VITAMIN D DEFICIENCY AND AIR POLLUTION: EFFECTS IMPACTING THE CARDIOVASCULAR SYSTEM

Kimberly M. Stratford

A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Toxicology in the School of Medicine.

Chapel Hill
2018

Approved by:

Mehdi Hazari,

Mirek Styblo,

David Diaz-Sanchez,

Saroja Voruganti,

Li Qian

ABSTRACT

Kimberly M. Stratford: Vitamin D Deficiency and Air Pollution: Effects Impacting the Cardiovascular System
(Under the direction of Mehdi Hazari)

Human studies have shown that air pollution is associated with cardiovascular morbidity, yet the role of nutrition in modifying this susceptibility is unclear. Vitamin D deficiency (VDD) is a public health concern and the adverse consequences of its interaction with other stressors is understudied. VDD is linked to low levels of klotho, an anti-aging protein with a critical role in sinoatrial function. Transient receptor potential (TRP) channels are regulated by klotho and mediate cardiac effects to air pollution. TRPC6 is a cation channel that is physiologically inactive under normal conditions but upregulated with cardiovascular disease. I hypothesized that VDD changes cardiovascular function through mechanisms involving klotho and TRPC6 that mediate the responses to air pollution. First, I determined the effect of early-life VDD on cardiovascular responses to photochemical smog in mice. Three-week-old mice were placed on either a VDD or normal diet for 16-19 weeks and surgically implanted with biopotential radiotelemeters to continuously measure electrocardiogram, heart rate (HR) and whole-body plethysmography (WBP) for arrhythmias, heart rate variability (HRV) and ventilatory function. This study shows that VDD mice had decreased HR/tidal volume and increased HRV compared to controls and smog modulated the response. Second, I determined the role of klotho in VDD-induced adverse cardiac response to acrolein exposure. Mice were treated with recombinant klotho to determine if the

potentiated effects are blocked. This study demonstrates that compared to controls, HR/HRV was decreased in VDD mice. When compared to air exposure, acrolein increased HR/HRV and klotho blocked acrolein-induced effects in both diets. Lastly, I investigated the role of TRPC6 in VDD-induced cardiovascular mechanical responses. Dobutamine stress test was used to increase heart rate and reveal latent cardiac effects due to VDD and the involvement of TRPC6 in VDD mice was determined using an antagonist. Compared to controls, VDD mice have higher blood pressure and blunted HR response to dobutamine which was restored by TRPC6 antagonist. Future studies are needed to evaluate klotho and TRPC6 as potential therapeutic interventions. This project is the first to characterize the role of VDD as a nutritional modifiable factor in relation to cardiovascular toxicological responses to stressors.

I dedicate this work to my parents:

The late Mr. Arthur W. Stratford and Mrs. Madie H. Stratford, for their unconditional love, guidance and prayers as well as All that I have lost along the way.

ACKNOWLEDGEMENTS

Completion of this dissertation was not a solo project and required the help of many wonderful people along the way and I am forever grateful for them. I would like to especially acknowledge my advisor, Dr. Mehdi Hazari who graciously agreed that I could join the laboratory and tackle a project with little precedence. He has been patient and kind all the while guiding me scientifically. A special thank you is due to Dr. Aimen Farraj for his guidance in study design, review of fellowship applications and manuscript drafts throughout the years. I would like to thank my committee members Drs. Mirek Styblo, David Diaz-Sanchez, Li Qian and Saroja Voruganti not only for their considerable time and expertise but also thoughtful and scientifically-sound critiques.

My time at the Environmental Protection Agency in Research Triangle Park has certainly been enhanced by the support of staff and scientists that have been integral to my training. Najwa Haykal-Coates has not only provided statistical guidance but also helped me in more ways that I can ever explicitly describe. With deep gratitude and appreciation, I would also like to thank Randy Harrison, Crystal Walden, Judy Richards, Allen Ledbetter, Photochemical Smog Exposure Team, Dr. Leslie Jarrell, Patty Dilliard, Wanda Williams and Lisa Copeland. Past and current members of the Hazari and Farraj laboratory's including Drs. Nicole Kurhanewicz and Leslie Thompson, Fatiha Malik, Kyle Martin and Brandi Martin.

Through my time in graduate school, my cohort has been consistent in their support from the beginning and I look forward to our friendships continuing to grow

afterwards. The Initiative for Maximizing Student Diversity at UNC led by Dr. Ashalla Freeman has provided a community of like-minded individuals that was my strength during tough times. I would also like to acknowledge Dr. Ilona Jaspers and Julie Cannefax for always answering my frantic questions with grace.

Lastly, a very special thank you to my family. Although my father died when I was a young age, my mother (Mrs. Madie Stratford) has provided unconditional love and advice when I needed it the most. Unfortunately, I have lost many members of my family during this time but the memories will never be forgotten.

PREFACE

The first manuscript presented in this dissertation (Chapter 2) is a pre-copy-editing, author produced version of an article accepted for publication in *Environmental Science and Technology*. The definitive publisher-authorized version of this manuscript is available online at https://pubs.acs.org/doi/full/10.1021/acs.est.7b04882 and is cited as follows:

Kimberly Stratford, Najwa Haykal-Coates, Leslie Thompson, Q. Todd Krantz, Charly King, Jonathan Krug, M. Ian Gilmour, Aimen Farraj, and Mehdi Hazari. Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice. *Environmental Science & Technology.* **2018** *52* (5), 3054-3061.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

ACE - Angiotensin converting enzyme

AHA - American Heart Association

ANOVA - Analysis of Variance

ANS - Autonomic nervous system

dP/dT (-) - Negative Derivative of left ventricular Pressure over Time

dP/dT (+) - Positive Derivative of left ventricular Pressure over Time

EPA - Environmental Protection Agency

FA - Filtered air

FGF23- Fibroblast growth factor 23

HF - High frequency

HR - Heart rate

HRV - Heart rate variability

LF - Low frequency

MRC - Mobile reaction chamber

ND - Normal Diet

NHANES - National Health and Nutrition Examination Survey

NO - Nitric oxide

NOS - Nitric oxide synthase

PAH - Polycyclic aromatic hydrocarbons

Penh - Ventilatory timing

PM_{10} - Particulate matter <10µm

$PM_{2.5}$ - Particulate matter <2.5µm

PTH - Parathyroid hormone

RAAS - Renin-angiotensin-aldosterone system

RMSSD - Foot mean square of the standard deviation of the normal RR

RXR - Retinoid X receptor

SDNN - Standard deviation of the normal RR

THC - Total hydrocarbons

TRP - Transient receptor potential

TRPC6 - Transient receptor potential canonical 6

U.S. - United States

VDD - Vitamin D Deficient

VDR - Vitamin D receptor

VDRE - Vitamin D Response Elements

VOC - volatile organic compounds

* significantly different from ND

◊ significantly different from pre-exposure or baseline of exposure

† significantly different from immediately post-exposure

‡ significantly different from filtered air exposure

¥ significantly from 3-week assessment

Chapter 1: Introduction

Health effects of air pollution

The London Smog of 1952 and other such extreme events ignited the public's awareness of air pollution and a scientific interest in studying the deleterious health outcomes of air pollution exposure.(Dooley 2002) London has had numerous episodes of terrible smog but the one in 1952 was the worst in terms of adverse human health effects, which resulted in approximately 12,000 "unnecessary" deaths. For five days in early December, increased industrial and fire emissions for heating, diesel combustion from buses combined with meteorological conditions created a perfect recipe for dense smog. All segments of the population were impacted, including the young, but especially the elderly who succumbed to bronchitis, pneumonia, and other respiratory diseases. Yet, although the relative number of cases presenting with respiratory symptoms was greater than any other complications, heart related adverse effects contributed to more deaths.(Dooley 2002) Most of this mortality was considered to be untimely because the overall risk in this population was not high.(Dooley 2002, Hunt, Abraham et al. 2003) In addition to mortality, retrospective studies have even linked early-life exposure to the London smog of 1952 to the development of asthma during adulthood.(Bharadwaj, Zivin et al. 2016) Unfortunately, complex multi-pollutant air pollution atmospheres like smog are still an environmental issue worldwide to this day, particularly for those with underlying health conditions.

Smog is a complex multi-pollutant mixture comprised of particulate matter (PM) of various sizes, gaseous components like ozone, volatile organic compounds (VOCs) and other chemicals. One of the greatest challenges to assessing the health effects of air pollution and assigning causality is due to the complexity and variability of composition, which can vary from place to place, and even from one season to another in a given area. Among the most ubiquitous gaseous pollutants, and indeed one of the most irritating to the airways, are the reactive aldehyde gases like acrolein (C_3H_{40}), which are produced by the combustion of any hydrocarbon-based fuel, fatty foods, biomass, cigarettes and other organic compounds to form a toxic, volatile unsaturated aldehyde.(Ghilarducci and Tjeerdema 1995, Kehrer and Biswal 2000, Conklin, Haberzettl et al. 2017) Acrolein inhalation causes nasal and pulmonary inflammation, (Snow, McGee et al. 2017) eye, nose and throat irritation, (Sim and Pattle 1957, Esterbauer, Schaur et al. 1991) respiratory distress (Ben-Jebria, Marthan et al. 1994, Bein and Leikauf 2011) and asthma exacerbations (Leikauf, Leming et al. 1989). Very high levels of acrolein (100-275 parts per million) have been shown to cause cardiac dysfunction and even mortality (Conklin, Haberzettl et al. 2017) and recent data suggest that even lower levels of the gas can lead to subtle cardiovascular changes, not only in people with underlying disease but healthy individuals as well.

Air pollution is associated with cardiovascular mortality

Although air pollution was long considered a direct threat to the health of the respiratory system, especially the lungs, it has become clear that its effects go beyond the airways. In fact, the American Heart Association (AHA) has released a statement in 2010 alerting people to the hazardous effects of air pollution. (Brook, Rajagopalan et al.

2010) In the last twenty years, cardiovascular morbidity and mortality have been demonstrated to be strongly correlated with short and long term ambient air pollution exposure (Brook, Rajagopalan et al. 2010), particularly in the presence of underlying cardiovascular disease (e.g. hypertension). (Hazari, Callaway et al. 2012, Rajagopalan and Brook 2012, Hazari, Griggs et al. 2014) Scientific data points to the fact that several major components of air pollution, particularly particulate matter (PM), can contribute to these effects. The Environmental Protection Agency (EPA) has identified and regulates six of these pollutants: ground level ozone, nitrogen dioxide, PM, lead, carbon monoxide and sulfur dioxide. Each of the criteria pollutants, along with the constituent polycyclic aromatic hydrocarbons (PAHs), VOCs and other environmental contaminants have the potential to react and cause a synergistic, or even antagonistic, effect on cardiac health. Therefore, the cardiovascular response to mixtures of air pollutants is still being characterized and requires a significant amount of work. (Brook, Rajagopalan et al. 2010)

Studies have conclusively linked fine PM, which is less than 2.5 μm ($PM_{2.5}$), and coarse PM (PM_{10}) to adverse health effects. In particular, $PM_{2.5}$ is produced by combustion of fossil fuels for human activities and short and long-term exposure to it has been shown to contribute to the early death of thousands of Americans every year. Similarly, hospitalization rates for arrhythmias, heart failure and cerebrovascular disease have been shown to increase for 24 hours after high $PM_{2.5}$ days and for 48 hours in individuals with previous myocardial infractions. (Brook, Rajagopalan et al. 2010) Although the focus of many studies of the adverse cardiovascular effects of air pollution has traditionally been on PM, gaseous environmental contaminants also

contribute to cardiovascular dysfunction.(Chiu, Weng et al. 2017, Coogan, White et al. 2017, Costa, Hoek et al. 2017, Day, Xiang et al. 2017, Mirowsky, Carraway et al. 2017, Yang, Qian et al. 2017) For example, ozone exposure causes systemic inflammation, (Mirowsky, Carraway et al. 2017) platelet stimulation, (Day, Xiang et al. 2017) hypertension (Coogan, White et al. 2017, Day, Xiang et al. 2017) and hospital admissions due to myocardial infarctions (Chiu, Weng et al. 2017) which are all major contributing factors to cardiovascular morbidity and mortality.

There are three biological mechanisms that have been proposed as mediating the cardiovascular response to air pollution. This includes 1) pulmonary oxidative stress and inflammation which can spread beyond the lungs, 2) autonomic nervous system imbalance, which has the potential to disrupt the normal function of not only the respiratory and cardiovascular systems, but also the digestive and endocrine systems as well, and the 3) translocation of PM and/or other components into the blood towards secondary target sites. Epidemiological, human and animal studies have greatly contributed to the weight of evidence for these biological mechanisms. Each of these pathways is highly complex and not likely taking place independently. In fact, vascular dysfunction, increased coagulation, and hypertension combined have been demonstrated to elicit adverse cardiovascular events in human and animal studies. (Brook, Rajagopalan et al. 2010)

Heart disease prevalence

So, why is an inhaled exposure to air pollution so concerning when it comes to the cardiovascular system, particularly if it is short-term? Part of the answer to this question lies in the prevalence of cardiovascular disease in the developed world.

Cardiovascular disease is the leading cause of mortality in the United States and is an all-encompassing term that includes atherosclerosis, hypertension, myocardial infarction, stroke, heart failure, and arrhythmia. Cardiovascular mortality rates in 2007 were 259.4 per 100,000 people and decreased to 223.9 per 100,000 people in 2013 which is an improvement of about 13.7%. (Mozaffarian, Benjamin et al. 2016) However, cardiovascular disease still affects 30% of the US population and is an immense economic and societal burden. (Mozaffarian, Benjamin et al. 2016, Trehan, Afonso et al. 2017) In addition, lifestyle and other factors which are not always fully appreciated are contributing to the more rapid progression of the disease. This suggests that there is a heightened susceptibility in a certain percentage of the population due to the presence of these factors.

Autonomic nervous system and heart rate variability

Cardiovascular function is intrinsically regulated by the autonomic nervous system (ANS), which is comprised of two major branches, the parasympathetic and sympathetic, that dynamically interact and simultaneously modulate function depending on the physiological state of the body and the environmental conditions or stimuli. (Thayer, Yamamoto et al. 2010, Shen and Zipes 2014) Parasympathetic tone of the autonomic nervous system is typically characterized as the “rest and digest” state and is critical for everyday basal bodily functions. In contrast, sympathetic tone represents the “fight or flight” state, which causes heart rate accelerations, regulation of blood pressure to accommodate stress and reduction of activities normally occuring during rest (e.g. digestion). Nerves of the autonomic nervous system originating from the brainstem innervate the heart to maintain sinus heart rhythm and when necessary modulate it to

respond to a given stressor (e.g. exercise). (Shen and Zipes 2014) Abnormal dominance of one branch over the other or inappropriate modulation during a given circumstance is called autonomic imbalance and it generally reflects the body's inability to either maintain homeostasis or compensate for change. (Tsuji, Larson et al. 1996, Brook and Julius 2000, Thayer, Yamamoto et al. 2010) Furthermore, the ANS is also modulated by chemoreflexes which respond to decreases in blood oxygen or increases in carbon dioxide by increasing sympathetic modulation, and baroreflexes, which are activated by blood pressure changes and result in parasympathetic activation. These control mechanisms alter heart rate, contractility and vasoconstriction/dilatation, as well as breathing patterns. (Kara, Narkiewicz et al. 2003, Heusser, Tank et al. 2005) Therefore, any persistent changes to normal autonomic function would impact the regulatory outflow of these reflexes and thereby impact the response of the target tissues.

Heart rate variability (HRV) is used clinically to assess autonomic tone of the heart and is based on the evaluation of beat-to-beat or R-R interval, which represents the duration of cardiac cycle. (Tsuji, Larson et al. 1996) It is not only determined by normal physiological signals (e.g. renin-angiotensin cascade, release of neurotransmitters and metabolic hormones) but also pathological conditions (e.g. cardiomyopathy and myocardial infractions) as well. (Tsuji, Larson et al. 1996, Brook and Julius 2000) Heart rate variability is measured using time and frequency domain parameters that are commonly used to represent the two branches, these parameters are constantly changing based on fluctuations of bodily activity. The time domain measures of HRV are standard deviation of the normal R-R interval (SDNN) and root

mean square of the standard deviation of the normal R-R interval (RMSSD). SDNN is commonly used to evaluate overall autonomic function while RMSSD indicates parasympathetic modulation. The frequency domain measures of HRV are low frequency (LF) and high frequency (HF). There is considerable debate about the interpretation of LF, however, in general current literature suggests that it represents both the sympathetic and parasympathetic influence on the heart. On the other hand, HF is a reliable indicator of parasympathetic tone. Some studies report the ratio of LF/HF as an indicator of the balance of sympathetic and parasympathetic activity. (Thayer, Yamamoto et al. 2010)

Heart rate variability and cardiovascular disease

Abnormal HRV represents a latent change in cardiac function particularly in instances when symptoms of dysfunction are not otherwise evident. (Stein, Domitrovich et al. 2005) Therefore, clinicians commonly use HRV to assess cardiac health or prognosis and risk of mortality, especially after an adverse cardiac event.(Thayer, Yamamoto et al. 2010) On the other hand, it is important to note that changes in HRV may not solely be due to ANS modulation but could also be explained by modified cardiac receptor and myocardium function. (Rowan, Campen et al. 2007) As such, epidemiological studies have repeatedly linked cardiac abnormalities with low HRV (i.e. increased sympathetic modulation), which indicates the heart is unable to respond to changing circumstances (i.e. lower variability and ability to modulate). (Brook, Rajagopalan et al. 2010) In addition, low HRV especially at rest, suggests that there is greater risk of an adverse cardiac event because the heart is in a higher state of persistent stress than normal. In contrast, many animal studies link greater HRV to air

pollution exposure and a shift away from the normal homeostatic cardiac response. (Brook, Rajagopalan et al. 2010, Farraj, Hazari et al. 2011, Hazari, Callaway et al. 2012, Carll, Lust et al. 2013, Hazari, Griggs et al. 2014, Kurhanewicz, McIntosh-Kastrinsky et al. 2014, Stratford, Haykal-Coates et al. 2018) Therefore, it is clear that a change in HRV alone is not sensitive enough to diagnose cardiac disease or dysfunction and should be evaluated in combination with other clinical tests.

