance. As 0.75% and 7.5% bone CM exhibited protective effects against long-term hypoxia/reoxygenation and CoCl_2 treatment in cell culture models, respectively, the impact of these concentrations on cardiac contractility after acute I/R injury *ex vivo* was investigated. In contrast to the more prolonged cell studies, these whole heart experiments were aimed at determining if bone CM was protective against the initial intracellular changes occurring during I/R, such as altered ion handling and free radical generation, and its relation to contractile performance. Langendorff perfused, spontaneously beating hearts from mice were pre-treated with 0.75% or 7.5% bone CM for 30 minutes and then challenged with an acute I/R protocol to elicit functional decline. Ischemia for 30 minutes resulted in complete cessation of cardiac contractility which was only partially restored during the reperfusion period (Figure 10A). Pre-treatment with bone CM at either the 0.75% or 7.5% concentration did not result in any statistically significant improvements in recovery of heart contractile systolic and diastolic force, rate of force development/relaxation, or heart rate during the 60-minute reperfusion period (Figure 10 and Figure 11).

Figure 10.--Bone CM (0.75%) does not protect against *ex vivo* cardiac contractility impairment from acute ischemia/reperfusion injury



A) Representative cardiac contractility record showing force measurements captured during baseline, ischemia and reperfusion. The inset displays a magnified view of the individual contractile waveforms produced by the heart. Average contractile measurements of B) percent recovery of peak systolic contractile force, C) absolute change in diastolic baseline force, D) percent recovery of maximal rate of force development, E) percent recovery of maximal rate of force relaxation, and F) percent recovery of heart rate during the reperfusion period. Mixed Model ANOVA, n=6 mice per group. Mean \pm SD.

Figure 11.--Bone CM (7.5%) does not protect against *ex vivo* cardiac contractility impairment from acute ischemia/reperfusion injury



Average contractile measurements of A) percent recovery of peak systolic contractile force, B) absolute change in diastolic baseline force, C) percent recovery of maximal rate of force development, D) percent recovery of maximal rate of force relaxation, and E) percent recovery of heart rate during the reperfusion period. Mixed Model ANOVA, n=5-6 mice per group. Mean \pm SD.

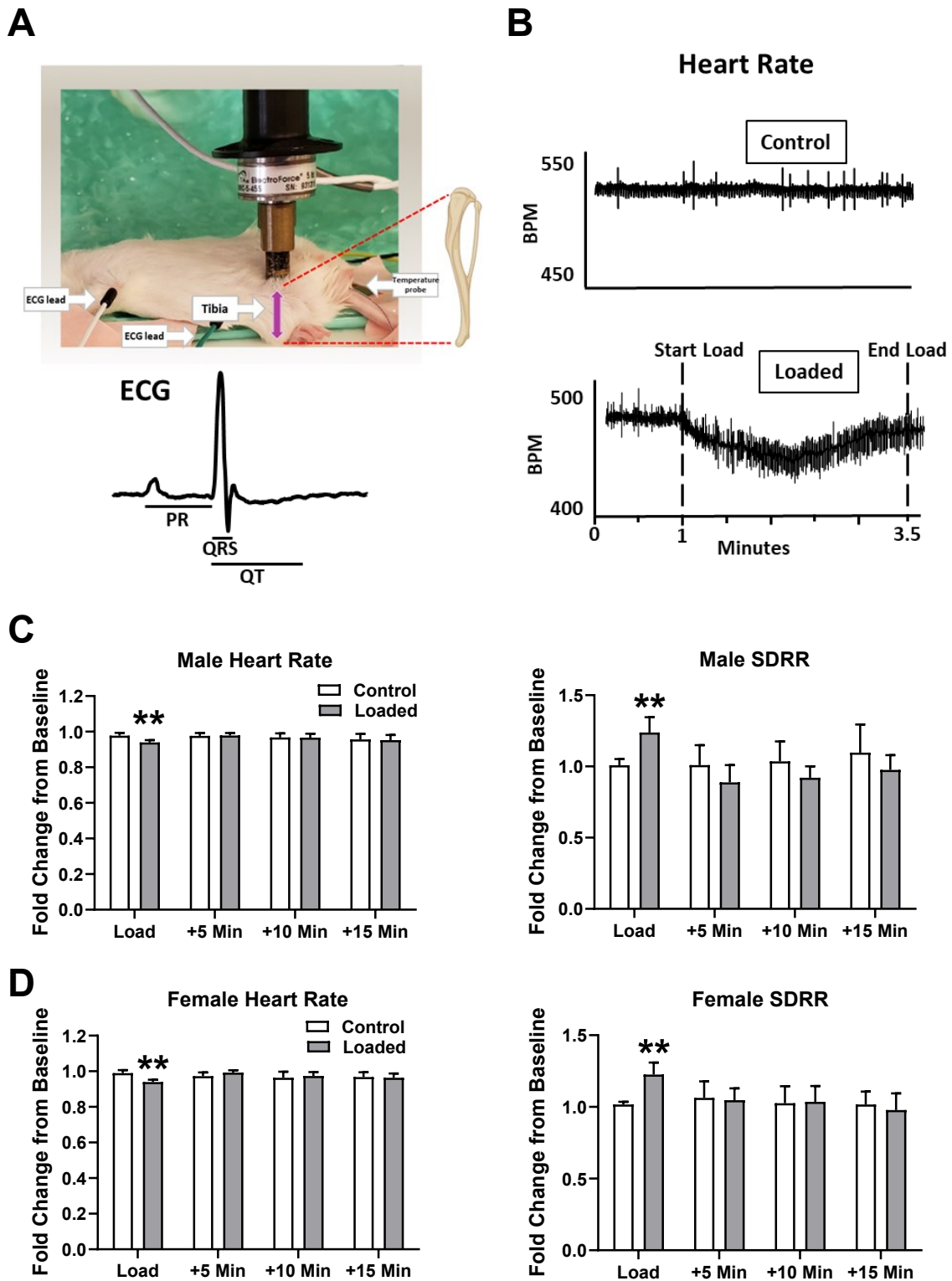
Animal Studies

Acute bone loading

The *in vitro* results were suggestive of an endocrine bone-heart link which may serve to protect the heart against injury during prolonged scenarios of hypoxia. *In vivo* the skeleton is under dynamic loading through daily activities as well as physical exercise. However, the physiological impact of bone mechanical loading on cardiac function has not been documented. To address this point, cardiac functional properties were determined using

electrocardiogram (ECG) in anesthetized male and female TOPGAL mice before, during, and after a two-and-a-half-minute cyclical compressive loading protocol in the tibia of a single hindlimb (Figure 12A). Resting heart rates of anesthetized mice varied from ~430-625 beats per minute at baseline before the loading protocol. Control animals, which were placed into the loading apparatus but received no mechanical loading, showed no significant change in heart rate during the entirety of the experiment. Conversely, within seconds after initiation of compressive loading in the tibia at 9 N, a significant immediate and transient 5%-8% decrease in heart rate was observed, followed by its restoration to approximately baseline levels near the end of the two and a half minute loading period (Figure 12B). This loading-dependent decrease in relative heart rate was observed in both male mice (control: 0.98 ± 0.02 vs. loaded: 0.94 ± 0.01 fold change from baseline; $p < 0.01$) and female mice (control: 0.99 ± 0.02 vs. loaded: 0.94 ± 0.01 fold change from baseline; $p < 0.01$) and followed a similar temporal pattern in both sexes (Figure 12C-D). Concomitant to the initial reduction in heart rate with loading, standard deviation of the RR interval (SDRR), a measure of overall heart rate variability (HRV), was significantly enhanced by 9%-44% during loading in both male mice (control: 1.01 ± 0.04 vs. loaded: 1.24 ± 0.01 fold change from baseline; $p < 0.01$) and female mice (control: 1.02 ± 0.02 vs. loaded: 1.23 ± 0.08 fold change from baseline; $p < 0.01$) (Figure 12C-D). Apart from the initial heart rate and HRV changes during loading, there were no sustained changes in heart rate or HRV relative to baseline during a 15-minute rest period subsequent to the end of loading (Figure 12C-D).

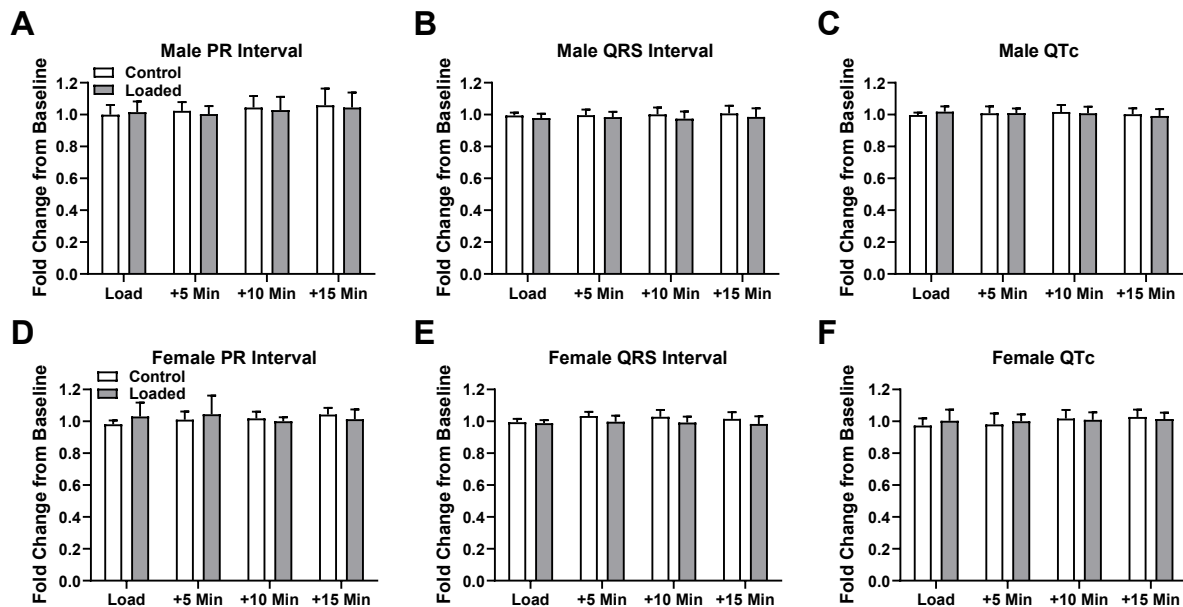
Figure 12.--Heart rate decreases and HRV increases in response to acute tibia mechanical loading in adult mice



A) Photograph of the *in vivo* bone loading setup showing animal positioning, direction of loading of the tibia and placement of ECG and body temperature recording leads. B) Tachograms of the resting heart rate in anesthetized control mice and in mice during the tibia mechanical loading protocol. C) Average male resting heart rate and standard deviation of the RR interval (SDRR) relative to baseline during mechanical loading and during 15 minutes after loading. D) Average female resting heart rate and standard deviation of the RR interval (SDRR) relative to baseline during mechanical loading and during 15 minutes after loading. ** $p < 0.01$ vs. control, Mixed Model ANOVA with Bonferroni post hoc analysis, $n = 6-7$ mice per group. Mean \pm SD. (Data originally presented in UMKC M.S. Thesis for Mark Gray)

Raw ECG signals were also examined during the loading experiments for determination of cardiac depolarization and repolarization properties. In contrast to alterations in heart rate and HRV, there were no significant changes to atrial or ventricular conduction parameters, including PR interval, QRS interval, or QTc interval during tibia loading or during the subsequent 15 minutes, which suggests no effect of bone loading on atrioventricular coupling, ventricular depolarization, or ventricular repolarization, respectively (Figure 13).

Figure 13.--ECG intervals are not affected during and after acute tibia mechanical loading in adult mice

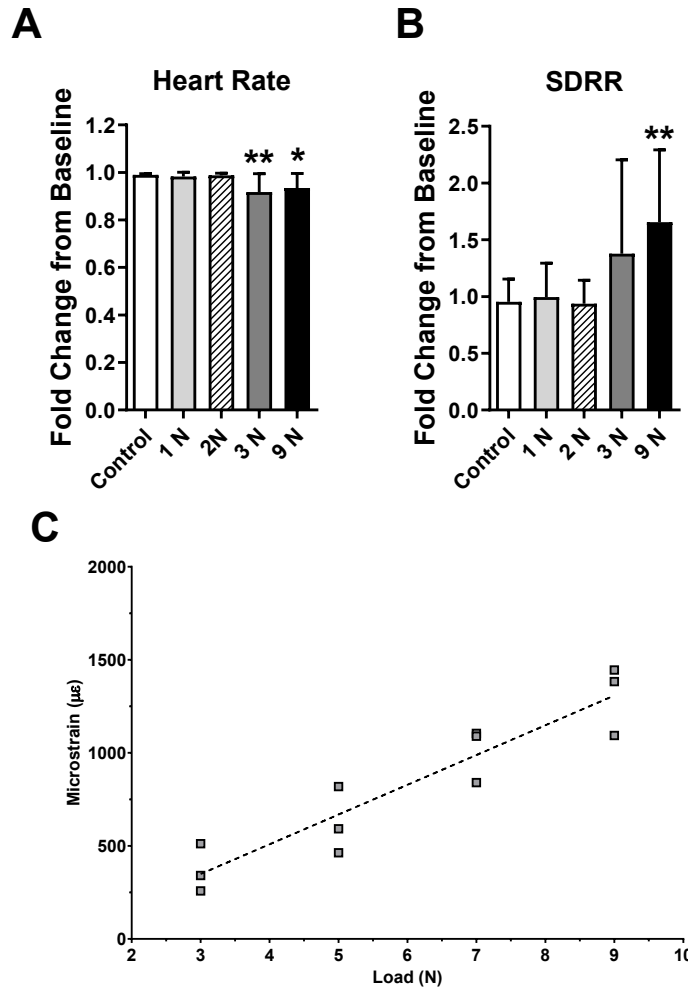


Male PR interval (A), QRS interval (B), and corrected QT interval (QTc) (C) relative to baseline during mechanical loading and during 15 minutes after loading. Female PR interval (D), QRS interval (E), and corrected QT interval (QTc) (F) relative to baseline during mechanical loading and during 15 minutes after loading. Mixed Model ANOVA, n=6-7 mice per group. Mean \pm SD. (Data originally presented in UMKC M.S. Thesis for Mark Gray)

Given that bone experiences a variety of loading forces and strains that are dependent upon the activity type, the responsiveness of the heart in adult CD-1 mice to physiological loads ranging from 1 N - 9 N was measured. These loading parameters cover a range of physiologically relevant strain inputs. It was found that loading of the tibia with 1 N or 2 N did not produce a significant change in heart rate and HRV, while loading at 3N and above activated a significant decrease in heart rate (Figure 14A). Interestingly, compared to heart rate in control mice (0.99 ± 0.01 fold change from baseline) the relative reductions in heart rate were similar at 3 N (0.92 ± 0.08 fold change from baseline; $p < 0.05$ vs control) and 9 N (0.93 ± 0.06 fold change from baseline; $p < 0.05$ vs control) suggesting that a specific threshold level of load

must be attained to activate these changes in heart rate (Figure 14A). Additionally, HRV became elevated during loading with 3 N (1.38 ± 0.83 fold change from baseline; $p > 0.05$ vs control) and 9 N (1.66 ± 0.64 fold change from baseline; $p < 0.05$ vs control) compared to control mice (0.95 ± 0.20 fold change from baseline), although this was only statistically significant for the 9 N load (Figure 14B). To determine the levels of strain produced within the tibia by loading, strain gauge measurements were captured in isolated tibiae from CD-1 adult mice and showed that the loading magnitudes of 3 N - 9 N corresponds with an average strain of $370 \mu\epsilon$ - $1307 \mu\epsilon$, respectively ((Figure 14C).

Figure 14.--Effect of mechanical loading at various magnitudes on heart rate and HRV in adult 2-6-month-old mice

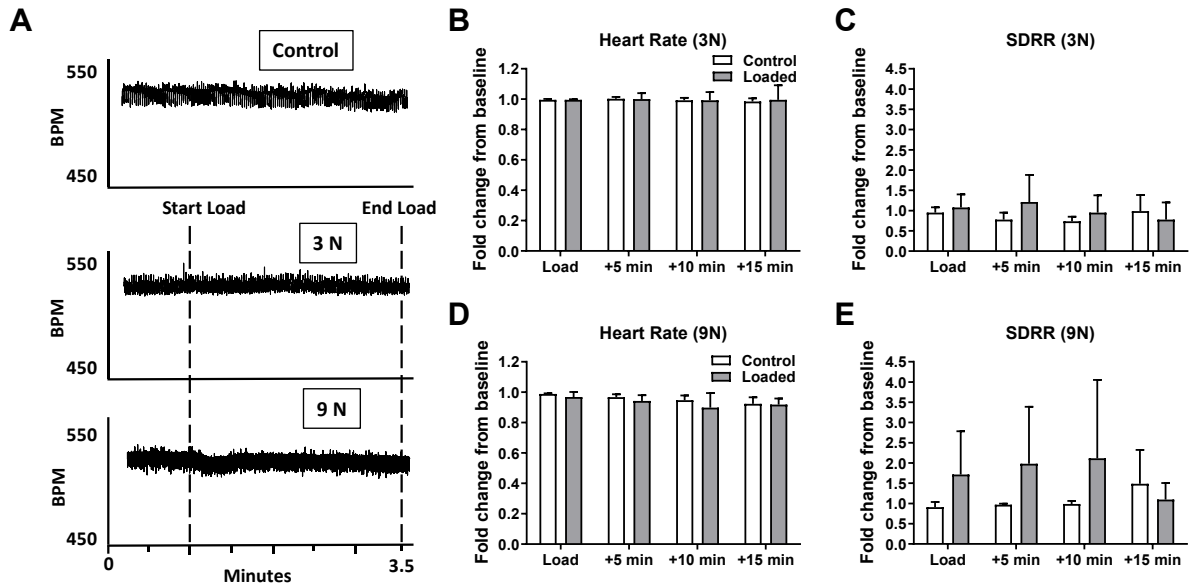


Average change in heart rate (A) and standard deviation of the RR interval (SDRR) (B) in response to tibia mechanical loading at 1-9 N. ** $p < 0.01$, * $p < 0.05$, one-way ANOVA with Bonferroni post hoc analysis, $n = 7-13$ mice per group. C) Strain gauge measurements in isolated tibia from adult mice during mechanical loading at 3-9 N. $n = 3$ mice. Mean \pm SD.

Considering the important impact of aging on many physiological and pathological processes, further loading experiments on middle-aged mice (11 months old) were performed. Intriguingly, loading of the tibia in this older age group did not result in significant changes to heart rate at either 3 N (control: 0.99 ± 0.0058 vs. loaded: 0.99 ± 0.0041 fold change from baseline; $p > 0.05$) (Figure 15A-B) or at 9 N (control: 0.99 ± 0.0048 vs. loaded: 0.97 ± 0.034 fold

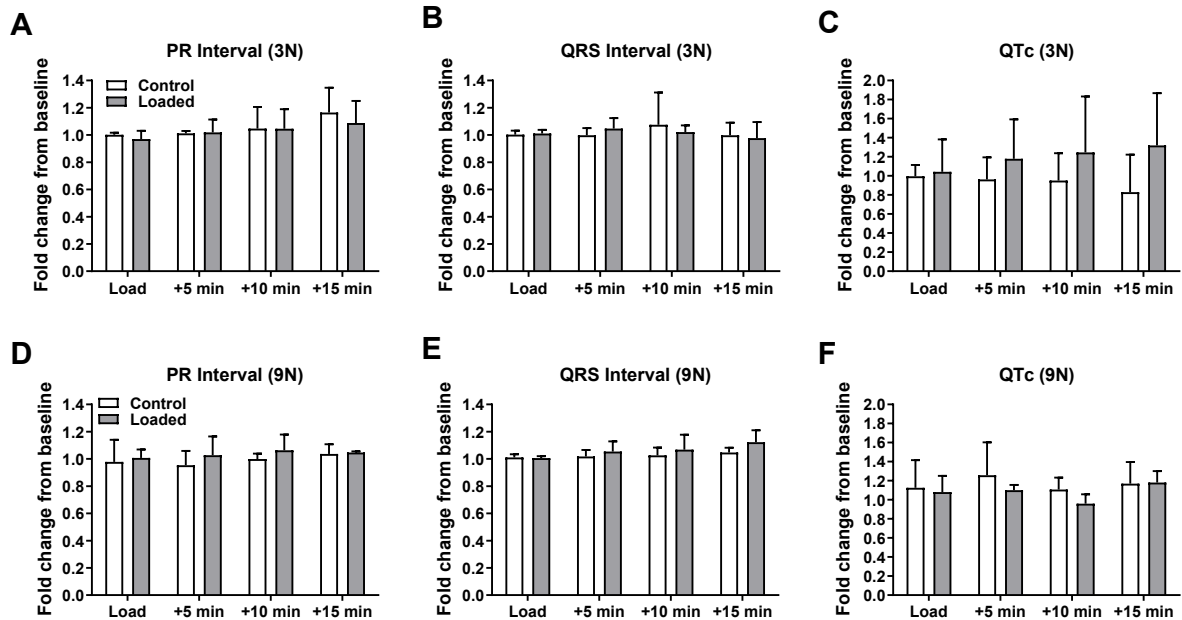
change from baseline; $p>0.05$) (Figure 15A & D) compared to control. Similarly, HRV was not significantly altered during loading at either 3 N (control: 0.94 ± 0.13 vs. loaded: 1.08 ± 0.32 fold change from baseline; $p>0.05$) (Figure 15C) and at 9 N (control: 0.91 ± 0.13 vs. loaded: 1.71 ± 1.07 fold change from baseline; $p>0.05$) (Figure 15E) compared to control mice, although there was an observable non-significant increase in the average HRV during loading at 9 N. These data suggest that the responses of heart rate and HRV to mechanical loading may weaken with age. With regard to the effect of aging on overall autonomic control of the heart, it was observed that average resting heart rate was not different when comparing adult mice to middle age mice (adult: 530.9 ± 57.9 vs. middle-age: 526.9 ± 59.0 BPM; $p>0.05$, data not shown) but average resting HRV was significantly reduced in the older animals (adult: 1.46 ± 0.72 vs. middle-age: 1.00 ± 0.49 SDRR; $p<0.05$, data not shown) indicating possible reduced autonomic control in this age group. Lastly, as was the case in the younger mice, loading at 3 N and 9 N in the middle-aged mice also did not produce significant changes to any of the ECG intervals measured (PR, QRS, QTc) (Figure 16).

Figure 15.--Heart rate and HRV responses to acute tibia mechanical loading are diminished in middle aged mice



A) Tachograms of resting heart rate derived from ECG signals in 11 month old, anesthetized control mice and mice during a tibia mechanical loading protocol at 3 N and 9 N. B) Average male resting heart rate and C) standard deviation of the RR interval (SDRR) relative to baseline during mechanical loading at 3 N and during 15 minutes after loading in 11 month old mice. D) Average male resting heart rate and E) standard deviation of the RR interval (SDRR) relative to baseline during mechanical loading at 9 N and during 15 minutes after loading in 11 month old mice. Mixed Model ANOVA with Bonferroni post hoc analysis, n=2-7 mice per group. Mean \pm SD.

Figure 16.--ECG intervals are not affected in response to acute tibia mechanical loading in middle aged mice

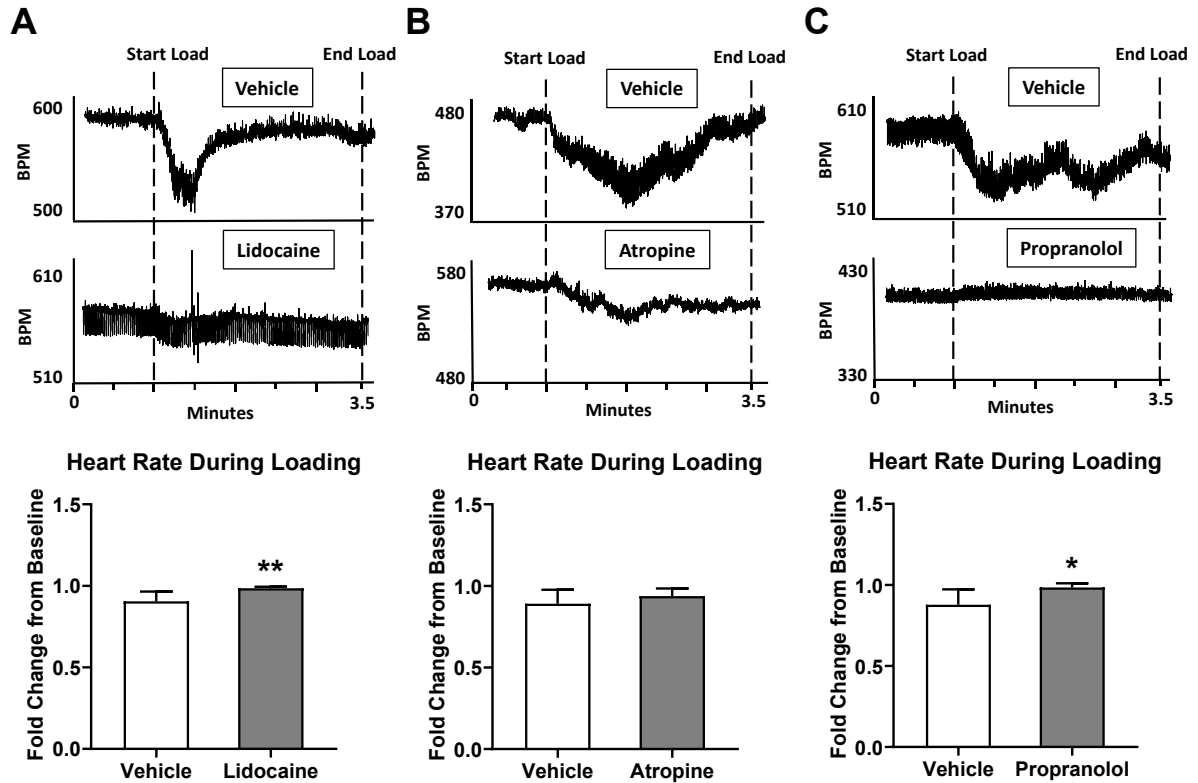


Male PR interval (A), QRS interval (B), and corrected QT interval (QTc) (C) relative to baseline during mechanical loading and during 15 minutes after loading in 11 month old mice. Female PR interval (D), QRS interval (E), and corrected QT interval (QTc) (F) relative to baseline during mechanical loading and during 15 minutes after loading in 11 month old mice. Mixed Model ANOVA, n=2-7 mice per group. Mean \pm SD.