Rodent HRV effects can be extrapolated to human HRV if the responses are assessed in light of differences in basic physiology (i.e. determinants of resting heart rate), sampling duration, circadian rhythm effects, usage of anesthesia and thermal responses. In such a case, the utility of evaluating HRV in rodents is due to the fundamental mechanisms of the cardiac regulation being similar to humans. (Rowan, Campen et al. 2007)

Given the two branches are essential to the proper function of the heart and that a dynamic balance must be maintained, extremes in either parasympathetic or sympathetic activity can also increase mortality. (Gold, Litonjua et al. 2000) Sometimes, this autonomic imbalance is only evident if the body is exposed to a stressor (e.g. air pollution exposure), which forces the heart to adapt and change its function accordingly. (Thayer, Yamamoto et al. 2010, Carll, Crespo et al. 2017)

Heart rate variability and air pollution exposure

Even under acute conditions, ambient air pollution can cause relatively subtle or latent changes in HRV or electrical disturbances (i.e. arrhythmia) indicative of increased cardiovascular risk. Even in healthy young subjects, exposure to air pollution increases parasympathetic tone of the ANS compared to clean air suggesting autonomic

imbalance. (Davoodi, Sharif et al. 2010) Additionally, animal studies have consistently demonstrated that air pollution induces autonomic modulation that contributes to alterations in cardiac electrophysiology which are further exacerbated following stress. (Farraj, Hazari et al. 2011, Hazari, Callaway et al. 2012, Kurhanewicz, McIntosh-Kastrinsky et al. 2014) Therefore, HRV might be thought of less as a marker of cardiovascular toxicity and more as an indicator of the internal state of the cardiovascular system and its relative ability to adapt to changing conditions. This is a critical point to understand because there are numerous factors that have the potential to influence autonomic regulation of the cardiovascular system.

Modifiable factors influence responsiveness to air pollution exposure

Historically, the paradigm for air pollution toxicology involved measuring the response of a given target tissue to a certain dose of pollutant still remains the case today. However, the field has evolved due to the recognition that changing lifestyles and other factors have the potential to change the way the body responds to an insult. In particular, the cardiovascular health effects of air pollution may differ from one person to another due to modifiable factors, despite similarities in age, gender and background, and may help explain divergent responses. (Requia, Adams et al. 2017) The American Heart Association defines ideal cardiovascular health as the absence of disease and the presence of seven key health factors that they term life's simple seven: smoking status, body mass index, physical activity, healthy diet, total cholesterol, blood pressure and blood glucose. (Mozaffarian, Benjamin et al. 2016) Life's simple seven are common modifiable factors that influence cardiovascular health and maintenance of a healthy diet is particularly important. In fact, data from the National Health and Nutrition

Examination Survey (NHANES) points to the prevalence of cardiovascular health factors in U.S. children and adults, and it shows that 63.4% of U.S. kids have poor diets that extends into adulthood among whom 30-50% will also have poor diets. (Mozaffarian, Benjamin et al. 2016) This suggests that children with poor diets (i.e. nutritional deficiencies) might continue on that path and become adults with poor diets; thus, chronically influencing the state of the body and responsiveness to air pollution exposure.

Vitamin D

One of the essential micronutrients that affects various functions throughout the body is vitamin D, which is endogenously synthesized by the skin and obtained from the diet (e.g. salmon, tuna, eggs, cheese, milk and fortified foods).(Holick 2007, Trehan, Afonso et al. 2017) As shown in Figure 1-1, 7-dehydrocholesterol is produced by UV light radiation and converted to cholecalciferol (vitamin D_3) which is also obtained through dietary intake of animal products. The liver then hydrolyzes vitamin D precursors using a cytochrome P450 enzyme (CYP27A1) to produce calcidiol or 25-hydroxyvitamin D, which is a key circulating biomarker of vitamin D. Calcidiol is hydrolyzed in the kidneys by 25-hydroxyvitamin D-1-alpha hydroxylase or CYP27B1 to produce the active vitamin D hormone termed calcitriol or 1-alpha, 25-dihyroxyvitamin D ($1,25(OH)_2D$). (Holick 2007, Berridge 2015, Rai and Agrawal 2017, Trehan, Afonso et al. 2017) The main function of calcitriol is to maintain calcium balance in the body. Calcitriol is tightly regulated by secretion of parathyroid hormone (PTH) which responds to blood calcium and phosphorus levels. (Holick 2007, Trehan, Afonso et al. 2017)

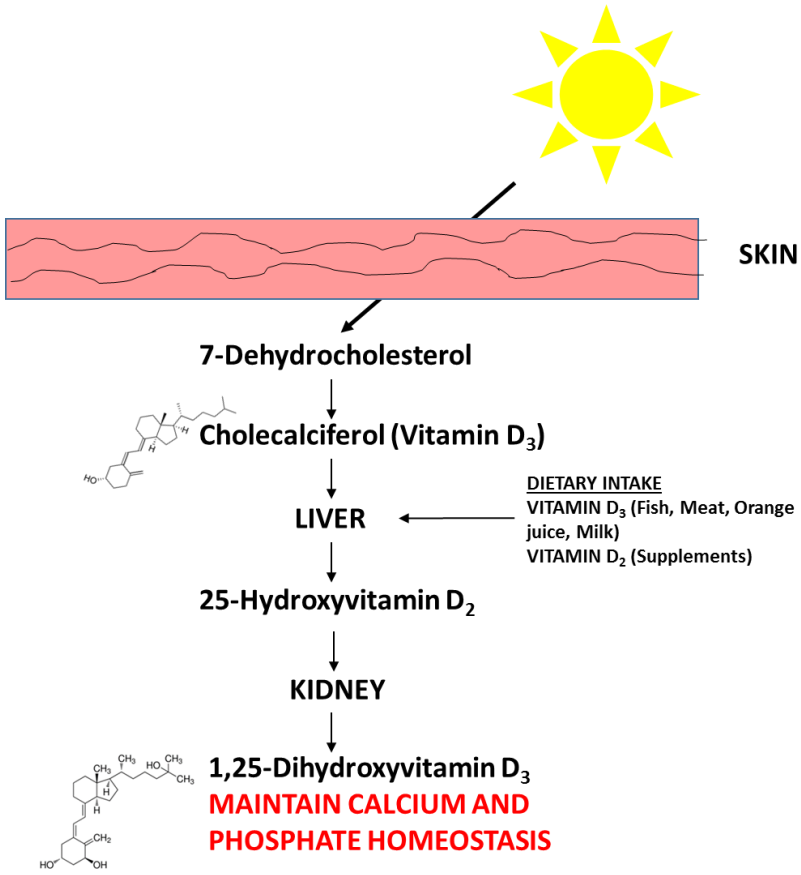


Figure 1-1. Comprehensive vitamin D synthesis pathway.

The vitamin D receptor (VDR) is a steroid nuclear receptor found throughout the body including osteoblasts, intestinal enterocytes and cells of immune and nervous systems. (Wang, Pencina et al. 2008) The VDR was identified *in vivo and in vitro* using the rat heart and atria-cultured cardiac cells, respectively. (Baksi , Walters) Vitamin D exerts its effects through the VDR and upon activation forms a complex with the retinoid X receptor (Figure 1-2), (Holick 2007, Fetahu, Höbaus et al. 2014, Norman and Powell 2014, Saccone, Asani et al. 2015), which then binds to vitamin D response elements (VDRE) on DNA and influences expression of a wide variety of genes covering numerous functions (e.g. calcium homeostasis, antioxidant production, protein

synthesis). (Sundar and Rahman 2011, Fetahu, Höbaus et al. 2014, Norman and Powell 2014, Saccone, Asani et al. 2015) Thus, vitamin D is essential for homeostasis and proper physiological function. For example, vitamin D regulates genes involved in bone turnover, detoxification, antioxidant defense and immune system modulation. Therefore, a deficiency in vitamin D leads to potentially adverse changes in the body, which may go unnoticed due to subtle alterations in physiology.

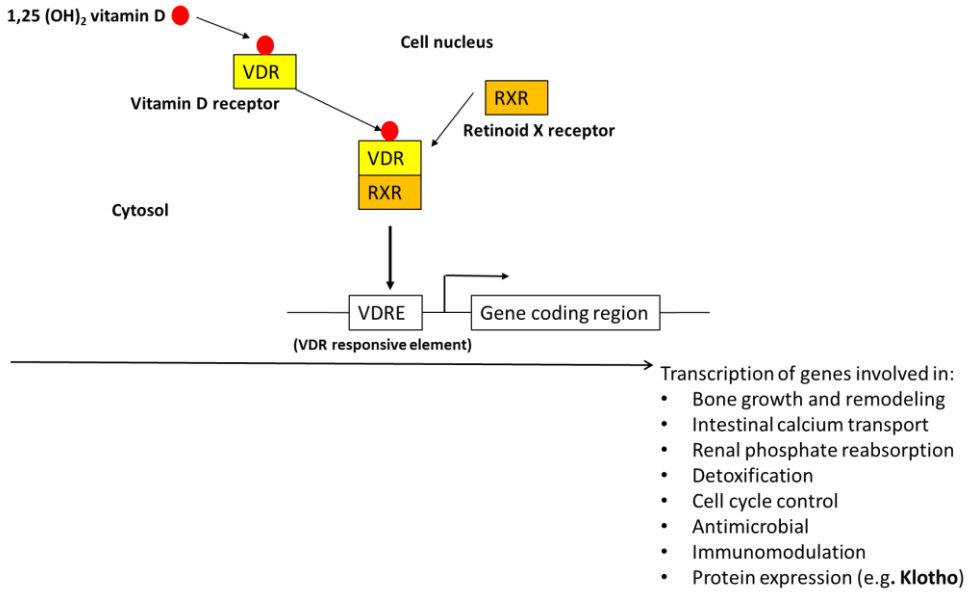


Figure 1-2. Schematic of the vitamin D receptor activation pathway that leads to gene transcription.

Vitamin D deficiency

Vitamin D deficiency (VDD) is a global epidemic affecting approximately one billion children and adults alike worldwide. (Trehan, Afonso et al. 2017) There is some contention regarding the serum level of vitamin D that is defined as “deficient” but typically it is accepted to be < 20-30 ng/ml (Figure 1-3). There are myriad of causes of VDD including diminished vitamin D synthesis in the skin, which is the most well-known

and decreased production of vitamin D precursors, renal/liver organ failure and malabsorption conditions. (Holick 2007) Low serum levels of vitamin D has been observed in chronic kidney disease suggesting an association between vitamin D status and organ dysfunction as well as impaired VDR activation. (Dusso 2011)

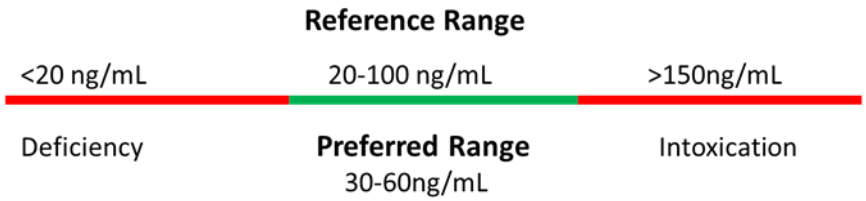


Figure 1-3. Recommended serum vitamin D reference range.

Vitamin D deficiency and cardiovascular disease

The consequences of VDD are present throughout the body. Traditionally, VDD has been associated with bone conditions but depression, infections, neuro-degenerative/neuromuscular diseases, diabetes and cancer are lesser known outcomes. (Holick 2007, DeLuca, Kimball et al. 2013, Muscogiuri, Annweiler et al. 2017) More recently, interest in VDD's contribution to cardiovascular disease has grown. (Holick 2007, Lee, O'Keefe et al. 2008, Muscogiuri, Annweiler et al. 2017) Previous studies have linked decreased serum vitamin D levels to stroke, myocardial infarction, heart failure and subsequent cardiovascular related death. (Pilz, März et al. 2008) In particular, epidemiological studies have demonstrated that VDD is associated with increased cardiovascular risk and subsequent myocardial infarction, atherosclerosis and stroke. In elderly stroke patients, VDD at the start of stroke has been shown to be correlated to increased likelihood of death after one year. (Muscogiuri, Annweiler et al. 2017) Similarly, data from NHANES suggests that VDD is a risk factor for

cardiovascular disease due to adverse effects in the heart. (Al-Khalidi, Kimball et al. 2017)

Vitamin D deficiency and the heart

One of the most common cardiovascular conditions in the U.S. is hypertension, and although many cases are linked to high salt intake, stress, or familial genetic disposition, vitamin D status also might play a role. People with high blood pressure are at increased risk of developing heart diseases (e.g. congestive heart failure, cardiac hypertrophy, stroke). When combined, hypertension and VDD further increase probability of cardiovascular disease compared to non-hypertensive VDD individuals as demonstrated by a prospective study (Figure 1-4). (Lee, O'Keefe et al. 2008) Previous experimental studies have demonstrated that vitamin D inhibits renin production and reduces activity of the renin-angiotensin-aldosterone system (RAAS) activity leading to lower blood pressure. However, VDD results in excess renin production and potentiates RAAS activity leading to hypertension.(Li, Kong et al. 2002, Li, Qiao et al. 2004, Trehan, Afonso et al. 2017) Additionally, PTH, which is also increased in VDD, also stimulates renin and aldosterone release to increase RAAS activity and vascular contractility and induce increased pressure. (Carbone, Mach et al. 2014) Interestingly, the incidence of hypertension and VDD is increasing in the United States, with evidence also pointing to increased risk for hypertension among VDD individuals. (Martins, Wolf et al. 2007, Scragg, Sowers et al. 2007, Forman, Curhan et al. 2008, Judd, Nanes et al. 2008, Lee, O'Keefe et al. 2008)

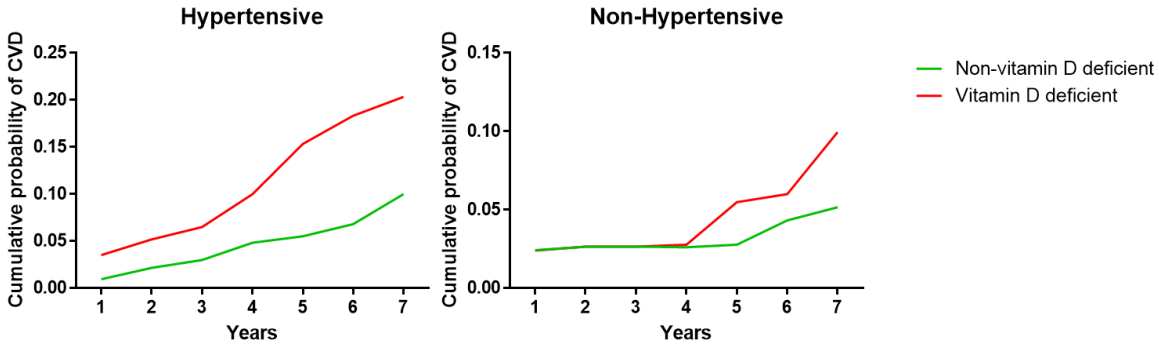


Figure 1-4. Hypertensive vitamin D deficient individuals have increased probability of cardiovascular disease compared to non-hypertensive vitamin D deficient individuals. (Reproduced from Lee et al., 2008)

Although many of the cardiovascular conditions observed in the United States involve intrinsic abnormalities in the heart or vasculature, extrinsic factors may also contribute to the development of disease. Vitamin D may influence the ANS, which is critical in maintaining and regulating cardiovascular function. The VDR is highly expressed on neurons in the brainstem suggesting a prominent role for the vitamin D. (Dimova, Tankova et al. 2017) This is highlighted by the fact that neurotransmitter synthesis is also regulated by vitamin D, particularly acetylcholine, serotonin and dopamine, which are important in the function of the ANS. (DeLuca, Kimball et al. 2013) During times of stress, the parasympathetic nervous system is typically inhibited but VDD has been shown to prevent this inhibition, possibly due to the vitamin's role in the central nervous system. (Dimova, Tankova et al. 2017) Thus, autonomic imbalance even in otherwise healthy VDD individuals may set the stage for cardiac dysfunction in the long-term. (Canpolat, Özcan et al. 2015) To better understand the relationship between VDD and cardiovascular disease, and how VDD causes these deficits, the

mediators and the pathways involved in the vitamin D effects on the cells of the body need to be studied.

Klotho

Recent studies have shown that the cardiac effects of vitamin D are mediated through the production and release of klotho. Klotho was identified as an anti-aging gene in 1997 using mutated mouse strains that developed atherosclerosis, osteopenia, emphysema, skin atrophy and shortened lifespans. (Matsumura, Aizawa et al. 1998) Due to the pre-mature aging phenotype associated with this gene, it was termed *klotho* after the Greek goddess spinning the thread of life. (Strewler 2007) It is an anti-aging transmembrane protein that is cleaved and enters systemic circulation and has been found in blood, serum and cerebrospinal fluid. (Imura, Tsuji et al. 2007, Strewler 2007) The soluble form of klotho, which can form a dimer, activates a myriad of cellular signaling pathways including those involved in antioxidant defense, enzymatic detoxification, inflammation and regulation of cationic channels like transient receptor potential (TRP). (Berridge 2015) Additionally, there are two forms, α- and β-klotho, the form used in these studies is α-klotho.(Imura, Tsuji et al. 2007) Klotho receptors have not been identified but binding to monosialogangliosides (acidic glycosphingolipid with sialic acid) in lipid rafts are thought to act as potential receptors for klotho. On the other hand, klotho also binds to sialic acid residues on certain cation channels (e.g. TRP channels) and exhibit hormone-like properties. (Dalton, An et al. 2017)

Klotho and calcium

Klotho is expressed in numerous tissues in the body including the kidneys, parathyroid gland and choroid plexus of the brain, all of which have substantial

involvement in calcium homeostasis. (Matsumura, Aizawa et al. 1998) Interestingly, it was noted that klotho deficient mice have elevated calcium, phosphate and vitamin D levels suggesting that the body tries to compensate for the lack of klotho by increasing vitamin D levels. (Strewler 2007, Berridge 2015) Regardless, klotho deficiency results in a premature aging phenotype (e.g. shortened lifespan, atherosclerosis, osteoporosis) and there are several mechanisms implicated in the subsequent development of dysfunction. For example, organ calcifications particularly in the kidneys can lead to typical symptoms of osteoporosis. (Strewler 2007) Moreover, klotho's role in calcium homeostasis is likely mediated by Na+-K+-ATPase, which is a membrane binding protein recruited to the plasma membrane in response to calcium signaling with klotho being required in the pathway. Klotho may also play a role in PTH secretion through the same Na+-K+-ATPase pathway described above. (Imura, Tsuji et al. 2007) This data suggests that the characteristic decrease in PTH in VDD could be mediated by disruption of Na+-K+-ATPase recruitment to the plasma membrane in response to calcium signaling. Additionally, klotho has a negative regulatory feedback mechanism to maintain calcium homeostasis.