The changes in heart rate in response to bone mechanical loading were rapid, occurring within several seconds of initiation of bone loading and suggested that this physiological phenomenon was possibly mediated by a neuronal mechanism. Therefore, the involvement of nerves in the hindlimb and the autonomic nervous system was next investigated through pharmacological blockade. First, to address activation of neural components in the loaded hindlimb, lidocaine was injected into the areas surrounding the tibia prior to the mechanical loading protocol while measuring ECG in anesthetized mice. Lidocaine pre-treated mice displayed a significant inhibition of the heart rate decrease during bone loading compared to

loaded mice treated with saline, suggesting that a neural afferent pathway mediates the signaling of bone loading to the heart (Figure 17A). Next, to determine if the autonomic nervous system was involved in this mechanism on heart rate, the inhibitor of parasympathetic muscarinic neurons, atropine, as well as sympathetic B1/B2 blocker propranolol by IP injections was utilized prior to bone loading in mice. Atropine pre-treatment did not result in a significant blockade of heart rate change in response to bone loading ($p=0.143$; Figure 17B). On the other hand, pre-treatment of propranolol effectively and significantly inhibited the heart rate decrease during bone mechanical loading (Figure 17C). These findings indicate reduced sympathetic tone as a major downstream mediator of heart rate reduction with bone loading in the efferent pathway of this bone-heart reflex.

Figure 17.--The heart rate changes in response to mechanical loading are mediated by afferent neurons in the hindlimb and reduced SNS tone



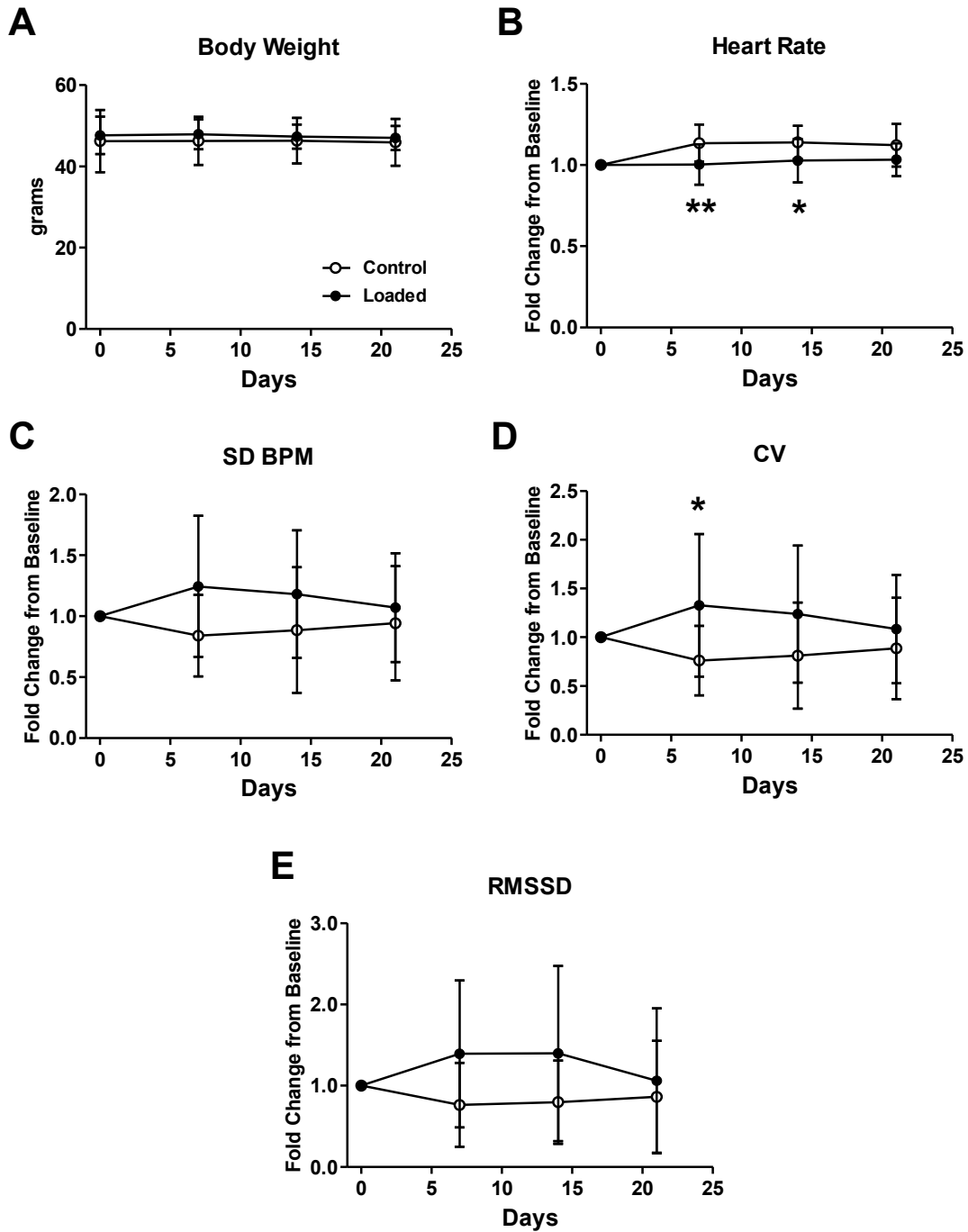
Representative tachograms of heart rate (Top) and quantification of average changes in heart rate relative to baseline (Bottom) in response to mechanical loading in mice pre-treated with lidocaine injected in the hindlimb (A), atropine injected IP (B), and propranolol injected IP (C). ** $p < 0.01$, * $p < 0.05$, two-tailed t-test, $n = 7-11$ mice per group. Mean \pm SD.

Chronic bone loading

It is challenging to isolate the dynamic loading experienced in bone during physical activity given the multiorgan involvement in this physiological process. However, previous work from our group has demonstrated that a repetitive bone loading regimen applied three-times per week over the course of three weeks is sufficient to increase bone formation⁸⁰. Given the findings that tibia loading can acutely modulate heart activity, the effect of regular tibia loading over three weeks on heart rate and ECG properties was investigated in a longitudinal

study. Average body weight did not differ among loaded and control groups over the three-week study (Figure 18A). Weekly ECG profiles captured in conscious mice at baseline and after each week of the loading regimen revealed a significantly lower resting heart rate relative to baseline after one week and two weeks of tibia loading compared to control mice (Figure 18B). Furthermore, loaded mice consistently showed increased HRV in relation to baseline values over the three-week period while control mice displayed reduced average HRV. A statistically significant enhancement of HRV was detected for the parameter of coefficient of variation (CV) in mice after one week of loading compared to control mice (Figure 18D), while root mean square of successive differences (RMSSD) showed a significant treatment effect ($p=0.0242$) but did not pass the post hoc analysis (Figure 18E). These results were consistent with the findings during acute loading, namely, the reduction in heart rate and enhancement in HRV, suggesting the effects of acute and chronic bone loading on the heart may be mediated by the same physiological mechanism involving the nervous system.

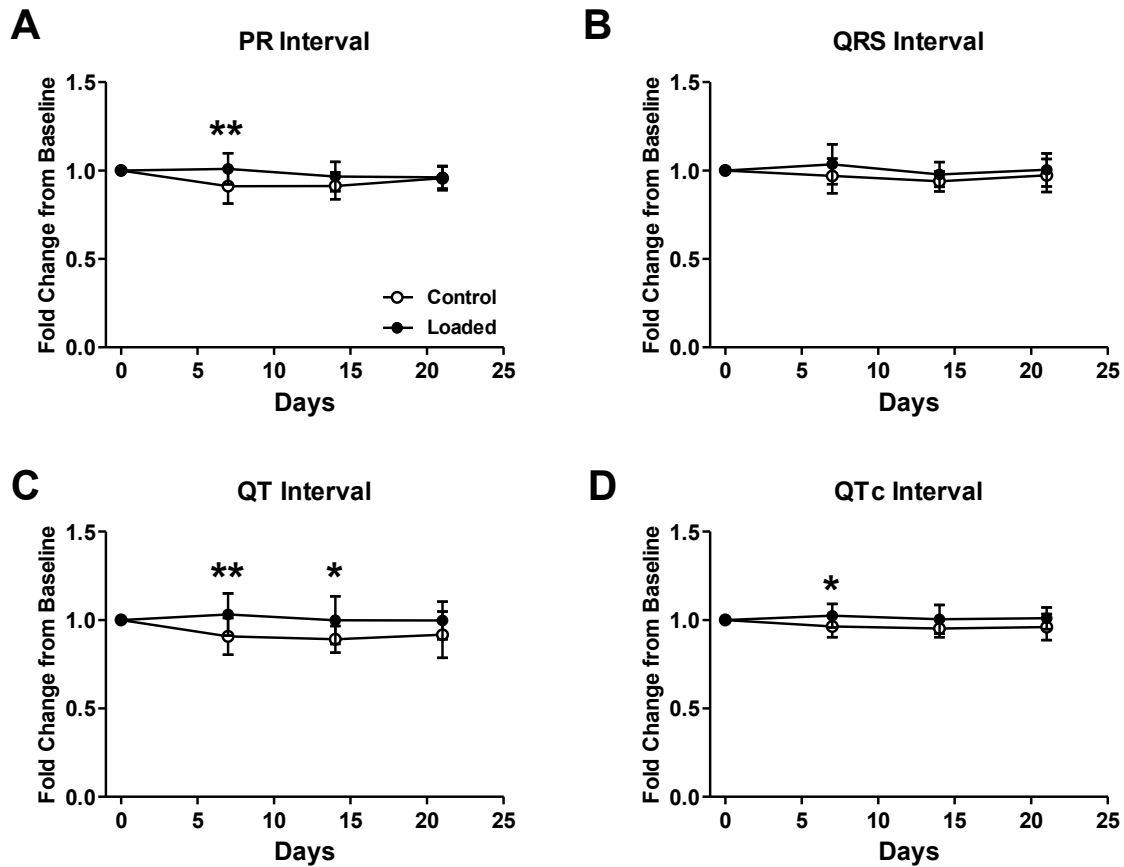
Figure 18.--Resting heart rate decreases and HRV increases in adult mice during three-weeks of tibia loading



Animal body weight (A) and weekly conscious ECG measurements of (B) resting heart rate, (C) standard deviation of heart rate (SD BPM), (D) coefficient of variation (CV), and (E) root mean square of successive differences (RMSSD) over the course of three-weeks in loaded and control mice. ** $p < 0.01$, * $p < 0.05$, Mixed Model ANOVA with Bonferroni post hoc analysis, $n = 14$ mice per group. Mean \pm SD.

With respect to the electrical properties of the heart, several ECG intervals were found to be significantly altered in response to loading. PR interval, representing atrioventricular coupling time, was significantly longer relative to baseline in mice after one week of loading in comparison to control mice (Figure 19A), while the ventricular depolarization time, QRS interval, did not display any differences among groups (Figure 19B). In addition, QT interval, an index of ventricular repolarization time, showed significantly longer values in mice receiving loading compared to control mice at both the one week and two-week time points (Figure 19C). Interestingly QT interval corrected for heart rate (QTc) was also significantly elevated in relation to baseline in mice after week one of loading (Figure 19D).

Figure 19.--ECG intervals are altered during three-weeks of tibia loading

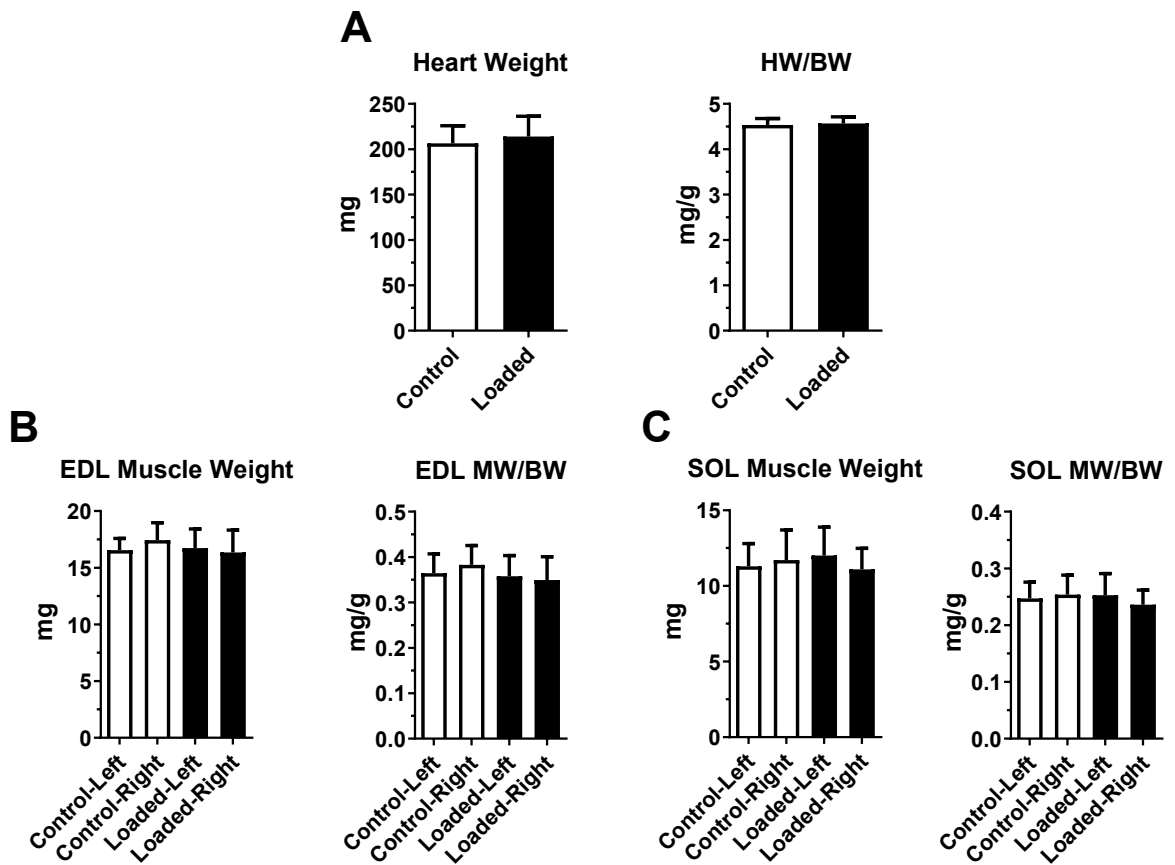


Weekly conscious ECG measurements of (A) PR interval, (B) QRS interval, (C) QT interval, and (D) corrected QT interval (QTc) over the course of three-weeks in loaded and control mice. ** $p < 0.01$, * $p < 0.05$, Mixed Model ANOVA with Bonferroni post hoc analysis, $n = 14$ mice per group. Mean \pm SD.

After the three-week loading protocol, the heart and skeletal muscles in the vicinity of the tibia, including extensor digitorum longus muscles (predominantly fast-twitch) and soleus muscles (predominantly slow-twitch) were isolated for size and muscle functional testing. As the EDL and soleus are anatomically positioned adjacent to the tibia, investigation of these skeletal muscles would provide some insight into potential changes to physical performance that may have occurred in hindlimb subjected to the three-week bone loading regimen. Heart

size, including wet weight and heart weight to body weight ratio (HW/BW), was not statistically different among control and loaded mice indicating that extended tibia loading did not induce cardiac hypertrophy or remodeling (Figure 20A). In the case of skeletal muscle, the EDL and soleus showed no significant differences in weight or muscle weight to body weight ratio (MW/BW) (Figure 20B-C).

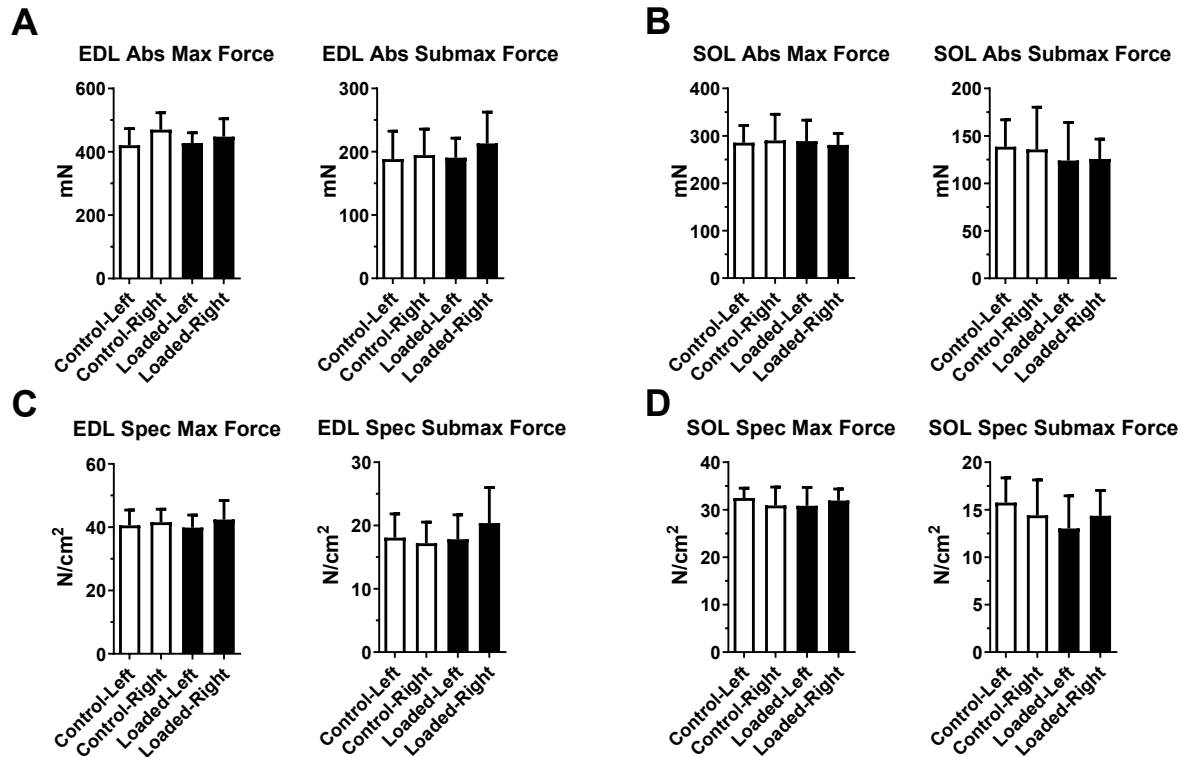
Figure 20.--Heart and skeletal muscle weights are not affected with three weeks of tibia loading



A) Heart weight and heart weight to body weight ratio (HW/BW) from three-week loaded or control mice. Two-tailed t-test, n=14 hearts per group. B) EDL muscle weight and muscle weight to body weight ratio (MW/BW), and C) SOL muscle weight and MW/BW from either the left or right limb of three-week loaded or control mice. One-way ANOVA, n=14 mice per group. Mean \pm SD.

Ex vivo skeletal muscle contractility analysis also showed no significant changes among groups in maximal or submaximal absolute contractile force production or in muscle specific force, which is the force normalized to the physiological cross-sectional area of the muscle (Figure 21). These findings showing that the regular tibia loading protocol did not alter hindlimb skeletal muscle size or strength in this model indicating that the mechanically loaded limbs of mice retained normal physical function during the study and were not damaged as a result of chronic loading. Thus, changes in mobility were likely not a contributing factor to the observed impacts of the three weeks of bone loading on the ECG data, further strengthening the direct causal association of bone loading to the cardiac findings.

Figure 21.--*Ex vivo* skeletal muscle contractile force is unaffected by three-weeks of tibia loading



A) EDL absolute maximal and submaximal force, B) soleus maximal and submaximal absolute force, C) EDL specific maximal and submaximal force, and D) soleus specific maximal and submaximal force in left and right limbs of three-week tibia loaded and control mice. One-way ANOVA, n=14 mice per group. Mean \pm SD.

CHAPTER 5

DISCUSSION

Bone exists in a dynamic interplay with external forces and other organ systems to maintain its homeostatic state within the body. On top of its elegant self-regulatory abilities, bone is now recognized to regulate the homeostasis of many other organ systems through endocrine/paracrine signaling functions and to communicate with the peripheral and central nervous systems. Previous studies from our group suggest a dynamic and delicate balance of maintenance of individual health by the skeleton. We have investigated the beneficial endocrine/paracrine reciprocal interactions between bone and skeletal muscle tissue and its implications for the aging process^{22, 89}. Our group has also examined the bone-heart-kidney endocrine axis of bone-secreted protein FGF23. FGF23 normally controls phosphate excretion in the kidney, but when excessively elevated in the circulation, as seen in chronic kidney disease, FGF23 can signal in the heart and contribute to the development of pathological cardiac hypertrophy, altered calcium signaling, and arrhythmias often presenting in patients with renal failure^{67, 90}. This dual role of bone in health and disease has prompted our laboratory to further investigate the clinical and physiological links of bone with the heart. Along these lines, it may be possible that disruption of the bone anabolic response and deterioration of the bone microenvironment as seen in osteoporosis and aging, could interfere with health-promoting bone-heart crosstalk and contribute to the progression of cardiac-related comorbidity as seen in numerous association studies of osteoporosis and CVD. To further

establish the clinical associations of bone with the heart and to investigate their molecular connection from the perspective of bone mechanical loading, which has largely been unexplored, a clinical investigation of novel associations of osteoporosis and CVD outcomes along with *in vitro* and *in vivo* mouse studies to explore the mechanisms of bone mechanical loading on heart physiology were undertaken. The results show for the first time that history of osteoporosis is associated with prolonged CVD-related LOS for index hospital admissions in both female and male patients and sex-specifically increases the biomarker of CVD, NT-proBNP, and mortality incidence. At the cellular level, mechanical loading in bone cells was associated with secretion of soluble factors that protected heart cells from ischemia. Moreover, the mouse studies revealed a novel bone-heart reflex mediated by bone mechanical loading and the autonomic nervous system that regulates heart rate and HRV and is diminished by middle age. These findings highlight novel connections between bone and the heart which have wide implications for the understanding of heart physiology in aging and disease. Lastly, the results from each of the study aims are collectively in support of the overall hypothesis that bone has a positive modulatory influence on heart physiology via the release of bone-derived hormones and neural signaling pathways, which are altered with aging and associated with the dual clinical manifestation of bone and cardiac pathology.

Clinical Studies

This clinical study draws from the large Cerner Health Facts database encompassing many different hospitals and patient populations to provide robust patient cohort sizes and statistical power to draw conclusions from focused populations such as male osteoporosis patients. Sex differences concerning negative effects of osteoporosis on CVD-related

outcomes were a common finding in this study. Studies examining sex differences with regard to osteoporosis patient outcomes are sparse, especially for associations specific to CVD, typically because the male osteoporosis patient groups relative to females are smaller and underpowered^{51, 52}. In this study, the patient group with a history of osteoporosis was 86% female and showed a 5.2% prevalence among CVD diagnoses (heart failure, ischemic heart disease, cardiomyopathy, arrhythmias, hypertensive-related disease) compared to 0.8% prevalence for males. Although females comprise the majority of osteoporosis diagnoses compared to males, the average length of hospital stay for females with osteoporosis was increased by 0.66 days (+25%) compared to a greater increase of 1.68 days (+55%) in males for all-cause CVD (Figure 1A). Males with history of osteoporosis also showed greater odds of prolonged LOS (Table 4, Figure 3) and higher mortality (Figure 6), suggesting history of osteoporosis is more detrimental to cardiac health outcomes in males. A recent large-scale study using the UK Biobank cohort also found sex differences showing that females with history of osteoporosis, but not males, had significantly higher risk for the development of CVD in adjusted models, while males with history of osteoporosis experienced worse outcomes related to CVD including higher risk for mortality, although history of CVD seemed to explain this association in males⁵⁰. On the other hand, the results of the current study showing increased mortality in male CVD patients with a history of osteoporosis were calculated from index encounters for CVD, meaning there was no prior history of admittance for CVD in these patients. Even without prior osteoporosis diagnosis, males with CVD had longer hospital stays (Figure 1), higher BNP/NT-proBNP levels (Figure 5) and greater mortality (Figure 6) than females, but these sex differences were greatly exacerbated by osteoporosis.