Klotho and the heart

Recent studies suggest that klotho has cardioprotective effects (Takeshita, Fujimori et al. 2004), either through the prevention of oxidative stress,(Yang, Wang et al. 2015) or inhibition of TRP channels (Xie, Cha et al. 2012) and RAAS activation (Eltablawy and Ashour 2018). Additionally, klotho deficient mice have increased left ventricular pressure and hypertrophy,(Hu, Shi et al. 2011) likely due to premature activation of components of RAAS,(Zhou, Mo et al. 2015, Yu, Meng et al. 2016, Yang

and Xu 2017) as well as neurological deficits which might contribute to autonomic dysfunction (Cararo-Lopes, Mazucanti et al. 2017). Klotho has been shown to be expressed in the sinoatrial node region (i.e. the pacemaker) of the heart and plays a critical role during stress (Takeshita, Fujimori et al. 2004) and even characterized as a biomarker of acute stress (Abdelmalik, Stevens et al. 2018). Wild-type mice at baseline have a normal heart rate and electrocardiogram, but have increased heart rate and normal electrocardiogram changes during restraint stress. In contrast, klotho deficient mice also have a normal heart rate response at baseline but during restraint stress these mice develop a sinoatrial node arrhythmia, which is likely due to altered cation channel function. Interestingly, the same sinoatrial node arrhythmia has been reported in mice exposed to air pollution. (Takeshita, Fujimori et al. 2004, Stratford, Haykal-Coates et al. 2018) Therefore, it appears that the bulk of VDD cardiovascular effects may revolve around the regulation and flux of calcium in and out of cells due to altered cation channel properties.

Transient receptor potential channel C6 (TRPC6)

One of the cation channels present in the heart and regulated by klotho is transient receptor potential C6 (TRPC6) cation channel. Transient receptor potential channels are non-selective, non-voltage gated cation channels that are important in cardiovascular disease (Figure 1-5). (Dietrich, Steinritz et al. 2017) (Watanabe, Murakami et al. 2009, Rowell, Koitabashi et al. 2010) The canonical TRP family includes TRPC6, under normal conditions has low expession, but it is upregulated in cardiac diseases and cardiac myopathy. (Rowell, Koitabashi et al. 2010, Xie, Cha et al. 2012, Loga, Domes et al. 2013, Watanabe, Iino et al. 2013, Seo, Rainer et al. 2014)

TRPC6 is activated by mechanical and oxidative stress. (Seo, Rainer et al. 2014) Mechanical stress is characterized by frictional force that causes muscular stretching, which in turn activates the channel. (Davies 2009) Activation of TRPC6 results in alterations of calcium membrane potentials that drive calcium transport into the cell, resulting in increased contractility. (Watanabe, Murakami et al. 2009, Watanabe, Iino et al. 2013) In VDD, higher blood pressure or mechanical stress overload might stimulate an increase in calcium due to the potentiated TRPC6 activation. All of this occurs because the myocardium must stretch to allow for blood to eject against the increased mechanical load. Consequently, if this process is sustained, there is a likelihood that cardiac hypertrophy and eventually heart failure will occur. (Yamaguchi, Iribe et al. 2017) In fact, increased expression of TRPC6 (and TRPC3) has been found in the heart as well as in the vascular smooth muscle and endothelium cells of humans and rodents prone to heart failure. (Loga, Domes et al. 2013, Watanabe, Iino et al. 2013, Yamaguchi, Iribe et al. 2017) Abnormal calcium signaling in the brain (Sawamura, Shirakawa et al. 2017) and kidneys (Wu, Xie et al. 2017) , due to other factors, also causes increased TRPC6 leading to dysfunction. Therefore, expression of TRPC6 increases due to stress (e.g. mechanical stress or exercise) and appears to result in increased calcium influx.

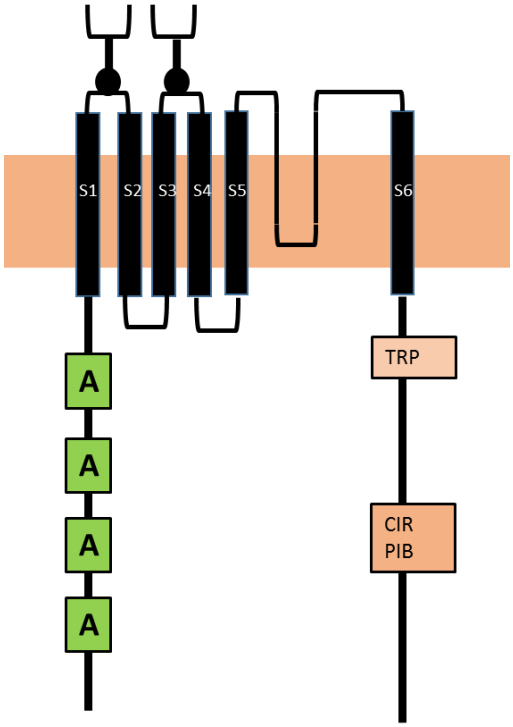


Figure 1-5. Schematic of transient receptor potential channel C6 (TRPC6). (Reproduced from Dietrich et al, 2017)

Klotho and TRPC6

Chronic high blood pressure and other similar cardiovascular conditions are dangerous not only because of the vascular effect but also because of the resulting mechanical changes on the heart due to sustained pressure overload. Studies have demonstrated that TRPC6 inhibition prevents cardiac hypertrophy from pressure load and is a potential therapeutic target. (Xie, Cha et al. 2012, Seo, Rainer et al. 2014) The stress that pressure overload puts on the heart is characterized by abnormal calcium signaling and upregulation of TRPC6, which can be considered a feedback loop. Under such circumstances, not only is the function of the heart altered at rest (i.e. baseline contractile characteristics) but also if the body encounters a stressor like exercise. Recent studies show that in klotho deficiency and subsequent pressure overload conditions, TRPC6 expression and cation influx are upregulated. (Frohlich and Susic

2012, Xie, Cha et al. 2012) This data suggests that the calcium signaling pathways in normal and pressure-overload hearts are distinct and that VDD may result in the latter due to the coexisting hypertension.

Cardiac stress test

The cardiovascular system operates just like all the other organ systems in that it maintains physiological processes and responds to the metabolic demands of the body. In particular, increases in heart rate and contractility, and changes in vasodilatation/constriction modify the flow of oxygenated blood to the tissues that need it the most (i.e. demand). Luckily the cardiovascular system is well equipped to respond to stress due to additional reserve capacity, when work rate, oxygen consumption and metabolic demand increase greatly. Evaluating this ability to compensate using a cardiac stress test can help clinicians diagnose the presence of underlying heart disease and at times the etiology of symptoms (e.g. angina). (Iyngkaran, Anavekar et al. 2017, Koyner and Chawla 2017) The purpose behind this approach is to uncover a latent change in the body which may be hidden or otherwise unnoticed. Although this is often achieved by an exercise challenge like walking or even running on a treadmill, dobutamine, whichi s beta-adrenergic receptor agonist, can be used to increase heart rate and contractility. Previous studies suggest that dobutamine administration mimics the cardiac effects of exercise and we have previously demonstrated that this form of challenge testing uncovers the harmful underlying effects of air pollution. (Hazari, Callaway et al. 2012, Kieu, Shaikh et al. 2017) Therefore, its use in this setting is not only suitable for the purposes of measuring cardiovascular toxicity but also mimicking what might be observed in a person.

Scope of dissertation

Epidemiological, human and animal data have linked adverse cardiovascular outcomes to air pollution exposure. Yet, it is only recently that toxicologists have begun to focus on the role of other factors due to the fact that the effects of today's ambient air pollution may not be perceivable. In other words, are there latent effects to the body despite the lower levels of air pollution in the United States and other developed countries and are those effects exacerbated in the presence of underlying deficiencies or disease. Although conditions like asthma and metabolic syndrome have been studied extensively in the context of air pollution, other conditions, like micronutrient deficiencies, have not. Part of the reason these maladies are not addressed with respect to cardiovascular dysfunction is the lack of conclusive evidence suggesting there is a link. Despite this, there is sufficient data suggesting that micronutrient deficiencies like VDD cause underlying physiological changes which alter the body's ability to respond to fluctuations in environmental conditions and insults even if they do not directly cause disease. Therefore, studies need to examine the impact of conditions like VDD or other public health concerns such as folate deficiency, in order to uncover whether there is a heightened susceptibility for an adverse response. Furthermore, characterization of the mechanism by which VDD causes these cardiac effects will provide biological plausibility and aid in the risk assessment process. The project described herein can begin to contribute to this effort. The impact of early-life VDD in this paradigm has not yet been determined and air pollution is likely the type of ubiquitous environmental stressor that causes adverse cardiovascular effects and overall decrements in public health. The goal of my dissertation is to understand how VDD alters cardiovascular responses to air pollution and further determine the role of

klotho and TRPC6 in mediating such responses (Figure 1-6). **Therefore, the central hypothesis of this project is that VDD changes the cardiovascular response to air pollution through mechanisms involving klotho and TRPC6.**

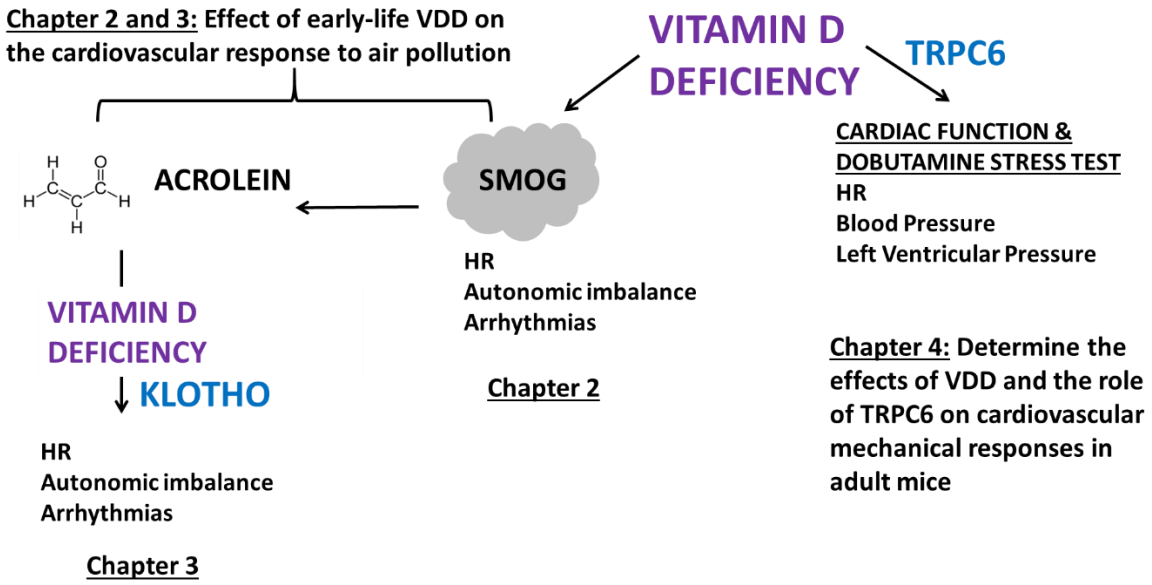


Figure 1-6. Schematic of dissertation approach. Chapter 2 and 3 will address the effect of early-life VDD on the cardiovascular response to air pollution exposure in adult mice. Chapter 3 will describe the role of klotho in VDD-induced acrolein exposure effects. Next, the effect of VDD on the mechanical function of the heart and the role of TRPC6 is explored in chapter 4.

To test this hypothesis, the following specific aims were examined.

1. Describe the effect of early-life VDD on the cardiovascular response to photochemical smog exposure in adult mice.
2. Describe the role of klotho in VDD-induced adverse cardiac response to acrolein in adult mice.
3. Describe the role of TRPC6 in VDD-induced cardiovascular mechanical responses in adult mice.

Specific Aim 1: Chapter 2 will describe the effect of early-life VDD on the cardiovascular response to photochemical smog exposure in adult mice.

There is growing evidence that VDD, which is now a worldwide public health concern, leads to an increased risk of cardiovascular disease. Yet it is still unclear whether it exacerbates the deleterious cardiovascular effects (e.g. arrhythmia, hypertension, fatal heart attacks) of environmental stressors like air pollution, particularly when it persists from early-life to adulthood. I hypothesize that persistent early-life VDD alters cardiac function and modifies the response of adult mice to smog.

Specific Aim 2: Chapter 3 will describe the role of klotho in VDD-induced adverse cardiac response to acrolein in adult mice.

Klotho is an anti-aging protein expressed in the sinoatrial node region of the heart with a critical role during stress, and is also suggested to be a mediator in vitamin D effects throughout the body. Klotho deficient mice have shortened lifespans, vascular calcifications and susceptibility to arrhythmia. Additionally, VDD mice have lower klotho mRNA expression in the heart. I hypothesize that treatment with klotho blocks the cardiovascular response of vitamin D deficient mice to acrolein.

Specific Aim 3: Chapter 4 will describe the role of TRPC6 in VDD-induced cardiovascular mechanical responses in adult mice.

Overexpression of TRPC6 has been shown to be associated with certain cardiovascular diseases, particularly those with pressure-overload, and is also believed to be increased in VDD. In this study, my goal is to determine the effect of VDD on the mechanical function of the heart both at rest and during dobutamine stress test. I hypothesize that

the changes in cardiovascular mechanical function in adult mice due to early-life persistent VDD are mediated by TRPC6.

Main objective of this dissertation

The results of this project are expected to improve our understanding of the role of VDD, a common worldwide public health problem, in the development of cardiovascular disease and air pollution-induced cardiovascular toxicity. The relevance of this work to public health will be further established through on-going collaborations with epidemiologists examining similar effects in human populations.

REFERENCES

Abdelmalik, P. A., R. D. Stevens, S. Singh, J. Skinner, J. R. Carhuapoma, S. Noel, R. Johns and R. J. Fuchs (2018). "Anti-aging factor, serum alpha-Klotho, as a marker of acute physiological stress, and a predictor of ICU mortality, in patients with septic shock." J Crit Care **44**: 323-330.

Al-Khalidi, B., S. M. Kimball, M. A. Rotondi and C. I. Ardern (2017). "Standardized serum 25-hydroxyvitamin D concentrations are inversely associated with cardiometabolic disease in U.S. adults: a cross-sectional analysis of NHANES, 2001-2010." Nutr J **16**(1): 16.

Baksi, S. N. "Deficiency in dietary vitamin D, not calcium, alters noradrenergic responsiveness in rat atria in vitro." Journal of molecular and cellular cardiology **18**(7): 653-656.

Bein, K. and G. D. Leikauf (2011). "Acrolein - a pulmonary hazard." Mol Nutr Food Res **55**(9): 1342-1360.

Ben-Jebria, A., R. Marthan, M. Rossetti, J. P. Savineau and J. S. Ultman (1994). "Human bronchial smooth muscle responsiveness after in vitro exposure to acrolein." Am J Respir Crit Care Med **149**(2 Pt 1): 382-386.

Berridge, M. J. (2015). "Vitamin D cell signalling in health and disease." Biochemical and Biophysical Research Communications **460**(1): 53-71.

Bharadwaj, P., J. G. Zivin and J. T. Mullins (2016). "Early-Life Exposure to the Great Smog of 1952 and the Development of Asthma." **194**(12): 1475-1482.

Brook, R. D. and S. Julius (2000). "Autonomic imbalance, hypertension, and cardiovascular risk." American journal of hypertension **13**(S4): 112S-122S.

Brook, R. D., S. Rajagopalan, C. A. Pope, J. R. Brook, A. Bhatnagar, A. V. Diez-Roux, F. Holguin, Y. Hong, R. V. Luepker, M. A. Mittleman, A. Peters, D. Siscovick, S. C. Smith, L. Whitsel, J. D. Kaufman, o. b. o. t. A. H. A. C. o. Epidemiology, C. o. t. K. i. C. D. Prevention, P. A. Council on Nutrition and Metabolism (2010). "Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement From the American Heart Association." Circulation **121**(21): 2331-2378.

Canpolat, U., F. Özcan, Ö. Özeke, O. Turak, Ç. Yayla, S. K. Açıkgöz, S. Çay, S. Topaloğlu, D. Aras and S. Aydoğdu (2015). "Impaired Cardiac Autonomic Functions in Apparently Healthy Subjects with Vitamin D Deficiency." Annals of Noninvasive Electrocardiology **20**(4): 378-385.

Cararo-Lopes, M. M., C. H. Y. Mazucanti, C. Scavone, E. M. Kawamoto and D. C. Berwick (2017). "The relevance of alpha-KLOTHO to the central nervous system: Some key questions." Ageing Res Rev **36**: 137-148.

Carbone, F., F. Mach, N. Vuilleumier and F. Montecucco (2014). "Potential pathophysiological role for the vitamin D deficiency in essential hypertension." World J Cardiol **6**(5): 260-276.

Carll, A. P., S. M. Crespo, M. S. Filho, D. H. Zati, B. A. Coull, E. A. Diaz, R. D. Raimundo, T. N. G. Jaeger, A. L. Ricci-Vitor, V. Papapostolou, J. E. Lawrence, D. M. Garner, B. S. Perry, J. R. Harkema and J. J. Godleski (2017). "Inhaled ambient-level traffic-derived particulates decrease cardiac vagal influence and baroreflexes and increase arrhythmia in a rat model of metabolic syndrome." Part Fibre Toxicol **14**(1): 16.