Previous studies have typically focused on the link between poor bone quality and the development of CVD ^{4, 50, 52, 69}. This retrospective study is the first to specifically measure the influence of history of osteoporosis with patient recovery from CVD as assessed by length of hospital stay, a clinical outcome which has received little attention among previous investigations of bone and cardiac health. Changes to the total number of days a patient remains hospitalized has multiple negative consequences including to the overall well-being of the individual, likelihood of acquiring nosocomial diseases, as well as the added economic burden, especially in older patients. Prolonged length of stay increases the time of bed rest experienced during hospital visits which is associated with negative changes in bone and skeletal muscle including sarcopenia ⁹¹ and compromised sensitivity to insulin ⁹². Thus, prolonged stays can exacerbate musculoskeletal decline leading to an array of adverse outcomes including heightened risk of disability ⁹³. It has been estimated that muscle strength declines by 5% per day of hospital bed confinement ⁹¹. Moreover, the amount of bone lost over 10 days of hospitalization would require four months to restore ⁹³. Thus, an increased length in hospital stay for CVD in those with history of osteoporosis would likely translate to worse outcomes in the acute setting as well as over the long-term.

Comorbidity burden is a known strong predictor of hospital stay duration and is an important covariable for statistical models predicting length of stay ^{94, 95}. The Charlson comorbidity index is a sophisticated diagnostic tool based on a variety of comorbidity parameters specific to a patient's individual clinical situation, which can aid in assessment of the risk of patient outcomes including mortality and hospital readmissions among other prognoses ^{96, 97}. Comorbidities accounted for by the Charlson index include congestive heart failure, pulmonary disease, diabetes, renal disease, dementia, liver disease, and cancer, with

cumulative scores categorized as mild (score of 1-2), moderate (score of 3-4) or severe (score of ≥ 5). Osteoporosis history was found to be an independent predictor even after controlling for Charlson comorbidity index suggesting that there are additional factors related to osteoporosis that extend beyond the comorbid disease burden which influence cardiac health outcomes.

BNP is a peptide hormone secreted by ventricular cardiomyocytes in response to stress in order to induce vasodilation and renal excretion of electrolytes and fluid. BNP is cleaved from its pro-peptide, proBNP, and released into the circulation as carboxy-terminal and amino-terminal fragments, BNP and NT-proBNP, respectively. Metabolism of BNP and NT-proBNP differs as NT-proBNP remains for longer periods in the circulation compared to active BNP fragment. The levels of BNP and NT-proBNP have demonstrated important clinical diagnostic value for heart disease and mortality risk assessment, especially in heart failure patients ⁹⁸. BNP and NT-proBNP have also been found to be increased in patients with atrial fibrillation ⁹⁹, left ventricular hypertrophy ^{100, 101}, or following myocardial infarction ¹⁰². In the current study, among all-cause CVD patients, females with a history of osteoporosis displayed significantly higher serum NT-proBNP concentrations than those with normal bone density (Figure 5), which significantly correlated with longer time spent in the hospital. Interestingly, the current findings are similar to other recent studies which have also observed elevations in the levels of BNP and NT-proBNP in individuals with poor bone quality. Bone mineral density in the lumbar spine has been reported to exhibit an inverse relationship with serum NT-proBNP levels in dialysis patients ^{103, 104}. A cross-sectional investigation of type II diabetes mellitus patients found a nearly 3-fold elevation of BNP concentration in those with osteoporosis, but not osteopenia, compared to those with normal bone mineral density ¹⁰⁵. Lastly, the presence

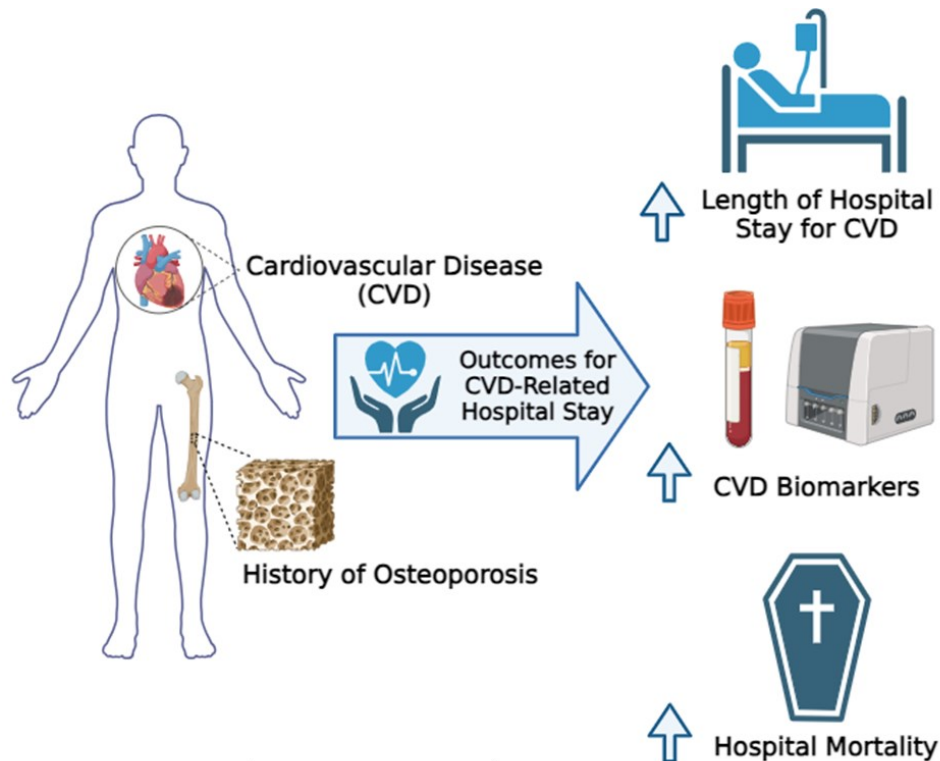
of osteoporosis in a cohort of chronic heart failure patients was associated with elevated NT-proBNP and higher mortality ¹⁰⁶. The findings of elevated heart disease biomarkers in this study indicate a worsening of cardiac tissue health status in those with a history of osteoporosis which may directly prolong recovery and could explain the observed prolongation of time spent hospitalized for CVD.

This retrospective cohort study contained some limitations which could affect the clinical interpretation of the findings. Firstly, this analysis provides only statistical associations among history of osteoporosis and CVD-related patient outcomes and cannot delineate direct causation of the relationships from the clinical data. Next, as the study relied on electronic health record data from the Health Facts database, the content of patient variables was somewhat limited in scope. For instance, diagnostic values of physiological measurements such as bone mineral density (g/cm^2), T-score (SD) and blood pressure (mmHg) were unavailable for inclusion in the statistical models, which would have allowed a more precise control and determination of association of bone loss and CVD patient outcomes, especially given that patients with more severe bone loss have been found to have greater associations with CVD parameters. Moreover, the study was unable to gauge personal health habits of patients including daily physical activity level and diet, which play an important role in patient outcomes. However, these lifestyle variables would also be reflected to some degree in CC index, obesity and smoking status which were included as covariates in the statistical models of this study. Lastly, medications used in the treatment of osteoporosis were not investigated as part of this study. Many traditional drugs for osteoporosis treatment such as bisphosphonates, selective estrogen receptor modulators, parathyroid hormone analogues, and denosumab (monoclonal antibody for RANKL) have shown little impact on the cardiovascular

system after much research¹⁰⁷. On the other hand, estrogen hormone replacement therapy has been found to carry some risk of CVD specifically related to blood clots in patients >60 years of age or >10 years after menopause¹⁰⁷. In addition, the more recently developed anabolic drug, Romosozumab, (monoclonal antibody for sclerostin) is not recommended for use in those with a history of myocardial infarction or stroke as a recent meta-analysis reported its use is significantly associated with increased risk of major adverse cardiac events, possibly due to altered Wnt signaling in the cardiovascular system by Romosozumab¹⁰⁷. However, Romosozumab was only recently approved in 2019 by the Food and Drug Administration for clinical use, and thus would likely not have been utilized in the patient set of the current study. Hence, further studies are needed to fully examine the cardiovascular impact of Romosozumab use in these patient groups.

In conclusion, the results of this large-scale clinical study, summarized in Figure 22, provide novel evidence that history of osteoporosis negatively impacts CVD-related hospital outcomes. These findings could have significant implications for considerations of optimal care of CVD and osteoporosis patients and to aid in the identification of CVD patients at elevated risk for adverse outcomes, particularly in men. These results are also suggestive for maintaining adequate bone quality for preventative care of the cardiovascular system.

Figure 22.--Clinical association of history of osteoporosis with CVD-related hospital outcomes



Retrospective analysis of the association of osteoporosis history with patient outcomes for CVD-related hospital admittance was performed utilizing the Cerner Health Facts database. History of osteoporosis independently predicted longer hospital stay for CVD in both men and women and was associated with increased patient mortality and elevated CVB biomarker levels of NT-proBNP in a sex-specific manner. Prolongation of hospital stay for CVD and hospital mortality was greatly exacerbated in men with a history of osteoporosis compared to women. Although both men and women with a history of osteoporosis displayed higher NT-proBNP levels, this increase was only statistically significant for women.

In Vitro Studies

The identification of osteoporosis as a significant modulator of cardiac health outcomes independent of traditional risk factors and comorbidity burden is suggestive of additional interactions between bone and the heart. As the documented endocrine ability of bone tissue continues to accumulate, it is imperative to measure the physiological role that bone endocrine

signaling may have in cardiac tissue, which could explain the clinical data. To explore this possible interaction from a bone endocrine perspective, CM from MLO-Y4 osteocytes containing secreted factors were tested and revealed that FFSS (i.e. mechanical loading) caused the release of cardioprotective factors from MLO-Y4 osteocyte cells which mitigated hypoxia-induced cardiac cell death *in vitro* (Figures 8 & 9). Myocardial hypoxia occurs when oxygen concentration falls below cellular consumption demand and can result as a consequence of reduced blood perfusion to heart muscle during acute myocardial infarction or ischemic injury, a major contributor for heart failure development ¹⁰⁸. During prolonged myocardial infarction cardiomyocytes undergo cell death in the areas of the myocardium experiencing hypoxia through both necrotic and apoptotic mechanisms ¹⁰⁹. The scale of the loss of viable heart cells after a major myocardial infarction in the human heart can be massive, encompassing more than a billion cardiomyocytes ¹¹⁰. In addition, the setting of chronic disease like heart failure is associated with increased cardiomyocyte apoptotic cell death markers within ventricular tissue indicating that loss of cardiomyocytes contributes to the overall heart contractile dysfunction and failure of the organ ¹¹¹. The findings with bone CM in this study demonstrate proof of concept that bone cell secreted factors can positively regulate cardiomyocytes and suggest it is not only having healthy bone cells *per se*, but the process of mechanical loading in healthy bone cells that is necessary for optimal release of bone factors that promote cardiomyocyte survival under hypoxic stress.