Carll, A. P., R. M. Lust, M. S. Hazari, C. M. Perez, Q. T. Krantz, C. J. King, D. W. Winsett, W. E. Cascio, D. L. Costa and A. K. Farraj (2013). "Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats." Toxicol Sci **131**(2): 583-595.

Chiu, H. F., Y. H. Weng, Y. W. Chiu and C. Y. Yang (2017). "Short-term effects of ozone air pollution on hospital admissions for myocardial infarction: A time-stratified case-crossover study in Taipei." J Toxicol Environ Health A **80**(5): 251-257.

Conklin, D. J., P. Haberzettl, G. Jagatheesan, M. Kong and G. W. Hoyle (2017). "Role of TRPA1 in acute cardiopulmonary toxicity of inhaled acrolein." Toxicol Appl Pharmacol **324**: 61-72.

Coogan, P. F., L. F. White, J. Yu, R. D. Brook, R. T. Burnett, J. D. Marshall, T. N. Bethea, L. Rosenberg and M. Jerrett (2017). "Long-Term Exposure to NO2 and Ozone and Hypertension Incidence in the Black Women's Health Study." Am J Hypertens **30**(4): 367-372.

Costa, A. F., G. Hoek, B. Brunekreef and A. C. M. Ponce de Leon (2017). "Effects of NO2 exposure on daily mortality in Sao Paulo, Brazil." Environ Res **159**: 539-544.

Dalton, G., S. W. An, S. I. Al-Juboori, N. Nischan, J. Yoon, E. Dobrinskikh, D. W. Hilgemann, J. Xie, K. Luby-Phelps, J. J. Kohler, L. Birnbaumer and C. L. Huang (2017). "Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling." Proc Natl Acad Sci U S A **114**(4): 752-757.

Davies, P. F. (2009). "Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology." Nat Clin Pract Cardiovasc Med **6**(1): 16-26.

Davoodi, G., A. Y. Sharif, A. Kazemisaeid, S. Sadeghian, A. V. Farahani, M. Sheikhvatan and M. Pashang (2010). "Comparison of heart rate variability and cardiac arrhythmias in polluted and clean air episodes in healthy individuals." Environ Health Prev Med **15**(4): 217-221.

Day, D. B., J. Xiang, J. Mo, F. Li, M. Chung, J. Gong, C. J. Weschler, P. A. Ohman-Strickland, J. Sundell, W. Weng, Y. Zhang and J. J. Zhang (2017). "Association of

Ozone Exposure With Cardiorespiratory Pathophysiologic Mechanisms in Healthy Adults." JAMA Intern Med **177**(9): 1344-1353.

DeLuca, G. C., S. M. Kimball, J. Kolasinski, S. V. Ramagopalan and G. C. Ebers (2013). "Review: the role of vitamin D in nervous system health and disease." Neuropathol Appl Neurobiol **39**(5): 458-484.

Dietrich, A., D. Steinritz and T. Gudermann (2017). "Transient receptor potential (TRP) channels as molecular targets in lung toxicology and associated diseases." Cell Calcium **67**: 123-137.

Dimova, R., T. Tankova and N. Chakarova (2017). "Vitamin D in the Spectrum of Prediabetes and Cardiovascular Autonomic Dysfunction." The Journal of Nutrition **147**(9): 1607-1615.

Dooley, E. E. (2002). "Fifty years later: clearing the air over the London smog." Environ Health Perspect **110**(12): A748.

Dusso, A. S. (2011). "Kidney disease and vitamin D levels: 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and VDR activation." Kidney International Supplements **1**(4): 136-141.

Eltablawy, N. and H. Ashour (2018). "Vitamin D protection from rat diabetic nephropathy is partly mediated through Klotho expression and renin-angiotensin inhibition." 1-7.

Esterbauer, H., R. J. Schaur and H. Zollner (1991). "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes." Free Radic Biol Med **11**(1): 81-128.

Farraj, A. K., M. S. Hazari, D. W. Winsett, A. Kulukulualani, A. P. Carll, N. Haykal-Coates, C. M. Lamb, E. Lappi, D. Terrell and W. E. Cascio (2011). "Overt and latent cardiac effects of ozone inhalation in rats: evidence for autonomic modulation and increased myocardial vulnerability." Environmental health perspectives **120**(3): 348-354.

Fetahu, I. S., J. Höbaus and E. Kállay (2014). "Vitamin D and the epigenome." Frontiers in Physiology **5**: 164.

Forman, J. P., G. C. Curhan and E. N. Taylor (2008). "Plasma 25-hydroxyvitamin D levels and risk of incident hypertension among young women." Hypertension **52**(5): 828-832.

Frohlich, E. D. and D. Susic (2012). "Pressure overload." Heart Fail Clin **8**(1): 21-32.
Ghilarducci, D. P. and R. S. Tjeerdema (1995). "Fate and effects of acrolein." Rev Environ Contam Toxicol **144**: 95-146.

Gold, D. R., A. Litonjua, J. Schwartz, E. Lovett, A. Larson, B. Nearing, G. Allen, M. Verrier, R. Cherry and R. Verrier (2000). "Ambient pollution and heart rate variability." Circulation **101**(11): 1267-1273.

Hazari, M., J. Griggs, D. Winsett, N. Haykal-Coates, A. Ledbetter, D. Costa and A. Farraj (2014). "A Single Exposure to Acrolein Desensitizes Baroreflex Responsiveness and Increases Cardiac Arrhythmias in Normotensive and Hypertensive Rats." Cardiovascular Toxicology **14**(1): 52-63.

Hazari, M. S., J. Callaway, D. W. Winsett, C. Lamb, N. Haykal-Coates, Q. Krantz, C. King, D. L. Costa and A. K. Farraj (2012). "Dobutamine" stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats." Environmental health perspectives **120**(8): 1088-1093.

Heusser, K., J. Tank, F. C. Luft and J. Jordan (2005). "Baroreflex Failure." Hypertension **45**(5): 834.

Holick, M. F. (2007). "Vitamin D Deficiency." New England Journal of Medicine **357**(3): 266-281.

Hu, M. C., M. Shi, J. Zhang, H. Quinones, C. Griffith, M. Kuro-o and O. W. Moe (2011). "Klotho deficiency causes vascular calcification in chronic kidney disease." J Am Soc Nephrol **22**(1): 124-136.

Hunt, A., J. L. Abraham, B. Judson and C. L. Berry (2003). "Toxicologic and epidemiologic clues from the characterization of the 1952 London smog fine particulate matter in archival autopsy lung tissues." Environ Health Perspect **111**(9): 1209-1214.

Imura, A., Y. Tsuji, M. Murata, R. Maeda, K. Kubota, A. Iwano, C. Obuse, K. Togashi, M. Tominaga, N. Kita, K. Tomiyama, J. Iijima, Y. Nabeshima, M. Fujioka, R. Asato, S. Tanaka, K. Kojima, J. Ito, K. Nozaki, N. Hashimoto, T. Ito, T. Nishio, T. Uchiyama, T. Fujimori and Y. Nabeshima (2007). "alpha-Klotho as a regulator of calcium homeostasis." Science **316**(5831): 1615-1618.

Iyngkaran, P., N. S. Anavekar, C. Neil, L. Thomas and D. L. Hare (2017). "Shortness of breath in clinical practice: A case for left atrial function and exercise stress testing for a comprehensive diastolic heart failure workup." World J Methodol **7**(4): 117-128.

Judd, S. E., M. S. Nanes, T. R. Ziegler, P. W. Wilson and V. Tangpricha (2008). "Optimal vitamin D status attenuates the age-associated increase in systolic blood pressure in white Americans: results from the third National Health and Nutrition Examination Survey." Am J Clin Nutr **87**(1): 136-141.

Kara, T., K. Narkiewicz and V. K. Somers (2003). "Chemoreflexes--physiology and clinical implications." Acta Physiol Scand **177**(3): 377-384.

Kehrer, J. P. and S. S. Biswal (2000). "The molecular effects of acrolein." Toxicol Sci **57**(1): 6-15.

Kieu, A., A. Shaikh, M. Kaeppler, R. J. Miles and M. E. Widlansky (2017). "Patients with hypertensive responses to exercise or dobutamine stress testing differ in resting hypertensive phenotype." J Am Soc Hypertens.

Koyner, J. L. and L. S. Chawla (2017). "Use of stress tests in evaluating kidney disease." Curr Opin Nephrol Hypertens **26**(1): 31-35.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, L. Walsh, A. K. Farraj and M. S. Hazari (2014). "Ozone co-exposure modifies cardiac responses to fine and ultrafine ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses." Part Fibre Toxicol **11**: 54.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D Deficiency: An Important, Common, and Easily Treatable Cardiovascular Risk Factor?" Journal of the American College of Cardiology **52**(24): 1949-1956.

Leikauf, G. D., L. M. Leming, J. R. O'Donnell and C. A. Doupnik (1989). "Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein." J Appl Physiol (1985) **66**(1): 171-178.

Li, Y. C., J. Kong, M. Wei, Z. F. Chen, S. Q. Liu and L. P. Cao (2002). "1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system." J Clin Invest **110**(2): 229-238.

Li, Y. C., G. Qiao, M. Uskokovic, W. Xiang, W. Zheng and J. Kong (2004). "Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure." J Steroid Biochem Mol Biol **89-90**(1-5): 387-392.

Loga, F., K. Domes, M. Freichel, V. Flockerzi, A. Dietrich, L. Birnbaumer, F. Hofmann and J. W. Wegener (2013). "The role of cGMP/cGKI signalling and Trpc channels in regulation of vascular tone." Cardiovasc Res **100**(2): 280-287.

Martins, D., M. Wolf, D. Pan and et al. (2007). "Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin d in the united states: Data from the third national health and nutrition examination survey." Archives of Internal Medicine **167**(11): 1159-1165.

Matsumura, Y., H. Aizawa, T. Shiraki-Iida, R. Nagai, M. Kuro-o and Y. Nabeshima (1998). "Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein." Biochem Biophys Res Commun **242**(3): 626-630.

Mirowsky, J. E., M. S. Carraway, R. Dhingra, H. Tong, L. Neas, D. Diaz-Sanchez, W. Cascio, M. Case, J. Crooks, E. R. Hauser, Z. Elaine Dowdy, W. E. Kraus and R. B. Devlin (2017). "Ozone exposure is associated with acute changes in inflammation, fibrinolysis, and endothelial cell function in coronary artery disease patients." Environ Health **16**(1): 126.

Mozaffarian, D., E. J. Benjamin, A. S. Go, D. K. Arnett, M. J. Blaha, M. Cushman, S. R. Das, S. de Ferranti, J. P. Despres, H. J. Fullerton, V. J. Howard, M. D. Huffman, C. R. Isasi, M. C. Jimenez, S. E. Judd, B. M. Kissela, J. H. Lichtman, L. D. Lisabeth, S. Liu, R. H. Mackey, D. J. Magid, D. K. McGuire, E. R. Mohler, 3rd, C. S. Moy, P. Muntner, M. E. Mussolino, K. Nasir, R. W. Neumar, G. Nichol, L. Palaniappan, D. K. Pandey, M. J. Reeves, C. J. Rodriguez, W. Rosamond, P. D. Sorlie, J. Stein, A. Towfighi, T. N. Turan, S. S. Virani, D. Woo, R. W. Yeh and M. B. Turner (2016). "Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association." Circulation **133**(4): e38-360.

Muscogiuri, G., C. Annweiler, G. Duval, S. Karras, G. Tirabassi, G. Salvio, G. Balercia, S. Kimball, K. Kotsa, L. Mascitelli, H. P. Bhattoa and A. Colao (2017). "Vitamin D and cardiovascular disease: From atherosclerosis to myocardial infarction and stroke." Int J Cardiol **230**: 577-584.

Norman, P. E. and J. T. Powell (2014). "Vitamin D and Cardiovascular Disease." Circulation Research **114**(2): 379-393.

Pilz, S., W. März, B. Wellnitz, U. Seelhorst, A. Fahrleitner-Pammer, H. P. Dimai, B. O. Boehm and H. Dobnig (2008). "Association of Vitamin D Deficiency with Heart Failure and Sudden Cardiac Death in a Large Cross-Sectional Study of Patients Referred for Coronary Angiography." The Journal of Clinical Endocrinology & Metabolism **93**(10): 3927-3935.

Rai, V. and D. K. Agrawal (2017). "Role of Vitamin D in Cardiovascular Diseases." Endocrinol Metab Clin North Am **46**(4): 1039-1059.

Rajagopalan, S. and R. D. Brook (2012). "Air Pollution and Type 2 Diabetes: Mechanistic Insights." Diabetes **61**(12): 3037-3045.

Requia, W. J., M. D. Adams, A. Arain, S. Papatheodorou, P. Koutrakis and M. Mahmoud (2017). "Global Association of Air Pollution and Cardiorespiratory Diseases: A Systematic Review, Meta-Analysis, and Investigation of Modifier Variables." Am J Public Health: e1-e8.

Rowan, W. H., 3rd, M. J. Campen, L. B. Wichers and W. P. Watkinson (2007). "Heart rate variability in rodents: uses and caveats in toxicological studies." Cardiovasc Toxicol **7**(1): 28-51.

Rowell, J., N. Koitabashi and D. A. Kass (2010). "TRP-ing up heart and vessels: canonical transient receptor potential channels and cardiovascular disease." J Cardiovasc Transl Res **3**(5): 516-524.

Saccone, D., F. Asani and L. Bornman (2015). "Regulation of the vitamin D receptor gene by environment, genetics and epigenetics." Gene **561**(2): 171-180.

Sawamura, S., H. Shirakawa, T. Nakagawa, Y. Mori and S. Kaneko (2017). Frontiers in Neuroscience TRP Channels in the Brain: What Are They There For? Neurobiology of TRP Channels. nd and T. L. R. Emir. Boca Raton (FL), CRC Press/Taylor & Francis (c) 2018 by Taylor & Francis Group, LLC.**:** 295-322.

Scragg, R., M. Sowers and C. Bell (2007). "Serum 25-hydroxyvitamin D, ethnicity, and blood pressure in the Third National Health and Nutrition Examination Survey." Am J Hypertens **20**(7): 713-719.

Seo, K., P. P. Rainer, V. Shalkey Hahn, D. I. Lee, S. H. Jo, A. Andersen, T. Liu, X. Xu, R. N. Willette, J. J. Lepore, J. P. Marino, Jr., L. Birnbaumer, C. G. Schnackenberg and D. A. Kass (2014). "Combined TRPC3 and TRPC6 blockade by selective small-molecule or genetic deletion inhibits pathological cardiac hypertrophy." Proc Natl Acad Sci U S A **111**(4): 1551-1556.

Shen, M. J. and D. P. Zipes (2014). "Role of the autonomic nervous system in modulating cardiac arrhythmias." Circ Res **114**(6): 1004-1021.

Sim, V. M. and R. E. Pattle (1957). "Effect of possible smog irritants on human subjects." J Am Med Assoc **165**(15): 1908-1913.

Snow, S. J., M. A. McGee, A. Henriquez, J. E. Richards, M. C. Schladweiler, A. D. Ledbetter and U. P. Kodavanti (2017). "Respiratory Effects and Systemic Stress Response Following Acute Acrolein Inhalation in Rats." Toxicol Sci **158**(2): 454-464.

Stein, P. K., P. P. Domitrovich, N. Hui, P. Rautaharju and J. Gottdiener (2005). "Sometimes higher heart rate variability is not better heart rate variability: results of graphical and nonlinear analyses." J Cardiovasc Electrophysiol **16**(9): 954-959.

Stratford, K., N. Haykal-Coates, L. Thompson, Q. T. Krantz, C. King and J. Krug (2018). "Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice."

Strewler, G. J. (2007). "Untangling klotho's role in calcium homeostasis." Cell Metab **6**(2): 93-95.

Sundar, I. K. and I. Rahman (2011). "Vitamin D and Susceptibility of Chronic Lung Diseases: Role of Epigenetics." Frontiers in Pharmacology **2**: 50.

Takeshita, K., T. Fujimori, Y. Kurotaki, H. Honjo, H. Tsujikawa, K. Yasui, J. K. Lee, K. Kamiya, K. Kitaichi, K. Yamamoto, M. Ito, T. Kondo, S. Iino, Y. Inden, M. Hirai, T. Murohara, I. Kodama and Y. Nabeshima (2004). "Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression." Circulation **109**(14): 1776-1782.

Thayer, J. F., S. S. Yamamoto and J. F. Brosschot (2010). "The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors." International Journal of Cardiology **141**(2): 122-131.

Trehan, N., L. Afonso, D. L. Levine and P. D. Levy (2017). "Vitamin D Deficiency, Supplementation, and Cardiovascular Health." Crit Pathw Cardiol **16**(3): 109-118.

Tsuji, H., M. G. Larson, F. J. Venditti, Jr., E. S. Manders, J. C. Evans, C. L. Feldman and D. Levy (1996). "Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study." Circulation **94**(11): 2850-2855.

Walters, M. R. "1,25-Dihydroxyvitamin D3 receptors identified in the rat heart." Journal of molecular and cellular cardiology **18**(1): 67-72.

Wang, T. J., M. J. Pencina, S. L. Booth, P. F. Jacques, E. Ingelsson, K. Lanier, E. J. Benjamin, R. B. D'Agostino, M. Wolf and R. S. Vasan (2008). "Vitamin D deficiency and risk of cardiovascular disease." Circulation **117**(4): 503-511.

Watanabe, H., K. Iino, T. Ohba and H. Ito (2013). "Possible involvement of TRP channels in cardiac hypertrophy and arrhythmia." Curr Top Med Chem **13**(3): 283-294.

Watanabe, H., M. Murakami, T. Ohba, K. Ono and H. Ito (2009). "The pathological role of transient receptor potential channels in heart disease." Circ J **73**(3): 419-427.

Wu, Y. L., J. Xie, S. W. An, N. Oliver, N. X. Barrezueta, M. H. Lin, L. Birnbaumer and C. L. Huang (2017). "Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho." Kidney Int **91**(4): 830-841.

Xie, J., S. K. Cha, S. W. An, O. M. Kuro, L. Birnbaumer and C. L. Huang (2012). "Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart." Nat Commun **3**: 1238.

Yamaguchi, Y., G. Iribe, M. Nishida and K. Naruse (2017). "Role of TRPC3 and TRPC6 channels in the myocardial response to stretch: Linking physiology and pathophysiology." Prog Biophys Mol Biol **130**(Pt B): 264-272.

Yang, B. Y., Z. M. Qian, M. G. Vaughn, E. J. Nelson, S. C. Dharmage, J. Heinrich, S. Lin, W. R. Lawrence, H. Ma, D. H. Chen, L. W. Hu, X. W. Zeng, S. L. Xu, C. Zhang and G. H. Dong (2017). "Is prehypertension more strongly associated with long-term

ambient air pollution exposure than hypertension? Findings from the 33 Communities Chinese Health Study." Environ Pollut **229**: 696-704.

Yang, K., C. Wang, L. Nie, X. Zhao, J. Gu, X. Guan, S. Wang, T. Xiao, X. Xu, T. He, X. Xia, J. Wang and J. Zhao (2015). "Klotho Protects Against Indoxyl Sulphate-Induced Myocardial Hypertrophy." J Am Soc Nephrol **26**(10): 2434-2446.

Yang, T. and C. Xu (2017). "Physiology and Pathophysiology of the Intrarenal Renin-Angiotensin System: An Update." J Am Soc Nephrol **28**(4): 1040-1049.

Yu, L., W. Meng, J. Ding and M. Cheng (2016). "Klotho inhibits angiotensin II-induced cardiomyocyte hypertrophy through suppression of the AT1R/beta catenin pathway." Biochem Biophys Res Commun **473**(2): 455-461.

Zhou, L., H. Mo, J. Miao, D. Zhou, R. J. Tan, F. F. Hou and Y. Liu (2015). "Klotho Ameliorates Kidney Injury and Fibrosis and Normalizes Blood Pressure by Targeting the Renin-Angiotensin System." The American Journal of Pathology **185**(12): 3211-3223.

Chapter 2: Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice[1]

Introduction

It is clear from epidemiological, human and animal studies that air pollution has a deleterious effect on cardiovascular health (e.g. ischemic heart disease, arrhythmia, stroke). (Brook, Rajagopalan et al. 2010) In fact, research from the last ten years has shown that there are several factors, which include not only those related to air pollution such as concentration, composition and chemistry, but also host factors like nutrition, that contribute to the overall response. Thus, characterization of these factors with the intent of understanding the underlying toxicological mechanisms as well as providing useable public health information about individual susceptibility is crucial to reducing the harmful effects of air pollution particularly given the prevalence of chronic diseases like asthma and heart disease.

Like all the organ systems, regulation and proper function of the cardiovascular system is dependent on adequate levels of micronutrients like vitamin D, which is one of a few molecules with a critical homeostatic role throughout the body. (Holick 2007, Lee, O'Keefe et al. 2008)

---

[1]This chapter previously appeared as an article in the Journal of Environmental Science and Technology. The original citation is as follows: Stratford, K., N. Haykal-Coates, L. Thompson, Q. T. Krantz, C. King and J. Krug (2018). "Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice." *Environmental Science & Technology.* 2018 52 (5), 3054-3061.

Although recent data supports the relationship between VDD and cardiovascular impairment (Wang, Pencina et al. 2008, Anderson, May et al. 2010, Masson, Agabiti et al. 2014), the link to cardiovascular disease development is still not firmly established nor is it clear whether it contributes to adverse responses due to air pollution. Vitamin D (Vitamin D_3) is a fat-soluble vitamin produced endogenously in the skin, after ultraviolent B radiation exposure, and acquired through the diet from a variety of foods like milk, fish, cheese and beans.(Lips 2006, Holick 2007) Vitamin D receptors are present on numerous tissues and cells throughout the body, including cardiomyocytes.(Holick 2007) Minimal sun exposure, obesity, improper nutrition as well as a myriad of other factors result in vitamin D deficiency (VDD)(Holick 2007, Lee, O'Keefe et al. 2008), which has become a public health concern affecting 8% of the pediatric population in the United States.(Kumar, Muntner et al. 2009) Early-life or childhood VDD can lead to vascular dysfunction, hypertension and other cardiac abnormalities(Carlton-Conway, Tulloh et al. 2004, Maiya, Sullivan et al. 2008, Tare, Emmett et al. 2011), yet it's precise role in electrocardiographic abnormalities and cardiac autonomic changes has not been extensively characterized. Vitamin D exerts its effects through a steroid nuclear receptor that(Holick 2007, Norman and Powell 2014) upon activation forms a complex with the retinoid X receptor.(Fetahu, Höbaus et al. 2014, Saccone, Asani et al. 2015) Activated vitamin D receptors (VDR) bind to vitamin D response elements (VDRE) on DNA and influence expression of a wide variety of genes covering numerous functions (ie. calcium homeostasis, antioxidant production, protein synthesis).(Sundar and Rahman 2011, Fetahu, Höbaus et al. 2014, Norman and Powell 2014, Saccone, Asani et al. 2015) For example, *klotho* is a membrane aging protein expressed in the sinoatrial node

region of the heart that is transcribed when vitamin D binds to VDRE. Decreased *klotho* has been demonstrated to be associated with shortened lifespan, vascular calcifications, arrhythmia as well as impaired vitamin D metabolism.(Song, Gao et al. 2013) Given that vitamin D alters gene expression it is not entirely unreasonable to assume that it would contribute to cardiopulmonary dysfunction.

Consequently, this is of interest to researchers given chronic VDD may cause subtle effects which increase the risk of a triggered adverse response to a stressor like air pollution. This is particularly true for complex, particulate matter (PM)-rich atmospheric photochemical smog, which is a mixture of not only PM but also ozone and other gaseous pollutants (e.g. acrolein and aldehyde isoforms) and represents the bulk of what people are exposed to in terms of ambient air pollution. It has long been demonstrated to be an environmental public health concern resulting in excess mortality.(Haagen-Smit 1952, Wilkins 1954, Lynne Page 1994) Therefore, the purpose of this study is to determine the effect of early-life persistent VDD in smog-induced cardiovascular toxicity. We hypothesized that low levels of vitamin D during early-life and persisting into adulthood would 1) induce electrical changes in the hearts of adult mice, 2) cause ventilatory alterations (i.e. altered breathing patterns), and 3) worsen the cardiopulmonary response to atmospheric smog.

Materials and Methods

Animals - Three-week old female C57Bl/6 mice (body weight = 9.6 ± 1.6g) were used in this study (Jackson Laboratory – Raleigh, NC). Mice were housed four-five per cage and maintained on a 12-hr light/dark cycle at approximately 22˚C and 50% relative humidity in an AAA-LAC-approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum during the quarantine

period (3 days) after arrival. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering.

Diet - Three days after the quarantine period ended, mice were maintained ad libitum on either a vitamin D deficient (VDD) (D10073001) or normal diet (ND) (D10012G-Research Diets Inc) for sixteen weeks. The VDD diet had no added vitamin D. The ND has 1000 IU per 10 grams of vitamin D. The diets had equal levels of all other vitamins and minerals including calcium, which was at the concentration specified by the American Institute of Nutrition.(Reeves, Nielsen et al. 1993) Water was provided ad libitum throughout the diet regimen.

Experimental Design and Groups – At the beginning of the study, mice were randomly assigned into a ND (n = 28) or VDD (n = 35) group and maintained on those diets for the extent of the study. Of those animals, 12 of the ND and 12 of the VDD mice were randomly chosen and implanted with radiotelemeters at 16 weeks of age. Each of the ND and VDD animal groups with radiotelemeters were then further randomly assigned to air (FA) or smog exposure groups as were the non-telemetered ND and VDD mice. The timeline of the experimental design is depicted in Figure 2-1.

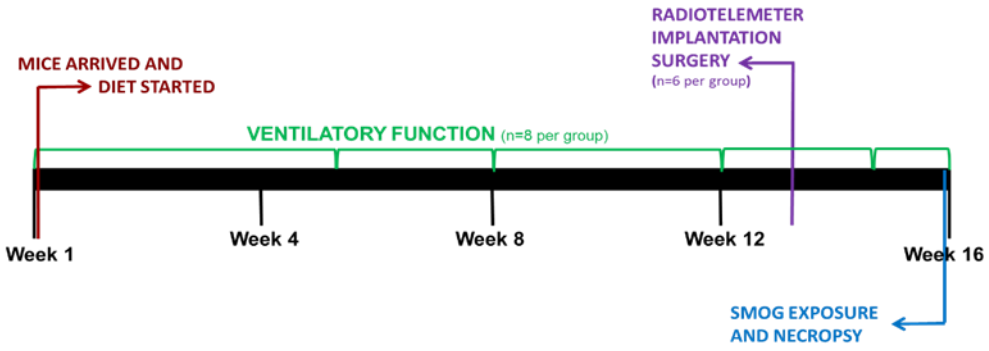


Figure 2-1. Experimental design of diet regimen, electrocardiographic/HRV analysis and photochemical smog exposure.

Surgical Implantation of radiotelemeters and data acquisition – Animals were implanted with radiotelemeters and monitored as previously described(Kurhanewicz, McIntosh-Kastrinsky et al. 2014). Briefly, animals were anesthetized using inhaled isoflurane (Butler Animal Health Supply, Dublin OH) and using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN) to approximate the lead II of a standard electrocardiogram (ECG). All animals were allowed 7-10 days to recover from the surgery and reestablish circadian rhythms. Signals from the radiotelemeters were used to monitor heart rate (HR), and ECG waveforms immediately following telemeter implantation, through exposure until 24hrs post-exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice. See Supplemental Information for specific details on radiotelemeter implantation, and HR and ECG analysis.

Heart Rate Variability Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RRs for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-

analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). HRV analysis was also conducted in the frequency domain using a Fast-Fourier transform. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions. The ratio of these two frequency domains (LF/HF) provides an estimate of the relative balance between sympathetic (LF) and vagal (HF) activity.

Whole-Body Plethysmography - Ventilatory function (e.g. enhanced pause, tidal volume and minute ventilation) was assessed in awake, unrestrained mice using a whole-body plethysmograph (Buxco, Wilmington, NC). Assessments were performed at 3, 8, 11 and 15weeks of age and 24hrs prior to the day of exposure, immediately post-exposure and 24hrs after exposure. The plethysmograph pressure was monitored using Biosystems XA software (Buxco Electronics Inc., Wilmington, NC). Using respiratory-induced fluctuations in ambient pressure, ventilatory parameters including tidal volume (VT), breathing frequency (f), inspiratory time (Ti), expiratory time (Te), minute volume (MV) and enhanced pause (Penh), which is a measure of ventilatory timing and can indicate airway irritation, were calculated and recorded on a breath-by-breath basis.

Tissue Collection and Analysis - Mice were euthanized 24 hours after exposure and blood and bronchoalveolar lavage fluid (BAL) were collected, processed and analyzed. Multiple biochemical markers (e.g. lactate dehydrogenase, protein, etc) were

assessed in the BAL. Vitamin D concentrations were determined in the serum spectro-photometrically using a Vitamin D EIA Kit (Cayman Chemical, Ann Arbor, Michigan).

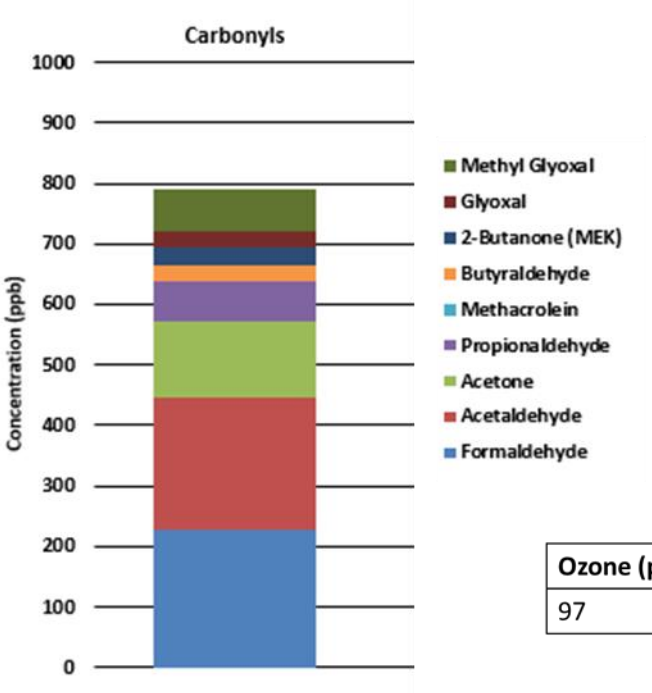
Photochemical Smog Exposures – A PM-enriched atmosphere with high particulate matter and low ozone and nitrogen oxide concentrations (PM-enriched) was generated in the Mobile Reaction Chamber (MRC) as described in detail by Krug et al. (Krug 2018)Briefly, PM-enriched atmosphere was artificially generated with 0.491 ppm nitrogen oxide, 0.528 ppm NOx, 29.9 ppmC total hydrocarbons (THC), 24 ppmC gasoline and 5.3 ppmC α -pinene as the initial conditions, which were then irradiated by ultraviolent light. PM-enriched smog atmosphere was transported under vacuum to 0.3 m^{-3} whole body inhalation chambers. Continuous gas and aerosol sampling for carbon monoxide, ozone, nitrogen oxides, THC and particle mass concentration were conducted at both the MRC unit as well as from the inhalation exposure systems. All PM was formed as secondary organic aerosol from the photochemical reactions in the MRC. Particle size distributions and gravimetric mass sampling was measured. Filter sampling for gravimetric analysis were conducted for the entire exposure time. Volatile organic compound (VOC) summa cannisters were periodically collected and analyzed by gas chromatography off-line to determine concentrations of various VOCs in the exposure atmosphere.

Statistics - All data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) software. Mixed-model ANOVAs, with Tukey's procedure for the post hoc comparisons, were used to examine the statistical differences between exposure and diet. To improve normality of the residuals and because the HRV variable distributions were highly skewed, each HRV parameter was natural- log transformed. Also, the delta values of

the variables from baseline were used in this analysis. The statistical significance was set at $P < 0.05$.

Results

Exposure characteristics - The measured criteria pollutants in PM-enriched atmosphere (MR044) were ozone (0.094 ppm), NO_x (0.154 ppb) and $PM_{2.5}$ (0.307 mg/m^3). The ten most abundant secondary compounds that were generated in the atmosphere were: ethanol (1.03 ppm), alpha-pinene (0.533 ppm), toluene (0.463 ppm), 2-methylpentane (0.330 ppm), n-hexane (0.222 ppm), isopentane (0.212 ppm), m-& p-xylene (0.201 ppm), 3-methylpentane (0.187 ppm), n-pentane (0.103 ppm) and n-butane (0.0748 ppm) (Figure 2-2) The Air Quality Health Index (AQHI), which provides a health-risk assessment of the smog, was 34.3. (Krug 2018)



Ozone (ppb)	NO_2 (ppb)	PM 2.5 ($\mu g/m^3$)
97	244	1070

Figure 2-2. Exposure characteristics of carbonyls and criteria pollutants in PM-enriched atmosphere.

Body weight and vitamin D levels - Body weight was determined on a weekly basis; there were no differences between ND and VDD mice (Figure 2-3). Vitamin D levels were confirmed to be lower in all VDD mice (1.06 ± 0.1 ng/mL) when compared to ND mice (21.1±2.2 ng/mL).

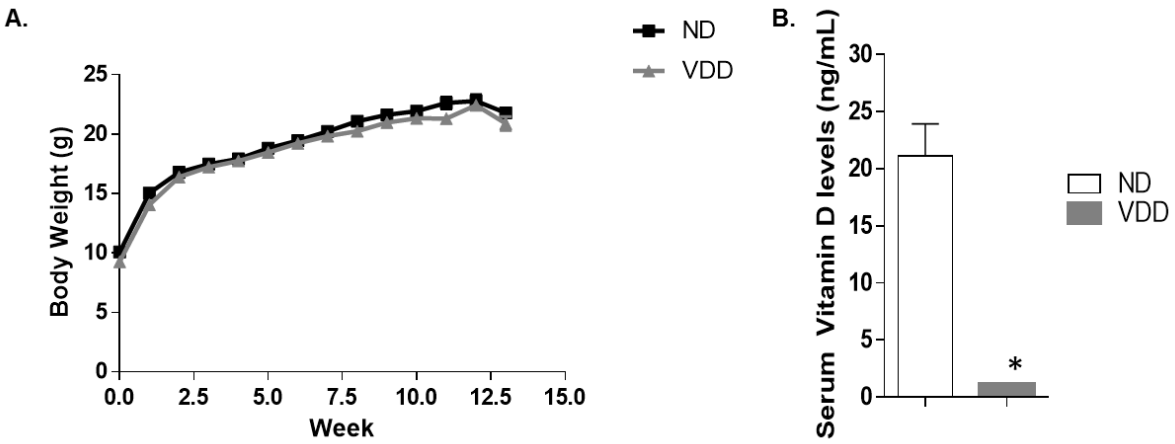


Figure 2-3. Body weight did not differ and VDD mice did become VDD during diet regimen. A. No significant differences in body weight. n = 28-35 B. VDD mice had significantly less serum vitamin D levels than ND mice. *significantly different from ND ($p < 0.05$). Values represent means ± SE.

Heart rate and heart rate variability – HR was decreased in VDD mice from the time of telemeter implantation up until exposure (Figure 2-4A), while HRV (SDNN, RMSSD, HF) increased during the same time period (Figure 2-4 B-D). Log-normal distribution was calculated for HRV because the data was not normally distributed. HR decreased significantly 24 hours after air exposure in ND animals and after PM-enriched exposure in VDD animals. ND animals exposed to PM-enriched smog and VDD animals exposed to air did not exhibit any changes (i.e. their HR did not decrease after exposure) (Figure 2-5A). SDNN was increased in all groups both immediately and 24 hours after exposure when compared to pre-exposure, yet it only decreased in the

24hrs after exposure in ND mice exposed to PM-enriched smog. VDD increased SDNN irrespective of the exposure, yet when combined, VDD and PM-enriched smog had a significantly greater effect on SDNN than either alone (Figure 2-5B). Similar trends were observed with RMSSD in which the combination of VDD and PM-enriched smog had a greater effect than either VDD or PM-enriched alone (Figure 2-5C). HF, which is the time frequency domain measure of HRV, decreased significantly due to VDD and PM-enriched exposure (Figure 2-5D); interestingly, this phenomenon was also observed in ND mice exposed to air but not in VDD mice exposed to air or the ND mice exposed to PM-enriched exposure. No significant changes were observed in LF (data not shown).