The process of mechanical loading in bone has been found to be an important stimulus for locally maintaining osteocyte cell survival through the actions of secreted factors ¹¹². These same factors may also travel outside the bone and positively regulate other nearby tissues. PGE₂ is an important rapid response molecule that accumulates in bone tissue immediately

following bone loading. FFSS applied to MLO-Y4 cells for two hours in culture (same conditions for CM as tested the current study), has been previously found to increase in PGE₂ concentration over 50-fold from baseline levels reaching approximately 1200 pg/ml in the CM²¹. The actions of PGE₂ include increasing bone formation and acting as a potent cell survival factor for osteocytes by signaling through the EP2 and EP4 receptors leading to activation of the cAMP/PKA and PI3K/Akt/GSK-3/β-catenin pathways¹¹³. Other studies suggest that skeletal muscle tissue may also benefit from bone-secreted PGE₂ as it has been shown that application of MLO-Y4 CM and PGE₂ both enhance muscle fiber formation and proliferation of muscle precursor cells *in vitro*^{22,23}. PGE₂ has also been found to be protective in heart tissue as increased PGE₂ levels achieved via cardiac overexpression of cyclooxygenase-2 (COX-2) reduced infarct size by around 50% following I/R injury in mice¹¹⁴. While PGE₂ shows potential for bone-heart protective signaling, its rapid turnover rate in tissue and in the circulation may dampen its effects on the heart *in vivo*¹¹⁵. Thus, more stable protein factors may play a greater role in mediating bone-heart crosstalk. Indeed, studies have identified several proteins secreted by MLO-Y4 cells specifically in response to mechanical loading including chemokine (C-C motif) ligand 7 (CCL7) and bone morphogenic protein 7 (BMP-7) that promote cell survival of osteocytes in the face of apoptosis-inducing conditions^{116, 117}. BMPs in particular are readily detectable in the serum at biologically active levels¹¹⁸ and BMP-7 is currently a potential treatment candidate for promoting protection against cardiac inflammation, apoptosis and fibrosis in heart disease patients¹¹⁹. The observations that aging and pathological conditions including osteoporosis and disuse are associated with substantial loss of viable osteocytes¹²⁰ and that conditions that promote osteocyte cell death also inhibit the release of protective factors from osteocytes¹¹³, in combination with the findings that these

factors also benefit cardiac cells, provide support that a bone-heart endocrine signaling axis may exist to promote positive regulation of cardiovascular system health.

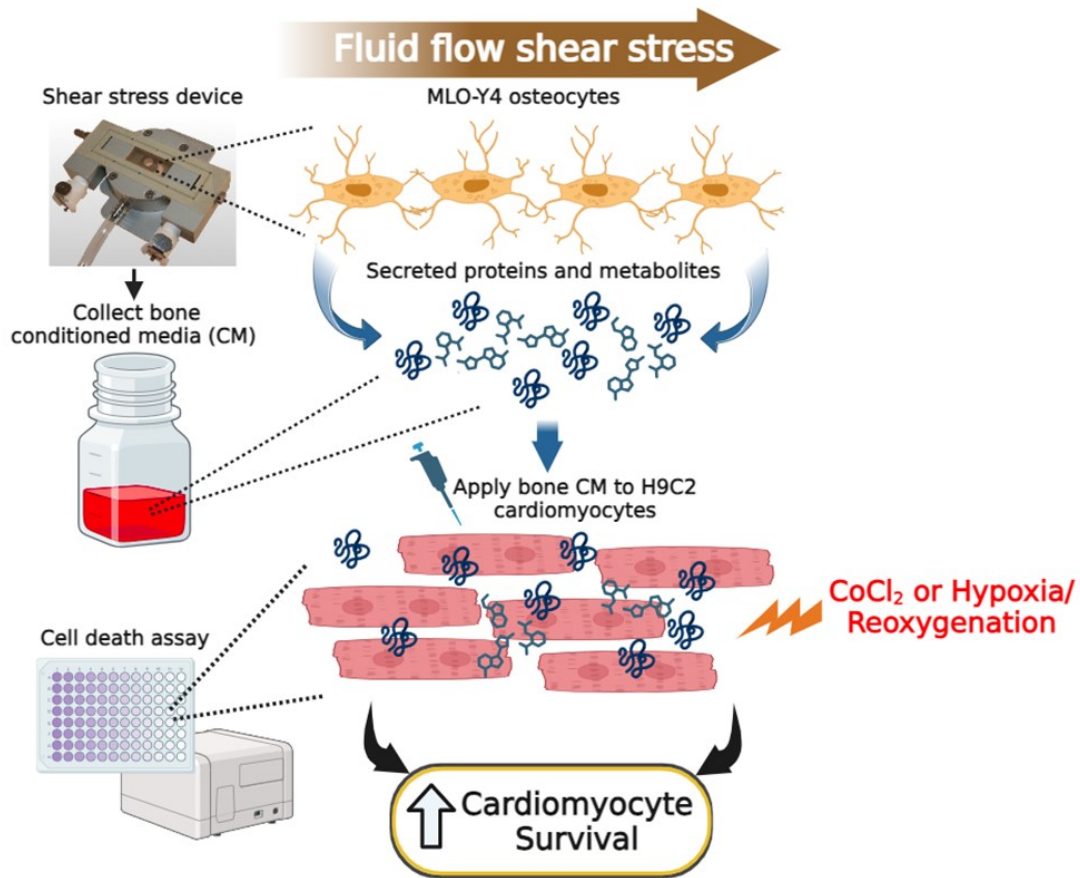
Although bone CM offered protection against cell death induced by hypoxia and reoxygenation in the cell culture models at 0.75% and 7.5%, the same concentrations did not translate to improved recovery of cardiac contractile function in Langendorff perfused mouse hearts subjected to acute global I/R injury (Figures 10 & 11). However, in contrast to the large-scale loss of viable cardiomyocytes during prolonged H/R as tested in the cell culture assays, the disruption of contractile performance in response to acute I/R injury is mainly orchestrated by a multitude of rapid cellular response mechanisms including metabolic dysfunction, dysregulation of calcium homeostasis, production of free radicals, acidosis, and inflammation. The acute drop in ATP production during oxygen depletion in ischemia leads to accumulation of calcium, hydrogen and sodium ions within the cytosol of the cardiomyocyte which persists into the reperfusion phase as coronary flow is restored causing further damage to the heart, known as reperfusion injury ¹²¹. Calcium overload is a major mechanism of myocardial injury mainly due to increased reverse mode activity of the membrane sodium-calcium exchanger (NCX) channel which directly triggers the development of sustained hypercontracture within the contractile filaments and impairs contractile functional output for prolonged periods after the original I/R event, known as cardiac stunning ¹²¹⁻¹²³. Moreover, the switch to anerobic respiration and reduction in cellular pH during I/R creates an inhibitory environment not suitable for cardiac contractility. The dysregulation of metabolic homeostasis induces production of free radicals during ischemia and especially in response to the sudden oxygen availability during reperfusion of the heart, that in turn inflict damage to proteins involved in excitation-contraction coupling, mitochondria and the phospholipids of the sarcolemma,

further exacerbating cardiomyocyte damage and contractile dysfunction¹²⁴. Although ion levels, energy balance and ultimately myocardial contractility can be eventually restored to viable areas of the heart with reperfusion techniques, the restoration takes time and the derangements in myocellular processes and reduced cardiac contractility can persist for hours during this recovery process¹²¹. Hence, the mechanisms of I/R injury in the acute setting that impact contractile function of the beating cardiac organ involve a complex interplay of intracellular factors that occur prior to the widespread induction of cell death as was assessed the cell culture models of prolonged hypoxia and reoxygenation. The results therefore suggest that bone secreted factors at the concentrations tested may chiefly protect cardiomyocytes from cell death during more prolonged periods of hypoxia and reoxygenation, but do not offer protection against the acute contractile dysfunction that is manifested immediately following I/R injury.

The *in vitro* studies were designed as proof of principle of bone-heart crosstalk pathways mediated by bone-secreted molecules, but additional future studies addressing some the inherent study limitations are important. One such limitation is that it remains unknown to what levels bone-secreted molecules accumulate in the circulation of animals that are subjected to skeletal loading stimulus. In the future, it will be important to perform metabolite, lipid and protein profiling on bone cell CM as well as on serum samples obtained from animals that have been subjected to mechanical bone loading for acute and prolonged periods of time. Next, there were inherent differences between the protocols and outcome variables of H/R in cultured cells and I/R in intact hearts which did not allow for direct comparisons of the results. For instance, the duration of bone CM pre-exposure was much more prolonged in cells (24 hours) compared to whole hearts (30 minutes) as was the overall duration of hypoxic stress (24 hours in cells

vs. 30 minutes in whole hearts). This was mainly due to the limitations inherent within the models themselves as cardiac tissue contractility cannot be maintained *ex vivo* for longer than several hours and primary cardiomyocytes do not survive more than 24 hours in culture. In conclusion, the findings of this study provide evidence that the endocrine capabilities of the skeleton, specifically of osteocytes within the context of bone loading, may extend towards offering protection to cardiac tissue during periods of pathological stress (Figure 23).

Figure 23.--Cardioprotective factors are released by bone cells mechanically loaded *in vitro*



In vitro studies were performed to investigate bone-heart crosstalk from a bone-endocrine perspective using conditioned media (CM) derived from MLO-Y4 osteocytes stimulated by fluid flow shear stress (FFSS), which mimics bone mechanical loading. Pretreatment of differentiated H9C2 cardiomyocytes with FFSS CM from bone cells promotes cardiomyocyte survival during prolonged CoCl₂ treatment (hypoxia mimetic) and hypoxia/reoxygenation as assessed by MTT cell viability assay. These data support a protective bone-heart endocrine connection that may contribute to cardiac health.

Animal Studies

At the level of the whole bone *in vivo*, the physiological output of mechanical loading involves multiple factors including the release of soluble proteins/metabolites as well as the stimulation of resident nerve fibers that activate the peripheral and central nervous system to promote bone anabolism. As the impact of acute and chronic bone loading on heart function *in vivo* has not been described previously, these studies were undertaken. The findings reveal

a novel bone-heart reflex mediated by bone mechanical loading and the autonomic nervous system. A rapid and transient lowering of resting heart rate and enhancement of HRV was documented during a cyclic compressive loading protocol in a single tibia in adult anesthetized mice regardless of sex (Figure 12). Parameters of intrinsic cardiac muscle conduction such as PR, QRS, and QTc remained unaffected with bone loading (Figure 13), suggesting this effect was mediated, likely, via changes in autonomic nervous system tone on the sinoatrial (SA) node and not a direct action on cardiac muscle or other cardiac conduction pathways. Inhibition of this bone-heart reflex with localized lidocaine (Figure 17A) confirmed the mechanism was of neural origin, specifically via afferent nerves in the hindlimb. The use of propranolol demonstrated the involvement of receptors associated with the sympathetic autonomic nervous system (Figure 17C). The exact processing of this bone loading-mediated signal in the brain is still not completely resolved but may involve a neural relay mechanism within the hypothalamus as described previously¹²⁵, and/or direct signaling with autonomic control centers in the medulla to lower sympathetic tone on the heart. This bone-heart neural mechanism is summarized in Figure 24.

Studies in transgenic and genetic deletion mouse models have identified a central control mechanism governing skeletal bone mass homeostasis involving the central nervous system and the sympathetic and parasympathetic arms of the autonomic nervous system^{25, 28, 32}. Other than relaying osteogenic cues, the SNS and PNS innervate and modulate functional output of nearly all major organ systems in the body including fat, liver, kidney, intestines, lungs, blood vessels, and heart. The nearly ubiquitous distribution of the autonomic nerves throughout the body ensures extensive connections among organ systems to maintain homeostatic balance. Therefore, it is possible that bone-derived neural signals that recruit

activation of the autonomic nervous system would also affect cardiovascular function. Accordingly, phenotypic changes in cardiac function, such as altered heart rate and HRV, would be expected to accompany the high and low bone mass presentations in mouse models where the bone-related endocrine-neuro mediators have been altered. In support of this notion, it has been previously shown that interleukin-1 (IL-1) signaling via the hypothalamic IL-1 receptor type 1 (IL-1RI) suppresses bone formation by ramping up osteoclast-mediated bone turnover through a PNS-related mechanism ¹²⁶. Silencing of the IL-1RI using a transgenic IL-1RI antagonist overexpression mouse model resulted in marked bone loss accompanied by a higher resting heart rate when SNS activity was masked ³¹. Another molecular mediator involved in the negative regulation of bone mass by the CNS is the peptide neuromedin U, which has been demonstrated to be an important downstream mediator of SNS-based control of bone mass by leptin signaling ¹²⁷. Intracerebroventricular injection of neuromedin U dose-dependently increased resting heart rate and mean arterial blood pressure in rats ¹²⁸. These findings demonstrate an intersection of shared neural pathways or mechanisms regulating both bone mass accrual and cardiac function, which are in line with the current findings showing a coupled responsiveness of heart rhythm properties with loading-induced strain in bone. However, whether this bone-heart connection plays a specific physiological role on the regulation of the heart or if it merely represents a generalized effect on autonomic tone to produce favorable conditions for the skeleton remains unclear.