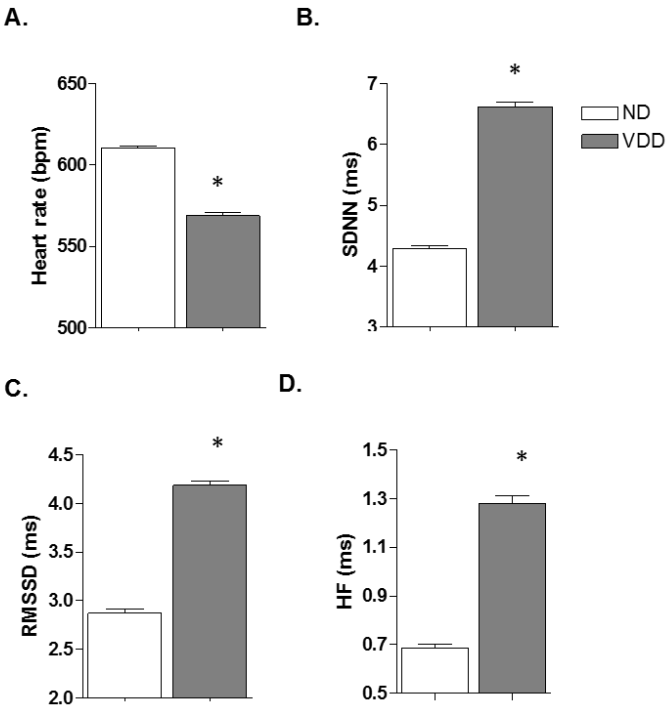


Figure 2-4. VDD mice had decreased heart rate and increased HRV prior to exposure. (**A.**) Heart rate was significantly decreased in VDD mice when compared to ND, whereas SDNN (**B.**), RMSSD (**C.**), and HF (**D.**) were significantly increased. Parameters were analyzed from the time of radiotelemeter implantation until exposure. * significantly different from ND ($p < 0.05$). Values represent means ± SE.

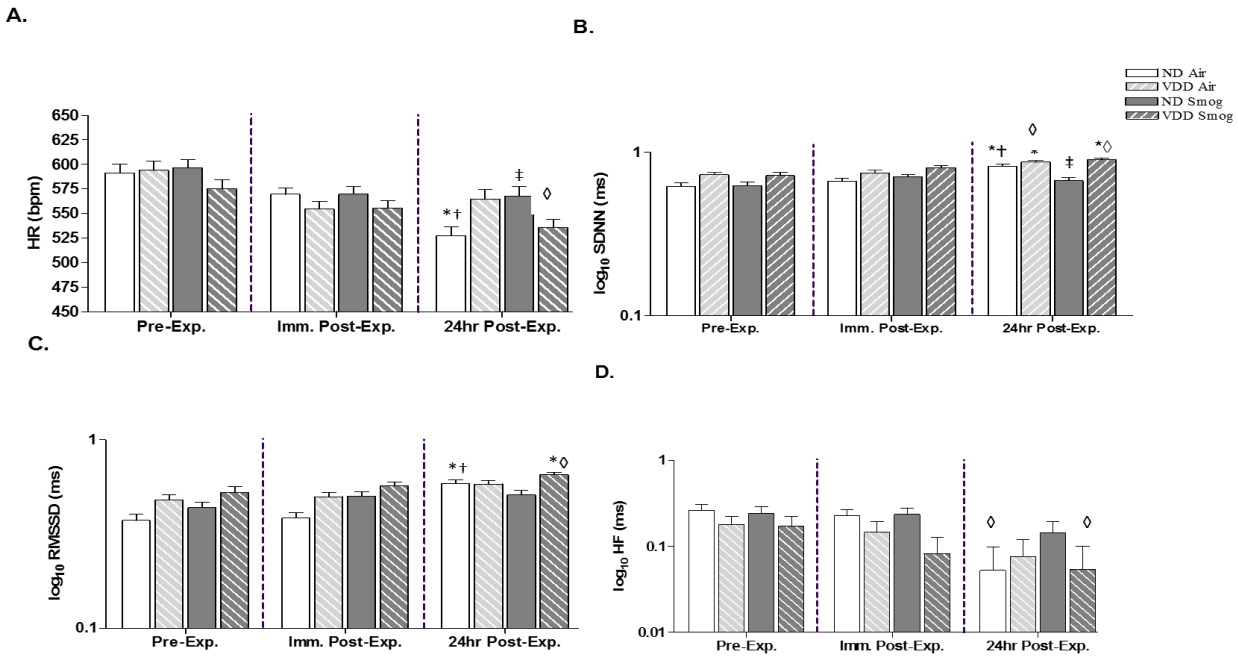


Figure 2-5. VDD alters heart rate and heart rate variability after PM-enriched exposure. There were no changes in HR or HRV immediately after exposure in both ND and VDD mice (**A - D**). SDNN was increased in all groups 24hrs after exposure when compared to pre-exposure except ND mice exposed to PM-enriched. VDD increased SDNN irrespective of the exposure, yet when combined, VDD and PM-enriched exposure had a significantly greater effect on SDNN than either alone (**B.**). Similar trends were observed with RMSSD for which the combination of VDD and PM-enriched smog had a greater effect than either VDD or PM-enriched exposure alone (**C.**). HF decreased significantly in VDD mice exposed to PM-enriched smog and ND mice exposed to FA but not in VDD mice exposed to FA or ND mice exposed to PM-enriched exposure (**D.**) *significantly different from ND ($p < 0.05$). ◊significantly different from pre-exposure ($p < 0.05$). † significantly different from immediately post-exposure. ‡ significantly different from filtered air exposure. Values represent means ± SE.

During exposure, HR decreased from baseline (30-minute acclimation period before exposure) in all animals regardless of diet or exposure. PM-enriched smog significantly blunted the decrease in HR in ND mice; a similar effect was seen in VDD mice but to a lesser degree (Figure 2-6A). SDNN increased in VDD mice during air exposure and in ND mice during smog exposure however there was no effect in VDD mice exposed to PM-enriched smog (Figure 2-6B). In addition, RMSSD increased

significantly in VDD mice during air exposure when compared to ND; this response was further increased if the animals were exposed to PM-enriched smog (Figure 2-6C). HF only increased in VDD mice during PM-enriched exposure (Figure 2-6D). No significant changes were observed in LF parameter during exposure (data not shown). Lastly, the number of arrhythmias significantly increased in ND mice during PM-enriched exposure and although VDD appears to also increase total arrhythmias the results were not significant (Figure 2-6E).

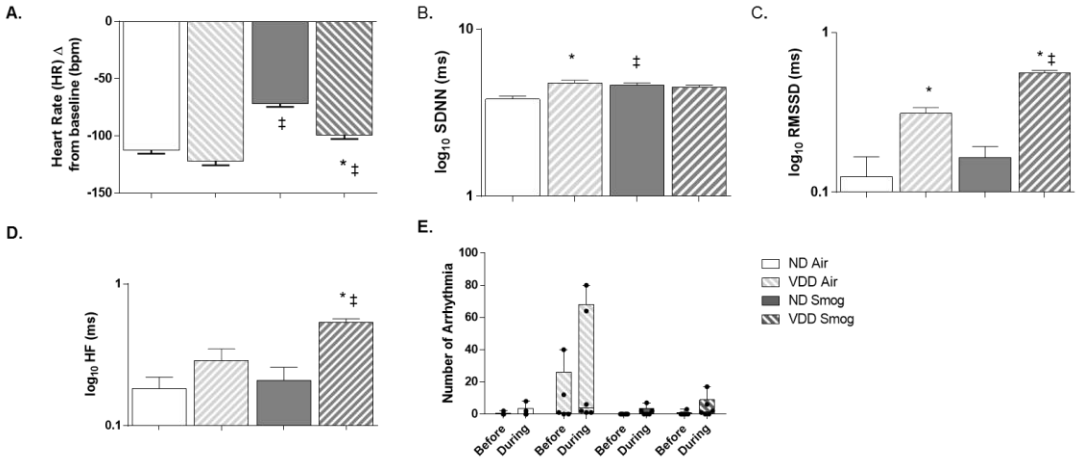


Figure 2-6. Exposure to PM-enriched smog prevents the recovery of resting heart rate in normal and VDD mice but only potentiates parasympathetic modulation in the latter. (**A.**) PM-enriched exposure significantly blunted the decrease in HR in both ND and VDD mice. (**B.**) SDNN increased in VDD mice during FA and in ND mice during PM-enriched exposure, however there was no effect in VDD mice exposed to PM-enriched smog. (**C.**) RMSSD increased significantly in VDD mice during FA when compared to ND; this response was further increased with PM-enriched smog. (**D.**) VDD mice exposed to PM-enriched smog had significantly increased HF when compared to ND and VDD mice exposed to FA. (**E.**) VDD mice exposed to air had significantly increased arrhythmia when compared to PM-enriched smog, but there was no effect of PM-enriched smog in VDD mice. * significantly different from ND ($p < 0.05$). ‡significantly different from filtered air exposure ($p < 0.05$). Values represent means ± SE.

Ventilatory function – Table 2-1, Table 2-2 and Figure 2-7 show the ventilatory data. Although the mice randomly placed on the ND had a lower *f* at 3 weeks of age

than those placed on the VDD the *f* was comparable at 8 weeks of age and continued to decrease with age for both groups (Figure 2-7A). Similarly, although V_T/MV were higher in the VDD group at the beginning of the diet regimen and up to 8 weeks of age, further increases in V_T/MV over time were blunted in the VDD mice when compared to ND mice (Figure 2-7B – VDD -72.4% increase; ND - 111.2% increase at 15wks). VDD mice had decreased Ti before and through the diet regimen when compared to ND mice while there were no differences in Te (Table 2-1).

Diet	Age (weeks)	Breathing Frequency (f)	Tidal Volume (mL)	Inspiratory Time (msec)	Expiratory Time (msec)	Minute Volume (mL/min)	Ventilatory Timing (penh)
ND	**3**	474.2±0.6	0.13±0.0	51.69±0.1	83.46±0.1	6.42±0.0	
	8	475.5±0.5 *	0.23±0.0 *	56.01±0.1 *	76.28±0.1 *	10.73±0.0 *	
	11	435.2±0.4 *	0.27±0.0 *	64.01±0.1 *	78.29±0.1 *	11.42±0.0 *	
	15	423.6±0.3 *	0.28±0.0 *	66.26±0.1 *	79.48±0.1 *	11.76±0.0 *	
	Pre-Exp.	432.8±0.6	0.29±0.0	61.97±0.1	81.69±0.2	12.37±0.0	1.26±0.0
VDD	**3**	503.0±0.5 ◊	0.15±0.0 ◊	50.40±0.1 ◊	74.72±0.1 ◊	7.41±0.0 ◊	
	8	483.5±0.5 *◊	0.23±0.0 *◊	54.57±0.1 *◊	75.29±0.1* ◊	10.56±0.0 *◊	
	11	439.5±0.4 *◊	0.24±0.0 *◊	62.35±0.1 *◊	79.56±0.1 *◊	10.55±0.0 *◊	
	15	426.7±0.41 *◊	0.26±0.0 *◊	65.17±0.1 *◊	80.86±0.1 *◊	10.64±0.0 *◊	
	Pre-Exp.	435.5±0.6	0.3±0.0	61.75±0.1	81.98±0.2	12.24±0.0	1.54±0.0

Table 2-1. VDD induced changes in ventilatory function parameters during diet regimen. * significantly different from 3-week assessment ($p < 0.05$).◊ significantly different from ND ($p < 0.05$). Values represent means ± SE.

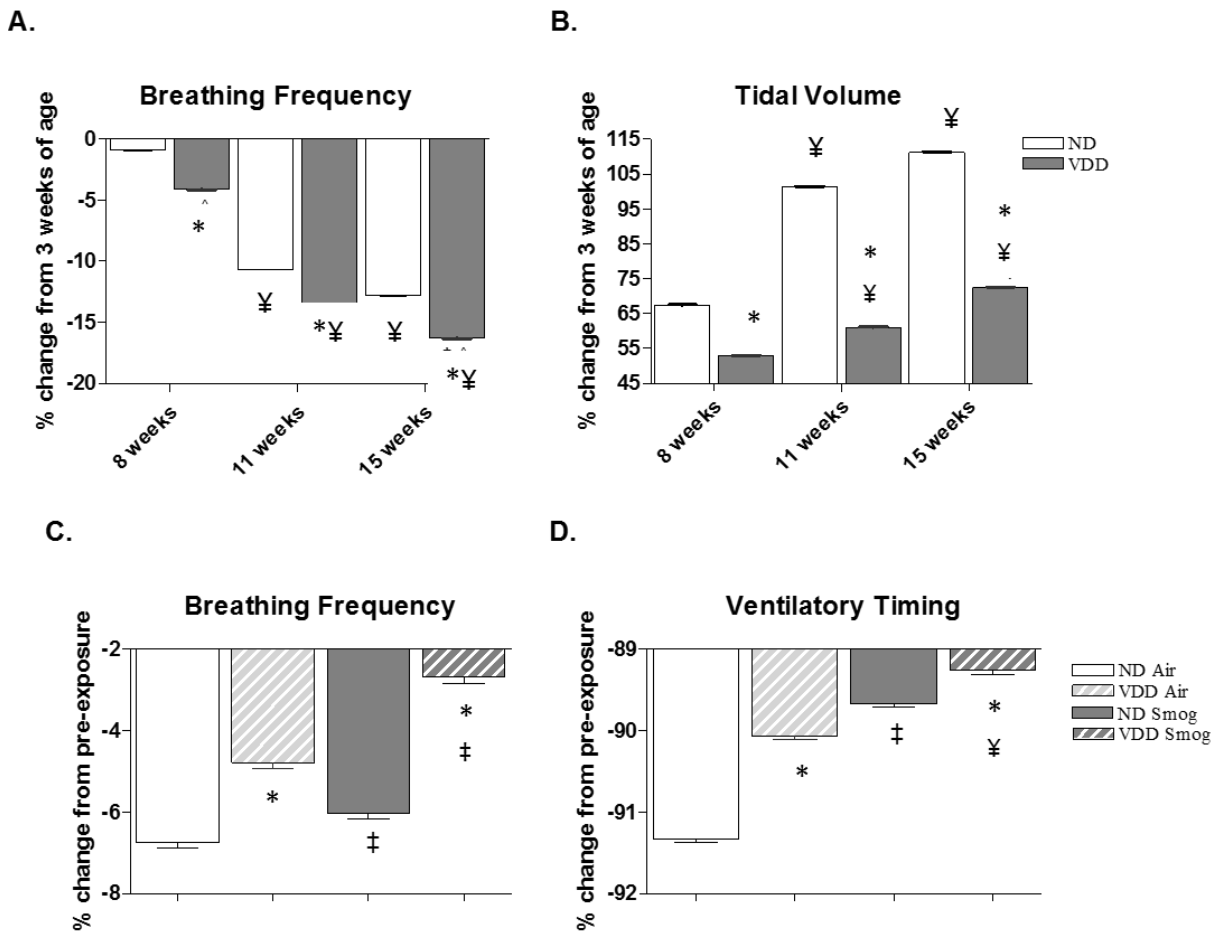


Figure 2-7. VDD mice have impaired development of normal breathing patterns and higher breathing frequency during PM-enriched exposure. VDD mice had significantly less decrease in *f* and increase in Vt during the 15 weeks of development when compared to ND (**A.** and **B.**). PM-enriched smog prevented *f* and penh from decreasing in both ND and VDD, but VDD mice had a significantly greater effect than ND (**C.** and **D.**). * significantly different from ND ($p < 0.05$). ¥ significantly different from 3-week assessment ($p < 0.05$). ‡ significantly different from filtered air exposure ($p < 0.05$). Values represent means ± SE.

Other than ventilatory timing (penh) being higher in VDD versus ND mice, there were no significant differences in the pre-exposure ventilatory parameters between the two diets (Table 2-2). All subsequent comparisons for exposure-related ventilatory effects were made within group. As such, all animals experienced a significant decrease

in *f* after exposure; however, this decrease enriched (i.e. *f* decreased the least in VDD mice after PM-enriched). Similar trends were observed with V_T, Ti, and Te however the values increased after exposure. Penh increased after PM-enriched exposure in ND mice when compared to pre-exposure and with respect to their controls; all the other groups including VDD mice exposed to PM-enriched had a decrease in penh post-exposure.

Diet	Exposure		Breathing Frequency (f)	Tidal Volume (mL)	Inspiratory Time (msec)	Expiratory Time (msec)	Minute Volume (mL/min)	Ventilatory Timing (penh)
ND	Air	Pre	437.9±0.6	0.29±0.0	61.89±0.1	79.64±0.1	12.59±0.0	1.38±0.0
		Post	410.8±0.6 ◊	0.31±0.0 ◊	66.07±0.1 ◊	86.93±0.2 ◊	12.53±0.0	1.15±0.0 ◊
	Smog	Pre	427.8±0.6	0.29±0.0	62.04±0.1	83.73±0.1	12.15±0.0	1.13±0.0
		Post	405.7±0.6 ◊	0.31±0.0 ◊‡	66.08±0.1 ◊	87.93±0.2 ◊‡	12.29±0.0 ◊	1.31±0.0 ◊‡
VDD	Air	Pre	436.2±0.6	0.31±0.0 *	61.26±0.1 *	81.03±0.1 *	12.14±0.0 *	1.43±0.0 *
		Post	415.6± 0.7 *◊	0.30±0.0 *◊	64.28±0.1 *◊	86.94±0.2 ◊	12.18±0.0 *	1.28±0.0 *◊
	Smog	Pre	434.9±0.6 *	0.29±0.0	62.24±0.1	82.93±0.2 *	12.33±0.0 *	1.65±0.0 *
		Post	424.4±0.6 *◊‡	0.30± 0.0 *◊‡	63.38±0.1 *◊‡	84.45±0.2 *◊‡	12.57±0.0 *◊‡	1.38±0.0 *◊‡

Table 2-2. Ventilatory function of ND and VDD mice is altered after photochemical smog exposure. * significantly different from ND ($p < 0.05$). ◊ significantly different from pre-exposure ($p < 0.05$). ‡ significantly different from filtered air exposure ($p < 0.05$). Values represent means ± SE.

Electrocardiogram analysis – The data is presented in Table 2-3. There were no significant differences in any parameters during the pre-exposure period. The PR interval decreased significantly from baseline during air exposure in both ND and VDD animals but there were no differences between the diets during, immediately and 24hrs after exposure. In contrast, the PR interval of VDD mice was increased compared to ND mice during PM-enriched exposure, immediately after and 24hrs later. PM-enriched exposure caused a decreased PR interval in ND mice and increased PR interval in VDD mice when compared to their respective air-exposed controls. VDD animals had a decreased QRS interval during air exposure when compared to ND; this difference between VDD and ND was also observed in animals exposed to PM-enriched and persisted 24hrs after that exposure. Smog exposure also caused a significant decrease in QRS complex in ND mice when compared to controls. Lastly, VDD caused a decrease in QTc when compared to ND with either air or PM-enriched exposure during either exposure or immediately after but there were no significant differences among any group for the most part.

Diet	Exposure	Timing	PR (ms)	QRS (ms)	QTcB (ms)
ND	**Filtered Air**	**Pre-Exposure**	37.21±0.28	10.38±0.11	56.23±0.65
		Exposure	36.62±0.10 §	7.66±0.14	57.16±0.55
		Immediately Post-Exposure	37.42±0.22	10.85±0.09	59.24±0.67
		24 Hr Post-Exposure	37.58±0.23	10.55±0.06	55.16±0.68
	Smog	**Pre-Exposure**	37.22±0.34	10.40±0.08	56.21±0.80
		Exposure	36.07±0.08 ‡	6.43±0.15 ‡	56.53±0.47
		Immediately Post-Exposure	36.27±0.30 ‡	10.60±0.07	54.82±0.79 ‡
		24 Hr Post-Exposure	36.06±0.25 ‡	10.34±0.06	55.11±0.86
VDD	**Filtered Air**	**Pre-Exposure**	37.27±0.45	10.39±0.10	55.20±0.71
		Exposure	36.49±0.10 §	6.31±0.14 §*	54.96±0.56
		Immediately Post-Exposure	36.49±0.24 *	10.40±0.08 *	54.82±0.44 *
		24 Hr Post-Exposure	36.31±0.21 *	10.42±0.09	55.02±0.57
	Smog	**Pre-Exposure**	37.34±0.22	10.38±0.10	56.37±0.46
		Exposure	37.46±0.08 ‡*	5.35±0.14 *	54.13±0.47 *
		Immediately Post-Exposure	38.11±0.23 ‡*	10.46±0.08	54.63±0.41
		24 Hr Post-Exposure	37.15±0.20 ‡*	10.01±0.06 ‡*	53.37±0.46

Table 2-3. Smog exposure and VDD induced alterations in electrocardiographic morphology in adult mice. * significantly different from ND ($p < 0.05$). † significantly different from immediately-post exposure ($p < 0.05$).◊ significantly different from pre-exposure ($p < 0.05$). ‡ significantly different from filtered air exposure ($p < 0.05$). § significantly different from baseline of exposure ($p < 0.05$). Values represent means ± SE.

Discussion

This study demonstrates that early life and persistent vitamin D deficiency into adulthood modifies the cardiopulmonary response to PM-enriched smog exposure. These results add to a growing body of research which shows that the degree and quality of health effects of air pollution are not only governed by concentration and composition but also non-environmental factors. Some of these factors are directly related to the host and aspects of daily living which likely accrue intrinsic changes in the body over many years and alter the degree of responsiveness to an environmental challenge or the ability to compensate for one. Such might be the case with VDD, which has become a growing public health concern in the United States with some estimates of prevalence reaching 41.6%.(Forrest and Stuhldreher 2011) This is of particular concern because millions of children may be deficient (Kumar, Muntner et al. 2009) and remain so into adulthood thus increasing the likelihood of chronic diseases. Although it has not been studied as extensively as bone-related maladies, the cardiopulmonary effects that result from VDD are increasingly being recognized as a cause, or promoter in the least, of several long-term diseases and heightened susceptibility to triggered adverse responses. Still this is the first study to show that VDD during development and into adulthood alters and potentially worsens the response to air pollution in mice.

Traditional toxicological investigations, particularly in rodents, have been using susceptible models (e.g. hypertension, metabolic syndrome, etc) to further characterize the risk of adverse responses to air pollution. Our own studies indicate that rodent strains with underlying cardiovascular disease have a worse response (e.g. arrhythmia) to air pollution than normal strains although the effects are often latent and can only be observed when a subsequent challenge or trigger is used to manifest them. (Hazari,

Haykal-Coates et al. 2009, Farraj, Hazari et al. 2011, Hazari, Haykal-Coates et al. 2011, Hazari, Callaway et al. 2012, Carll, Lust et al. 2013, Hazari, Griggs et al. 2014) This suggests that to some degree the adverse effects of air pollution may be due more to disruption of homeostasis than direct tissue toxicity. Imbalance in autonomic control of the cardiovascular system as measured by heart rate variability is an example of such changes and has been well documented not only in rodents but in humans as well. (Binkley, Nunziata et al. 1991, Liao, Creason et al. 1999, Brook and Julius 2000) Furthermore, the importance of such an assessment rests in the fact that it indicates a subtle shift in the underlying regulation of the body's dynamic systems which may not be manifested as clinical symptoms or overt signs of toxicity. Thus, the objective of this study was to investigate whether VDD acts as a modifiable factor in smog-induced cardiopulmonary dysfunction. We hypothesized that as a result of early-life VDD the homeostatic balance of the cardiopulmonary system would be altered, thus the cardiopulmonary response to stressors like smog would also change.

Although the VDD mice were maintained on the deficient diet immediately post-weaning there was no difference in the body weights or the growth of VDD animals after 15 weeks as determined by tibia length (not shown) when compared to ND. Even calcium levels were found to be in the normal range for all mice irrespective of diet. Despite this seemingly normal development, VDD deficient mice had increased heart rate variability, indicating altered cardiac autonomic function, when compared to controls in the two weeks prior to exposure. Although decreased HRV is generally thought of as the primary indicator of cardiac risk (Gold, Litonjua et al. 2000, Pope, Hansen et al. 2004), an increase in HRV may not necessarily be a positive sign. (Stein,

Domitrovich et al. 2005) Instead, it may be the change from normal which suggests an impaired regulation of the cardiovascular system but in a way that may not be entirely appreciated until the body is challenged and has to maintain homeostasis. Increases in vagal tone or HRV have been associated with adverse cardiovascular events in diabetes (Eguchi, Schwartz et al. 2010), and linked with increased mortality in heart failure patients and the elderly. (de Bruyne, Kors et al. 1999, Stein, Domitrovich et al. 2005) In addition, increased RMSSD has been found to be associated with elevated risk of air pollution-induced arrhythmia. (Davoodi, Sharif et al. 2010)

An increase in HRV suggests parasympathetic modulation of the cardiovascular system which was clearly seen as a significantly lower heart rate in the VDD animals when compared to ND. Vitamin D deficiency is linked to HRV changes in humans as well (Canpolat, Özcan et al. 2015, Jung, Jung et al. 2015, Mann, Hollenberg et al. 2015) and although the profile differs from rodents the overall conclusion that subtle underlying alterations are occurring in the deficient state holds true. It is likely the differences between humans and mice may be due to the fact that in contrast to humans, baseline heart rate in mice is influenced greatly by the sympathetic branch (Just, Faulhaber et al. 2000) and short-term HRV is under the control of parasympathetic modulation. (Gehrmann, Hammer et al. 2000, Pham, Bonham et al. 2009) Furthermore, the HRV effects of VDD in humans may be due to other disease processes (e.g. kidney disease) related to chronic deficiency which is still not confirmed to occur in mice. It may be that mice develop a similar HRV decrement if left on the deficient diet for a longer period; our future studies will address this issue. In any case, it is not entirely clear why VDD causes altered autonomic function in mice. One study

showed that VDD caused changes in calcium flux and cardiomyocyte contraction-relaxation, which was shown to be linked to vitamin D receptors (VDR) located on the T-tubule (Tishkoff, Nibbelink et al. 2008); the authors concluded that these effects would likely result in changes in heart rate. On the other hand, 24hrs before exposure there was still a trend of increased HRV (SDNN and RMSSD) in the VDD animals when compared to ND even though the HR among the groups was similar. This suggests that other factors may have contributed to the changes in HRV; in fact, VDD has been shown to affect blood pressure, which is a known determinant of HRV. (Artaza, Mehrotra et al. 2009)

We have consistently shown that heart rate tends to decrease gradually over a 3-4 hour air exposure as the animal calms down in the chamber. In the presence of air pollution, heart rate does not decrease as much (i.e. remains elevated). (Carll, Lust et al. 2013, Farraj, Walsh et al. 2015) In the current study, although PM-enriched smog prevented HR from decreasing in both ND and VDD groups, the effect was significantly less in the VDD mice (i.e. HR of VDD mice was less elevated), which along with the greater increase in RMSSD and HF appears to confirm the shift towards parasympathetic modulation in the VDD animals. This modulation was also reflected in the greater decrease in HR and increase in RMSSD in VDD animals exposed to air. These trends persisted immediately after exposure with HR continuing to decrease over the next 24hrs. Over the same period SDNN and RMSSD continually increased in all groups except ND animals exposed to PM-enriched smog, which consequently had the smallest decrease in HR. It is unclear whether this relatively smaller decrease in HR and increase in HRV in the ND mice exposed to PM-enriched reflects increased risk.

(Tsuji, Larson et al. 1996, La Rovere, Pinna et al. 2001) These responses are similar to what was observed and reported for PM-enriched by Hazari et al. in this issue with the difference in effects due to VDD being evident here. (Hazari and Stratford 2018) Indeed, this group had a significantly increased number of arrhythmia during exposure but these stopped after it ended. Similar increases in arrhythmia incidence were observed in the other three treated groups, especially VDD mice exposed to air which had a large number of atrial premature beats, but they were not significant. Yet, there is some indication from past studies that VDD is associated with arrhythmia (Ozcan, Gurlek et al. 2015) due to a number of mechanisms, either by activation of the RAAS axis and predisposition to hypertension (Lee, O'Keefe et al. 2008), through the enhancement of myocardial oxidative stress (Argacha, Egrise et al. 2011, Gradinaru, Borsa et al. 2013), or by increasing PTH levels which also affects blood pressure and myocardial contractility (Ogard, Sondergaard et al. 2005, Zittermann 2006). Whether these conditions existed in our animals is unknown and further does it worsen the response to air pollution likely remains to be clarified.

Assessments of ventilatory function were also performed from the beginning of the diet regimen to the end of the study and revealed some diet-induced effects. Although the animals were randomly assigned to either the normal or deficient diet, the VDD group had a higher breathing frequency than ND before commencing the diets. This difference did not exist at 15 weeks of age. In contrast, VDD animals had a significantly reduced increase in tidal volume over the 16 week diet regimen when compared to ND. Numerous studies have documented the negative effects of VDD on lung development in both humans and rodents. (Zosky, Hart et al. 2014, Foong, Bosco

et al. 2015, Chen, Wilson et al. 2016) Furthermore, it appears that VDD causes deficits in lung function that are primarily explained by differences in lung volume and also exacerbates the development of lung COPD-like characteristics (i.e. inflammation) in mice exposed to cigarette smoke. Two important points are relevant here given these conditions; first, it is clear that the dose of PM-enriched smog in VDD animals may have been different from ND animals, and second, it is likely the airway responsiveness of VDD mice to PM-enriched smog was also changed. We cannot address the first point since we were unable to measure ventilatory function during exposure. However, when compared to pre-exposure, VDD animals had a significantly smaller decrease in breathing frequency after exposure than ND and this effect was even greater when VDD and PM-enriched smog were combined. A similar trend was observed in ventilatory timing (penh), which is an indicator of airway irritation, suggesting VDD may have altered the ventilatory responses to the smog. This is relevant to the overall hypothesis of this study because ventilatory patterns not only affect HRV but also can play a role in adverse cardiac events (e.g. arrhythmia) when triggered by airway irritation. (Widdicombe and Lee 2001)

Finally, there were some notable changes in ECG parameters in this study. Although there were no pre-exposure differences in any of the ECG parameters between any of the groups, PM-enriched exposure caused a significant increase in PR interval and decrease in QRS complex duration when compared to air; this smog effect was altered by VDD and persisted for 24hrs after exposure only in that group. Even though these changes may just represent fluctuations in the heart rate and not any true electrical disturbances or pathology there is a clear impact of VDD by which the PM-

enriched-induced effect continued into the next day. In such a situation, if these ECG changes truly were due to an electrical disturbance then it would not be inconceivable for a subsequent adverse cardiac event to be triggered, even if hours after the exposure had ended. In addition, our animals were only kept on the diet for 16 weeks, which may not have been enough time for VDD to cause more tangible effects. For example, chronic changes in blood pressure are connected with vitamin D levels but this was not evaluated in this study. Thus, future experiments will be conducted on animals that are kept on VDD for more than 32 weeks and will include other physiological measures that will help to potentially clarify the ECG phenomenon.

Conclusions

In conclusion, persistent VDD that begins in early-life alters HR, HRV and ventilatory parameters and changes the response to smog. In particular, the changes in HRV or autonomic imbalance that were observed may represent altered homeostasis, which potentially suggests that the body is prone to an adverse response if an environmental trigger or stressor is encountered. Although this has yet to be proven, our previous studies clearly show that autonomic imbalance plays a role in triggered cardiac dysfunction. This study is relevant because it demonstrates how nutritional deficiencies can subtly modify the homeostatic balance of the cardiopulmonary system and increase risk from exposure to PM-enriched smog. This especially applies to children who are VDD and may be at heightened risk of developing cardiovascular disease later in life and so may also be particularly sensitive to the effects of air pollution. Regardless, if present in man such effect modification could have significant relevance to public health and the assessment of toxicological risk.

REFERENCES

Anderson, J. L., H. T. May, B. D. Horne, T. L. Bair, N. L. Hall, J. F. Carlquist, D. L. Lappé and J. B. Muhlestein (2010). "Relation of Vitamin D Deficiency to Cardiovascular Risk Factors, Disease Status, and Incident Events in a General Healthcare Population." The American Journal of Cardiology **106**(7): 963-968.

Argacha, J. F., D. Egrise, S. Pochet, D. Fontaine, A. Lefort, F. Libert, S. Goldman, P. van de Borne, G. Berkenboom and R. Moreno-Reyes (2011). "Vitamin D deficiency-induced hypertension is associated with vascular oxidative stress and altered heart gene expression." J Cardiovasc Pharmacol **58**(1): 65-71.

Artaza, J. N., R. Mehrotra and K. C. Norris (2009). "Vitamin D and the cardiovascular system." Clin J Am Soc Nephrol **4**(9): 1515-1522.

Binkley, P. F., E. Nunziata, G. J. Haas, S. D. Nelson and R. J. Cody (1991). "Parasympathetic withdrawal is an integral component of autonomic imbalance in congestive heart failure: demonstration in human subjects and verification in a paced canine model of ventricular failure." Journal of the American College of Cardiology **18**(2): 464-472.

Brook, R. D. and S. Julius (2000). "Autonomic imbalance, hypertension, and cardiovascular risk." American journal of hypertension **13**(S4): 112S-122S.

Brook, R. D., S. Rajagopalan, C. A. Pope, J. R. Brook, A. Bhatnagar, A. V. Diez-Roux, F. Holguin, Y. Hong, R. V. Luepker, M. A. Mittleman, A. Peters, D. Siscovick, S. C. Smith, L. Whitsel, J. D. Kaufman, o. b. o. t. A. H. A. C. o. Epidemiology, C. o. t. K. i. C. D. Prevention, P. A. Council on Nutrition and Metabolism (2010). "Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement From the American Heart Association." Circulation **121**(21): 2331-2378.

Canpolat, U., F. Özcan, Ö. Özeke, O. Turak, Ç. Yayla, S. K. Açıkgöz, S. Çay, S. Topaloğlu, D. Aras and S. Aydoğdu (2015). "Impaired Cardiac Autonomic Functions in Apparently Healthy Subjects with Vitamin D Deficiency." Annals of Noninvasive Electrocardiology **20**(4): 378-385.

Carll, A. P., R. M. Lust, M. S. Hazari, C. M. Perez, Q. T. Krantz, C. J. King, D. W. Winsett, W. E. Cascio, D. L. Costa and A. K. Farraj (2013). "Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats." Toxicol Sci **131**(2): 583-595.

Carlton-Conway, D., R. Tulloh, L. Wood and D. Kanabar (2004). "Vitamin D deficiency and cardiac failure in infancy." Journal of the Royal Society of Medicine **97**(5): 238-239.

Chen, L., R. Wilson, E. Bennett and G. R. Zosky (2016). "Identification of vitamin D sensitive pathways during lung development." Respir Res **17**: 47.

Davoodi, G., A. Y. Sharif, A. Kazemisaeid, S. Sadeghian, A. V. Farahani, M. Sheikhvatan and M. Pashang (2010). "Comparison of heart rate variability and cardiac arrhythmias in polluted and clean air episodes in healthy individuals." Environ Health Prev Med **15**(4): 217-221.

de Bruyne, M. C., J. A. Kors, A. W. Hoes, P. Klootwijk, J. M. Dekker, A. Hofman, J. H. van Bemmel and D. E. Grobbee (1999). "Both decreased and increased heart rate variability on the standard 10-second electrocardiogram predict cardiac mortality in the elderly: the Rotterdam Study." Am J Epidemiol **150**(12): 1282-1288.