A physiological bone loading protocol was employed in this study under a range of loading forces correlating with normal daily activity and up to a high impact style of activity. Utilization of the 9 N loading protocol in the tibia produces sufficient microstrain to activate osteogenic signaling in bone cells and a bone anabolic response as previously reported by our

group and others using this model ^{79, 129, 130}. Through strain gauging studies it was found that *ex vivo* tibial bone loading in 6-month-old CD-1 mice ranging from 3 N to 9 N resulted in mean tibial microstrain ranging from 370.25 $\mu\epsilon$ to 1307.08 $\mu\epsilon$, respectively (Figure 14C). For reference in humans, a study focusing on the relationship between physical activity in humans and microstrain on the tibia observed that the mean tibial microstrain during walking at 6.1 kilometers per hour was 658.11 $\mu\epsilon$, reaching as high as 1011.15 $\mu\epsilon$ when the subjects were carrying an additional 35 kg of weight ¹³¹. Incredibly, tibial microstrain reached magnitudes of greater than 2000 $\mu\epsilon$ in the middle of the tibia and greater than 3000 $\mu\epsilon$ in the distal third of the tibia when human subjects landed from a 45-centimeter vertical jump, while running on the treadmill at 13 kilometers per hour produced a range from 101-1950 $\mu\epsilon$ ¹³². Other activities for which human tibial microstrain data have been reported include stepmaster (1006 $\mu\epsilon$), bicycling (291 $\mu\epsilon$), and leg press (883 $\mu\epsilon$) ¹³³. In addition to these data, load forces of 1-9 N were selected based on Harold Frost's "Mechanostat Theory," in which Frost details the evidence for various thresholds of bone loading. Loads of 300-1500 $\mu\epsilon$ result in bone maintenance due to physiologic loading ¹³⁴ while loads of >3000 $\mu\epsilon$ result in damage to the bone microarchitecture due to non-physiologic strain ¹³⁴⁻¹³⁶. The experiments in this study maintain tibial microstrain in the physiologic loading range (300-1500 $\mu\epsilon$) representing the effects of typical daily exertion on the tibia while avoiding levels of microstrain that have been shown to cause harm.

The results suggest that the influence of bone loading on autonomic tone and heart function is diminished by as early as middle age (Figure 15). Since strain levels in the tibia during loading have been found to be similar in middle-aged mice compared to young mice ¹³⁷, this supports other mechanisms, such as a nervous system-based change, underlying these

findings with early aging. Moreover, the middle-aged mice in this study had evidence of altered autonomic control with significantly lower baseline HRV compared to younger adult mice. However, despite these findings, it remains unclear whether this altered autonomic tone at middle age is a cause or consequence of the reduced response of heart rate and HRV to bone loading. In the setting of aging and age-related disorders like osteoporosis, bone shows alterations to the osteocyte-dendritic network, increased osteocyte apoptosis, reduced nerve density, and a decrease in the ability to respond to mechanical loading¹³⁸⁻¹⁴⁰. While reduced nerve fiber content has been documented within the bones of aged animals compared to young animals¹⁴⁰, little is known of the changes to nerves in bone occurring at middle age, which could help interpret the current findings at this age. It is possible that the reduced cardiac responses to bone loading in mice at middle age may represent an early alteration in bone-neural pathways which could preface further aging-related bone loss or autonomic dysfunction. Interestingly, autonomic dysfunction has been previously documented in those with compromised bone. A study measuring the frequency domain parameters of HRV in osteoporosis patients revealed significant alterations, indicating lower parasympathetic and higher sympathetic tone compared to those with normal bone mass¹⁴¹. Furthermore, a retrospective study among the 2009-10 Korea National Health and Nutritional Examination Survey in hypertensive individuals found an inverse association between resting heart rate and bone mineral density¹⁴². The role of autonomic dysfunction in cardiovascular disease is of great clinical significance^{143, 144}, and cardiovascular disease remains the major cause of mortality and disability worldwide notwithstanding the progress made in cardiovascular disease treatment and prevention measures⁴⁹. Deterioration of bone structure and anabolic capacity, as experienced with osteoporosis, is strongly clinically associated with

cardiovascular dysfunction, suggesting a common etiology of these conditions. Incidence of cardiovascular disease and associated mortality are significantly inflated in patient populations with a history of osteoporosis ⁵⁰, and beta blockers show dual efficacy in treating cardiovascular dysfunction and improving bone quality ¹⁴⁵. Given that there is a link among bone, the SNS and cardiac function that is affected with aging, the clinical association of these two diseases might be explained, at least in part, by dysfunctional regulation of autonomic tone by the skeleton. A better understanding of the nature of bone-neuro-heart crosstalk pathways could lead to development of new and superior treatment strategies for the presently undertreated and widespread diseases of osteoporosis and cardiovascular dysfunction.

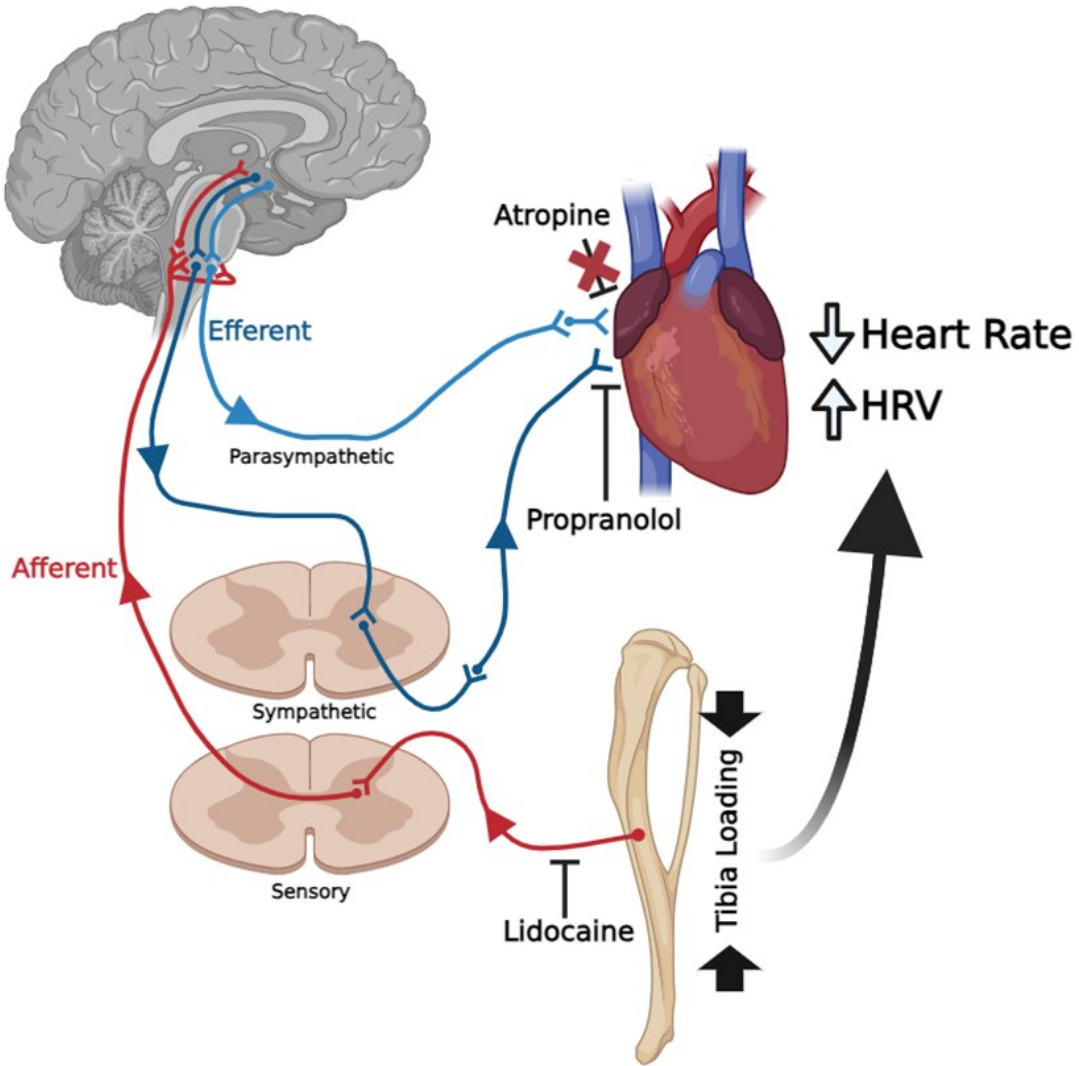
Repetitive physical activity *in vivo* involves both skeletal muscle contraction and mechanical loading of bone. The experimental approach employed in this study provides a unique insight into the effects on the heart solely from the perspective of bone loading in isolation from muscle contraction. In skeletal muscle, there are stretch-sensitive mechanoreceptors and metabolically-sensitive metaboreceptors (groups III & IV afferents) that relay afferent neuronal signals via the PNS and SNS, respectively, to stimulate cardiac activity during acute physical exercise ¹⁴⁶ whereas this study shows an opposite effect of bone loading via reduction of SNS tone on the heart. Intriguingly, the results from both the acute bone loading and three weeks of chronic bone loading studies, were strikingly similar with respect to lowered heart rate and enhanced HRV. These findings demonstrate on the one hand an immediate and transient cardiac response to acute bone loading, while on the other hand the emergence of this cardiac phenotype is maintained in baseline animal physiology during the three week loading period. These findings further suggest that daily bone loading may in itself promote changes in the heart that are consistent with exercise adaptation. Regular

exercise training is associated with a number of physiological adaptations in the heart including lowering of resting heart rate and elevated HRV parameters¹⁴⁷ mediated by increases in PNS activity and reduced SNS tone^{148, 149}. Inversely, SNS activity has been observed to be pathologically elevated in sedentary and obese human populations^{150, 151}. Considering that the SNS was found in this study to mediate the immediate cardiac response to acute bone mechanical loading, the same pathway could be a key player in the alterations in baseline heart rate and HRV with the three-week bone loading regimen. Moreover, bone loading also prolonged the ECG intervals of PR and QT (Figure 19) which can also be modulated by sympathetic tone^{152, 153} and QT in particular is known to have an inverse relationship with heart rate¹⁵⁴. These changes in cardiac rhythm and ECG properties were statistically different only during the first two weeks of the loading protocol, with no significant differences found by the end of the study. This may be explained by structural remodeling and accumulation of bone mineral content that is expected to occur in the tibia using this bone loading protocol, translating to decreasing local strains within the tibia over time using the same magnitude and direction of loading stimulus, and ultimately resulting in diminished activation of the resident nerve fibers and reduced responsiveness of the heart.

There are several limitations which should be considered when interpreting the results of this study. In order to maintain a controlled loading environment without the influence of skeletal muscle contraction, bone mechanical loading was performed on mice under isoflurane anesthesia, which is known to depress autonomic nervous control¹⁵⁵. Therefore, it is possible that nerve responses and signaling may differ in conscious animals or with the use of different anesthetics. Next, changes in blood pressure or cardiac filling were not measured to avoid disturbing the protocol and to avoid physiological variables that these procedures may

introduce. Nevertheless, changes in blood pressure were likely not reflexively inducing changes in heart rate since the response was eliminated by localized injection of lidocaine to the tibia. While this association of bone loading and heart rate has been documented, the response may involve additional physiological components. A mechanical loading protocol previously shown to activate anabolic signaling in the tibia and ulna of mice was employed. However, it remains unknown if loading of different bone types other than the tibia produces similar responses in the heart. Lastly, the impact of different loading protocol variables such as frequency, rest period, and dwell time on the bone-heart response was not addressed in this study. In conclusion, the findings that bone loading in the tibia age-dependently modulate heart rate and HRV support the idea of the skeletal and cardiovascular systems as tightly intertwined entities in physiology, health, and aging.

Figure 24.--Mechanistic overview of the impact of tibia mechanical loading on cardiac function *in vivo*



The findings of the animal study reveal a novel bone-heart reflex mediated by bone mechanical loading and the sympathetic autonomic nervous system. A rapid and transient lowering of resting heart rate and enhancement of HRV was documented during a cyclic compressive loading protocol in a single tibia in adult anesthetized mice. Pharmacological blockade with lidocaine and propranolol, but not atropine, blocked the responsiveness of the heart to bone mechanical loading implicating sensory neural afferents in the hindlimb and sympathetic efferents as the major neural mediators of this reflex.

Conclusion

Through previous studies from our laboratory and others it can be concluded that normal functioning bone promotes the systemic homeostasis and health of many organ systems whereas the dysfunction of bone at least facilitates if not induces their pathology. These observations speak to the purpose of bone as an essential element for dually serving the physical and physiological integrity of vertebrate species. In contrast to the older notions that the skeleton merely serves as a static and passive structural entity, more recent scientific revelations have uncovered its true diverse functional capacity and far-reaching influence. In fact, new ideas have been proposed by others regarding skeletal evolution in light of the recent advances to the knowledge of bone cell biology. Theories spearheaded by Dr. Gerard Karsenty's laboratory, for example, provide a unique perspective on the possible ancient biological importance of recently discovered bone endocrine mechanisms. Karsenty's group has demonstrated that a single bone hormone, undercarboxylated osteocalcin, regulates fertility, glucose metabolism, muscular performance, memory, and the acute stress response¹⁵⁶. With this evidence they argue that although this plethora of targets may seem non-related at first, its evolutionary purpose becomes coherent when considering that all these processes are inherent to the escape from predation¹⁵⁶, which is consistent with bone's classic view of allowing movement and protection of internal organs.

Along these lines, the new findings within the studies performed here now extend the network of roles bone may serve as a regulatory organ in the body and contribute to organism survival. In these studies, it has been demonstrated for the first time that cardioprotective molecules are expelled from bone cells in response to mechanical stimuli (fluid flow shear stress). Many of these secreted molecules would theoretically be transported out of the bone

via the lacunocanalicular system and into the general circulation where they may travel to the heart and provide survival signaling cues to cardiomyocytes. Since the heart comes in contact with the totality of the body's blood at a high rate, it would likely be a major site of bone-derived endocrine hormone actions. As it has been shown that osteocalcin hormone levels decline with aging ⁴³, it can be speculated that release of cardioprotective factors from bone may follow a similar pattern. Thus, the diminished ability of bone to respond to mechanical loading and to produce factors that protect the heart from stress would place aging induced pathology of bone, such as osteoporosis, in clearer context with the worse CVD-related outcomes in patients with a history of osteoporosis that were discovered in the current clinical study and by other previous studies. This proposed bone-heart endocrine connection is described in Figure 25.

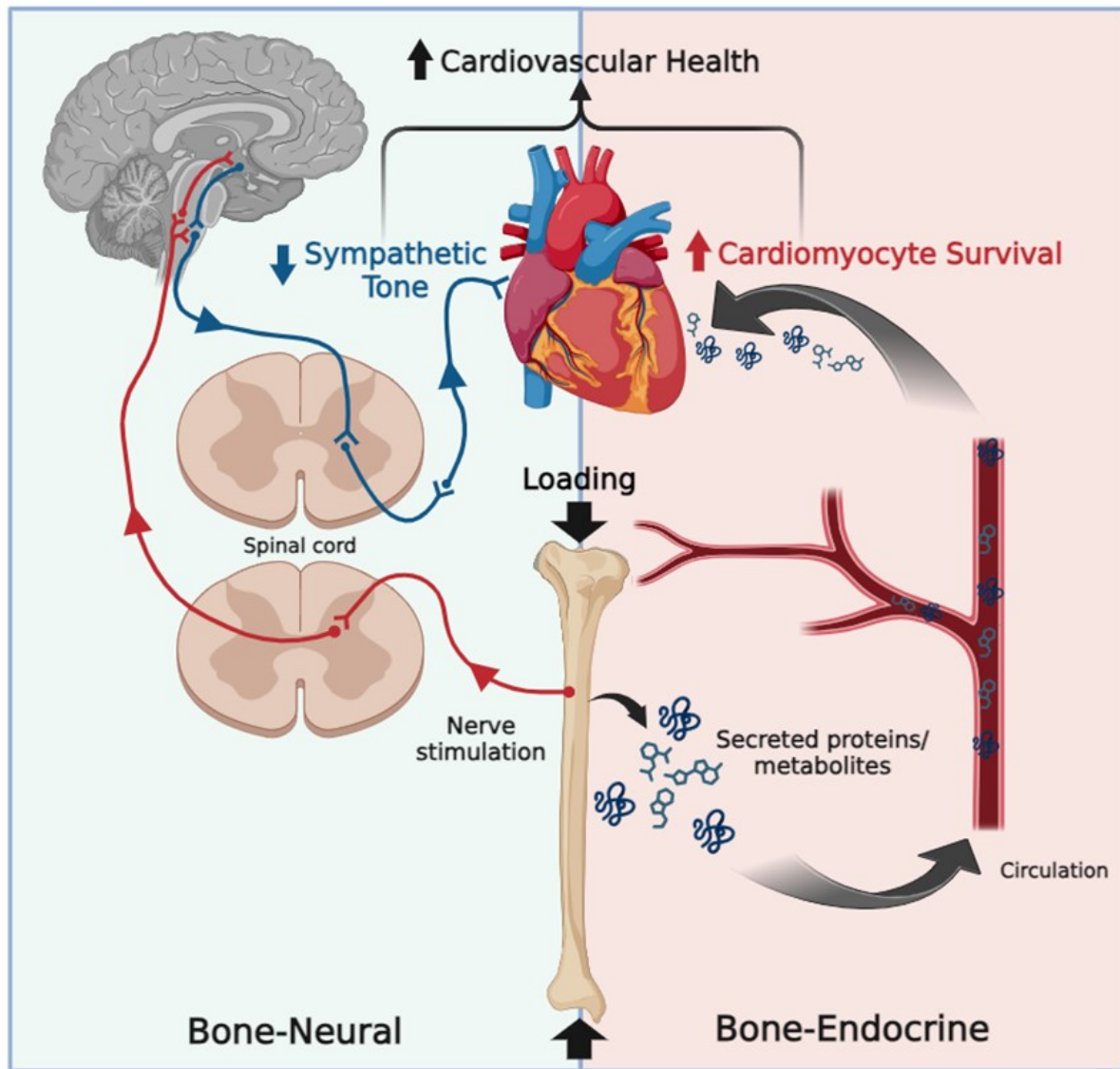
In addition to the humoral communication transmitted from bone through the blood, the current findings also reveal a novel neuronal interface of bone mechanical loading with the cardiovascular system. This suggests that bone utilizes the circulation and also the nervous system to deliver its regulatory cues throughout the body. No other studies have described a neural connection between bone and other organs apart from the brain. The fact that bone derived neural signals recruit or downregulate the autonomic nervous system, particularly the SNS, to create conditions favorable for bone growth, is further suggestive of a link among bone loading and cardiovascular disease, a setting commonly associated with overactive SNS tone. One line of evidence for this proposal is that middle-aged mice in this study compared to younger adult mice exhibited a loss of the influence of bone loading on heart rate and HRV, which was concomitant with altered baseline autonomic control of the heart, as evidenced by lower HRV values at middle age. As bone mechanical loading reduces SNS tone leading to

increased HRV, a powerful predictive marker of improved cardiovascular and overall health outcomes, this may indicate that bone loading actively promotes cardiovascular health. On the other hand, as bone-mediated withdrawal of SNS tone becomes diminished, such as during aging or with bone disorders like osteoporosis, this pathological cardiac environment may contribute to worse CVD-related hospital outcomes. Figure 25 depicts this novel bone-heart neural connection.

In conclusion, the heart may benefit from both signaling by humoral protective factors originating from bone and from autonomic nervous system tuning associated with bone mechanical loading. Like many other physiological processes that lose vigor with aging and age-related disorders, bone endocrine and neural pathways may be subjected to the same changes, leading to the fragmentation of these regulatory influences on the heart and eventual progression into cardiac pathology. Studies performed here provide the needed groundwork exploring the physiological mechanisms linking bone and heart health and can serve as a springboard for the further exploration of bone-heart crosstalk pathways as therapeutic tools in novel drug discovery for CVD. Accordingly, future studies based on this work should be aimed at characterizing the molecular identities of the protective factors released during bone mechanical loading, their target receptors in the heart, and the effect of the aging process on the bone secretory profile. This could lead the development of new drugs modeled after bone-secreted hormones for prevention or treatment of CVD. It is also important for future studies to delineate the mechanism by which the bone-heart neural reflex becomes diminished with aging and if this neural pathway can be targeted in a therapeutic sense as treatment for autonomic dysregulation and/or CVD. Moreover, from a clinical standpoint, further exploration is warranted to understand the drivers of the sex differences seen in CVD outcomes

such as prolonged hospital stay and higher mortality in men versus women with a history of osteoporosis, which may be due to biological factors or biases in care. Finally, implementing higher consideration of bone-heart interactions into current medical practice standards could immediately help clinicians consider high risk patients and formulate more effective health care plans for preventing and treating CVD based on bone-centered care.

Figure 25.--Proposed endocrine and neural based mechanisms linking bone mechanical loading and cardiovascular health



The comprehensive model based on the findings of this study illustrates the bone contribution to heart health through two distinct physiological mechanisms. (Right) Mechanical loading of bone (e.g., during physical activity) induces the secretion of signaling molecules that protect heart cells from hypoxia-induced cell death suggesting that the bone may regulate the heart in an endocrine fashion. (Left) Heart function is influenced by bone via the central nervous system. Mechanical loading in bone activates nerve fibers that relay signals through the autonomic nervous system to downregulate sympathetic tone on the heart leading to lower heart rate and higher HRV. Together, promotion of heart cell survival by bone-endocrine factors and reduction of sympathetic tone on the heart through bone-neural signaling during mechanical loading may provide a beneficial stimulus for maintenance of cardiovascular health. Changes in these bone-heart crosstalk pathways with aging or bone disease may contribute to the clinical relationships between poor bone quality and CVD.

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VITA

Julian Alfredo Vallejo is a Bolivian American born in Austin, Texas on July 20, 1987, to parents Karen Palmer and Luis Vallejo. He attended Liberty High School in Liberty, Missouri and graduated in 2006 with a scholarship to attend Maple Woods Community College to begin studying biological sciences. Mr. Vallejo subsequently transferred to the University of Missouri – Kansas City (UMKC) where he earned his Bachelor of Science degree in Biology in 2011.

After graduating, Mr. Vallejo joined the research laboratory of Dr. Marco Brotto at the UMKC School of Nursing and Health Studies as a research assistant working with other members of the Muscle Biology Research Group (MUBIG) investigating bone-muscle crosstalk, exercise and the aging process. In 2014, Mr. Vallejo was awarded a Diversity Supplement Grant (2014-2016, P01 AG039355-01A1) from the National Institutes of Health - National Institute on Aging (NIH-NIA) to pursue graduate studies at UMKC while simultaneously working as full time research staff in the Brotto Lab to support his family. In 2016, Mr. Vallejo graduated with his Master of Science degree in Cellular & Molecular Biology, performing his thesis work over bone-muscle crosstalk mechanisms. In addition to the comprehensive research experience acquired during his time with the Brotto Lab, Mr. Vallejo developed specialized expertise in microdissection and *ex vivo* contractility analysis of mouse skeletal muscles, a method which he was routinely recruited to perform for studies

under two consecutive five-year NIH-NIA Program Project Grants investigating bone-muscle crosstalk at UMKC (2013-2017 & 2018-2023, P01 AG039355, PI: Dr. Lynda Bonewald).

In 2015, Mr. Vallejo joined the research laboratory of Dr. Michael Wacker in the UMKC School of Medicine where he applied his research experience in skeletal muscle towards the study of the cardiovascular system, particularly in the investigation of cardiac pathology mechanisms associated with chronic kidney disease, such as those mediated by the bone-derived hormone, fibroblast growth factor 23 (FGF23), and gut-microbiome derived metabolite, trimethylamine-n-oxide (TMAO). Through his research position in the Wacker Lab, Mr. Vallejo assumed new roles as a research mentor to numerous medical and graduate students and as a laboratory manager. Mr. Vallejo was promoted to Senior Research Assistant in 2019 and was subsequently accepted into the UMKC Ph.D. program in 2020 to study bone-heart crosstalk in Dr. Wacker's laboratory aided by funding from a second NIH-NIA Diversity Supplement Grant (2021, P01 AG039355-08).

Over the course of his research career, Mr. Vallejo has been privileged to publish research findings spanning diverse fields including that of exercise, aging, bone-muscle/bone-heart crosstalk, nutritional supplementation, and cancer. He has published four peer-reviewed first author or co-first author research papers, has co-authored another nine research papers, and has been selected to present 12 research abstracts at various local and national research conferences including the American Society for Bone and Mineral Research (ASBMR) annual meeting, the American Society of Nephrology (ASN) Kidney Week, Experimental Biology, and the Biophysical Society (BPS) annual meeting. Mr. Vallejo has also received recognition for his research presentations as he was awarded 1st Prize for Best Presentation at the American

Physiological Society Joint Meeting of the Missouri and Iowa Chapters in 2014 and was the winner of the Diversity Poster Competition at the ASBMR Annual Meeting in 2017.

Upon completion of his Ph.D. in Biomedical and Health Informatics & Molecular Biology and Biochemistry, Mr. Vallejo plans to continue pursuing basic, translational and clinical postdoctoral research and accomplish his goal of becoming Principal Investigator and Professor.