Eguchi, K., J. E. Schwartz, T. G. Pickering, S. Hoshide, J. Ishikawa, K. Shimada and K. Kario (2010). "Increased heart rate variability during sleep is a predictor for future cardiovascular events in patients with type 2 diabetes." Hypertens Res **33**(7): 737-742.

Farraj, A. K., M. S. Hazari, D. W. Winsett, A. Kulukulualani, A. P. Carll, N. Haykal-Coates, C. M. Lamb, E. Lappi, D. Terrell and W. E. Cascio (2011). "Overt and latent cardiac effects of ozone inhalation in rats: evidence for autonomic modulation and increased myocardial vulnerability." Environmental health perspectives **120**(3): 348-354.

Farraj, A. K., L. Walsh, N. Haykal-Coates, F. Malik, J. McGee, D. Winsett, R. Duvall, K. Kovalcik, W. E. Cascio, M. Higuchi and M. S. Hazari (2015). "Cardiac effects of seasonal ambient particulate matter and ozone co-exposure in rats." Part Fibre Toxicol **12**: 12.

Fetahu, I. S., J. Höbaus and E. Kállay (2014). "Vitamin D and the epigenome." Frontiers in Physiology **5**: 164.

Foong, R. E., A. Bosco, A. C. Jones, A. Gout, S. Gorman, P. H. Hart and G. R. Zosky (2015). "The effects of in utero vitamin D deficiency on airway smooth muscle mass and lung function." Am J Respir Cell Mol Biol **53**(5): 664-675.

Forrest, K. Y. and W. L. Stuhldreher (2011). "Prevalence and correlates of vitamin D deficiency in US adults." Nutr Res **31**(1): 48-54.

Gehrmann, J., P. E. Hammer, C. T. Maguire, H. Wakimoto, J. K. Triedman and C. I. Berul (2000). "Phenotypic screening for heart rate variability in the mouse." American Journal of Physiology - Heart and Circulatory Physiology **279**(2): H733-H740.

Gold, D. R., A. Litonjua, J. Schwartz, E. Lovett, A. Larson, B. Nearing, G. Allen, M. Verrier, R. Cherry and R. Verrier (2000). "Ambient pollution and heart rate variability." Circulation **101**(11): 1267-1273.

Gradinaru, D., C. Borsa, C. Ionescu and D. Margina (2013). "Advanced oxidative and glycoxidative protein damage markers in the elderly with type 2 diabetes." J Proteomics **92**: 313-322.

Haagen-Smit, A. J. (1952). "Chemistry and physiology of Los Angeles smog." Industrial & Engineering Chemistry **44**(6): 1342-1346.

Hazari, M., J. Griggs, D. Winsett, N. Haykal-Coates, A. Ledbetter, D. Costa and A. Farraj (2014). "A Single Exposure to Acrolein Desensitizes Baroreflex Responsiveness and Increases Cardiac Arrhythmias in Normotensive and Hypertensive Rats." Cardiovascular Toxicology **14**(1): 52-63.

Hazari, M. S., J. Callaway, D. W. Winsett, C. Lamb, N. Haykal-Coates, Q. Krantz, C. King, D. L. Costa and A. K. Farraj (2012). "Dobutamine" stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats." Environmental health perspectives **120**(8): 1088-1093.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, D. L. Costa and A. K. Farraj (2009). "A single exposure to particulate or gaseous air pollution increases the risk of aconitine-induced cardiac arrhythmia in hypertensive rats." Toxicol Sci **112**(2): 532-542.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, Q. T. Krantz, C. King, D. L. Costa and A. K. Farraj (2011). "TRPA1 and Sympathetic Activation Contribute to Increased Risk of Triggered Cardiac Arrhythmias in Hypertensive Rats Exposed to Diesel Exhaust." Environmental Health Perspectives **119**(7): 951-957.

Hazari, M. S. and K. M. Stratford (2018). "Comparative Cardiopulmonary Effects of Particulate Matter- And Ozone-Enhanced Smog Atmospheres in Mice."

Holick, M. F. (2007). "Vitamin D Deficiency." New England Journal of Medicine **357**(3): 266-281.

Jung, C. H., S. H. Jung, K. J. Kim, B. Y. Kim, C. H. Kim, S. K. Kang and J. O. Mok (2015). "The relationship between vitamin D status and cardiac autonomic neuropathy in patients with type 2 diabetes mellitus." Diab Vasc Dis Res **12**(5): 342-351.

Just, A., J. Faulhaber and H. Ehmke (2000). "Autonomic cardiovascular control in conscious mice." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **279**(6): R2214-R2221.

Krug, J. D. (2018). "Photochemical Conversion of Surrogate Emissions for Use in Toxicological Studies: Role of Particulate- and Gas-Phase Products." **52**(5): 3037-3044.

Kumar, J., P. Muntner, F. J. Kaskel, S. M. Hailpern and M. L. Melamed (2009). "Prevalence and Associations of 25-Hydroxyvitamin D Deficiency in US Children: NHANES 2001–2004." Pediatrics **124**(3): e362-e370.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, L. Walsh, A. K. Farraj and M. S. Hazari (2014). "Ozone co-exposure modifies cardiac responses to fine and ultrafine

ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses." Part Fibre Toxicol **11**: 54.

La Rovere, M. T., G. D. Pinna, S. H. Hohnloser, F. I. Marcus, A. Mortara, R. Nohara, J. T. Bigger, Jr., A. J. Camm and P. J. Schwartz (2001). "Baroreflex sensitivity and heart rate variability in the identification of patients at risk for life-threatening arrhythmias: implications for clinical trials." Circulation **103**(16): 2072-2077.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D Deficiency: An Important, Common, and Easily Treatable Cardiovascular Risk Factor?" Journal of the American College of Cardiology **52**(24): 1949-1956.

Liao, D., J. Creason, C. Shy, R. Williams, R. Watts and R. Zweidinger (1999). "Daily variation of particulate air pollution and poor cardiac autonomic control in the elderly." Environmental Health Perspectives **107**(7): 521.

Lips, P. (2006). "Vitamin D physiology." Progress in biophysics and molecular biology **92**(1): 4-8.

Lynne Page, S. (1994). ""The Death-Dealing Smog over Donora, Pennsylvania": Industrial Air Pollution, Public Health Policy, and the Politics of Expertise, 1948-1949." Environmental History Review **18**(1): 117-139.

Maiya, S., I. Sullivan, J. Allgrove, R. Yates, M. Malone, C. Brain, N. Archer, Q. Mok, P. Daubeney, R. Tulloh and M. Burch (2008). "Hypocalcaemia and vitamin D deficiency: an important, but preventable, cause of life-threatening infant heart failure." Heart **94**(5): 581-584.

Mann, M. C., M. D. Hollenberg, D. A. Hanley and S. B. Ahmed (2015). "Vitamin D, the autonomic nervous system, and cardiovascular risk." Physiol Rep **3**(4).

Masson, S., N. Agabiti, T. Vago, M. Miceli, F. Mayer, T. Letizia, U. Wienhues-Thelen, G. F. Mureddu, M. Davoli, A. Boccanelli, R. Latini and P. s. The Investigators of the (2014). "The fibroblast growth factor-23 and Vitamin D emerge as nontraditional risk factors and may affect cardiovascular risk." Journal of Internal Medicine: n/a-n/a.

Norman, P. E. and J. T. Powell (2014). "Vitamin D and Cardiovascular Disease." Circulation Research **114**(2): 379-393.

Ogard, C. G., S. B. Sondergaard, H. Vestergaard, H. Jakobsen and S. L. Nielsen (2005). "Myocardial perfusion defects and the left ventricular ejection fraction disclosed by scintigraphy in patients with primary hyperparathyroidism." World J Surg **29**(7): 914-916.

Ozcan, O. U., A. Gurlek, E. Gursoy, D. M. Gerede and C. Erol (2015). "Relation of vitamin D deficiency and new-onset atrial fibrillation among hypertensive patients." J Am Soc Hypertens **9**(4): 307-312.

Pham, H., A. C. Bonham, K. E. Pinkerton and C.-Y. Chen (2009). "Central neuroplasticity and decreased heart rate variability after particulate matter exposure in mice." Environmental health perspectives **117**(9): 1448.

Pope, C. A., 3rd, M. L. Hansen, R. W. Long, K. R. Nielsen, N. L. Eatough, W. E. Wilson and D. J. Eatough (2004). "Ambient particulate air pollution, heart rate variability, and blood markers of inflammation in a panel of elderly subjects." Environ Health Perspect **112**(3): 339-345.

Reeves, P. G., F. H. Nielsen and G. C. Fahey (1993). "AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet." The Journal of Nutrition **123**(11): 1939-1951.

Saccone, D., F. Asani and L. Bornman (2015). "Regulation of the vitamin D receptor gene by environment, genetics and epigenetics." Gene **561**(2): 171-180.

Song, S., P. Gao, H. Xiao, Y. Xu and L. Y. Si (2013). "Klotho Suppresses Cardiomyocyte Apoptosis in Mice with Stress-Induced Cardiac Injury via Downregulation of Endoplasmic Reticulum Stress." PLoS ONE **8**(12): e82968.

Stein, P. K., P. P. Domitrovich, N. Hui, P. Rautaharju and J. Gottdiener (2005). "Sometimes higher heart rate variability is not better heart rate variability: results of graphical and nonlinear analyses." J Cardiovasc Electrophysiol **16**(9): 954-959.

Sundar, I. K. and I. Rahman (2011). "Vitamin D and Susceptibility of Chronic Lung Diseases: Role of Epigenetics." Frontiers in Pharmacology **2**: 50.

Tare, M., S. J. Emmett, H. A. Coleman, C. Skordilis, D. W. Eyles, R. Morley and H. C. Parkington (2011). "Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats." J Physiol **589**(Pt 19): 4777-4786.

Tishkoff, D. X., K. A. Nibbelink, K. H. Holmberg, L. Dandu and R. U. Simpson (2008). "Functional vitamin D receptor (VDR) in the t-tubules of cardiac myocytes: VDR knockout cardiomyocyte contractility." Endocrinology **149**(2): 558-564.

Tsuji, H., M. G. Larson, F. J. Venditti, Jr., E. S. Manders, J. C. Evans, C. L. Feldman and D. Levy (1996). "Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study." Circulation **94**(11): 2850-2855.

Wang, T. J., M. J. Pencina, S. L. Booth, P. F. Jacques, E. Ingelsson, K. Lanier, E. J. Benjamin, R. B. D'Agostino, M. Wolf and R. S. Vasan (2008). "Vitamin D deficiency and risk of cardiovascular disease." Circulation **117**(4): 503-511.

Widdicombe, J. and L. Y. Lee (2001). "Airway reflexes, autonomic function, and cardiovascular responses." Environ Health Perspect **109 Suppl 4**: 579-584.

Wilkins, E. T. (1954). "Air Pollution and the London Fog of December, 1952." The Journal of the Royal Society for the Promotion of Health **74**(1): 1-21.

Zittermann, A. (2006). "Vitamin D and disease prevention with special reference to cardiovascular disease." Prog Biophys Mol Biol **92**(1): 39-48.

Zosky, G. R., P. H. Hart, A. J. Whitehouse, M. M. Kusel, W. Ang, R. E. Foong, L. Chen, P. G. Holt, P. D. Sly and G. L. Hall (2014). "Vitamin D deficiency at 16 to 20 weeks' gestation is associated with impaired lung function and asthma at 6 years of age." Ann Am Thorac Soc **11**(4): 571-577.

Chapter 3: The Effects of Klotho on Acrolein-Induced Heart rate and Heart Rate Variability Changes in Normal and Vitamin D Deficient Mice

Introduction

Cardiovascular morbidity and mortality are strongly correlated with short and long term ambient air pollution exposure,(Brook, Rajagopalan et al. 2010) which at times only manifests as subtle or latent changes especially in healthy individuals. These changes include autonomic imbalance or electrical disturbances (i.e. arrhythmia) which are indicative of increased cardiovascular risk even after acute exposure. Furthermore, research within the last ten years has shown that there are several factors that contribute to this effect. These include not only factors related to air pollution such as concentration, composition and chemistry, but also intrinsic host factors like nutrition. As far as the latter is concerned, homeostatic regulation and proper cardiac function is dependent on adequate levels of micronutrients. Vitamin D is one of the micronutrients needed by the body with a critical homeostatic role in several organ systems including the cardiovascular system.(Holick 2007, Lee, O'Keefe et al. 2008) Additionally, vitamin D receptors are present on numerous tissues and cells throughout the body, including cardiomyocytes.(Holick 2007)

Unfortunately, vitamin D deficiency (VDD) has become highly prevalent in the United States, as well as in the world, even affecting otherwise healthy individuals but there is not a consensus regarding the threshold of VDD. (Gordon, DePeter et al. 2004, Holick 2007, Martins, Wolf et al. 2007, Gordon, Feldman et al. 2008, Bener, Al-Ali et al.

2009, Kumar, Muntner et al. 2009) Although, VDD has been traditionally studied with respect to calcium regulation and bone-related conditions, it is now known that it also contributes to adverse cardiovascular outcomes.(Holick 2007, Mann, Exner et al. 2013, Masson, Agabiti et al. 2014) For example, during early-life or childhood, VDD can lead to vascular dysfunction and subsequent hypertension.(Gordon, Feldman et al. 2008, Tare, Emmett et al. 2011, Weng, Sprague et al. 2013) We previously showed that early-life persistent VDD induces autonomic imbalance in VDD mice that are otherwise healthy, similar findings have been demonstrated by others as well.(Canpolat, Özcan et al. 2015) (Stratford, 2018) Due to the fact that vitamin D is active throughout the body and elicits molecular and cellular effects in many different cell types, it is clear that VDD has the potential to induce numerous effects on the homeostatic controls of the body. Despite this understanding of VDD, a definitive mediator for these responses is still unknown.

Therefore, these studies focused on the role of klotho, which is an anti-aging factor regulated by vitamin D and known to play a role in controlling intracellular signaling, oxidative stress and cation transport in various cell types.(Hazari, Haykal-Coates et al. 2011, Ding and Ma 2015) In fact, klotho, which is upregulated with vitamin D receptor agonist supplementation,(Song, Gao et al. 2013) is highly conserved between humans and mice.(Ding and Ma 2015) Klotho has a direct effect on the heart by maintaining sinoatrial function during stress and its deficiency has been linked to arrhythmia(Takeshita, Fujimori et al. 2004) and certain cardiovascular diseases.(Song, Gao et al. 2013) Hence, klotho might be a potential mediator of vitamin D effects in the body, particularly when a stressor like air pollution is encountered.

Ambient air pollution is a mixture of many particulate, gaseous and volatile compounds that are usually produced by combustion of fossil fuels. For example, acrolein is a ubiquitous air pollutant and a common product of combustion of diesel fuel, fatty foods, cigarette smoke, wood and other organic compounds into a gaseous unsaturated aldehyde.(Ghilarducci and Tjeerdema 1995, Kehrer and Biswal 2000) In humans and mice, acrolein is typically an upper airway irritant and the health effects are mostly limited to the nasal passages.(Kehrer and Biswal 2000) Regardless, acrolein inhalation is linked to a myriad of respiratory conditions including eye, nose and throat irritation,(Sim and Pattle 1957, Esterbauer, Schaur et al. 1991) pulmonary edema(Kutzman, Popenoe et al. 1985, Hales, Barkin et al. 1989), bronchial hyper-responsiveness(Ben-Jebria, Marthan et al. 1994, Bein and Leikauf 2011), respiratory distress(Ben-Jebria, Marthan et al. 1994, Bein and Leikauf 2011) and asthma exacerbations(Leikauf, Leming et al. 1989). In addition, animal and in-vitro studies have demonstrated that acrolein exposure results in adverse cardiovascular responses including cytotoxicity in vascular endothelial cells and cardiac fibroblasts(Toraason, Luken et al. 1989, Kachel and Martin 1994) as well as left ventricular hypertrophy, which can lead to dilated cardiomyopathy(Ismahil, Hamid et al. 2011). It has also been demonstrated to cause cardiac autonomic dysfunction and electrical instability.(Hazari, Haykal-Coates et al. 2009, Conklin, Haberzettl et al. 2017)

However, the impact of VDD on these acrolein effects is unclear and warrants investigation, particularly because both vitamin D (Stratford, Haykal-Coates et al. 2018) and acrolein modulates autonomic function of the heart. Furthermore, vitamin D affects, potentially through klotho, the function of TRP channels, which are potently activated by

acrolein. Thus, the goal of this project was to determine how VDD modifies cardiac responses to acrolein in adult mice and clarify the role of klotho. We hypothesized that VDD-induced cardiopulmonary changes in acrolein responsiveness would be blocked by klotho. These studies seek to clarify whether treatment with klotho (potentially injectable) can mitigate the effects of VDD in this paradigm.

Materials and Methods

Animals - Three-week old female C57Bl/6 mice were used in this study (Jackson Laboratory, Raleigh, NC). Mice were housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22˚C and 50% relative humidity in an AAA-LAC-approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum during the quarantine period (3 days) after arrival. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering.

Diet - Three days after the quarantine period ended, mice were maintained ad libitum on either a vitamin D deficient (VDD) (D10073001-Research Diets Inc.) or normal diet (ND) (D10012G-Research Diets Inc.) for nineteen or forty weeks. The VDD diet had no added vitamin D but had vitamin mix V10037. The ND has 1000 IU per 10 grams of vitamin D which is the recommended amount for mice. The diets had equal levels of all other vitamins and minerals including calcium, which was at the concentration specified by the American Institute of Nutrition (Reeves, Nielsen et al. 1993). Water was provided ad libitum throughout the diet regimen.

Experimental Design and Groups – At the beginning of the study, mice were randomly assigned into a ND (n = 20) or VDD (n = 20) group and maintained on those diets for the extent of the study. Of those animals, 18 of the ND and 18 of the VDD mice were randomly chosen and implanted with radiotelemeters at 20 weeks of age and given klotho. All mice were randomly assigned to filtered air (FA) or acrolein exposure groups. Figure 3-1 demonstrates the experimental design.

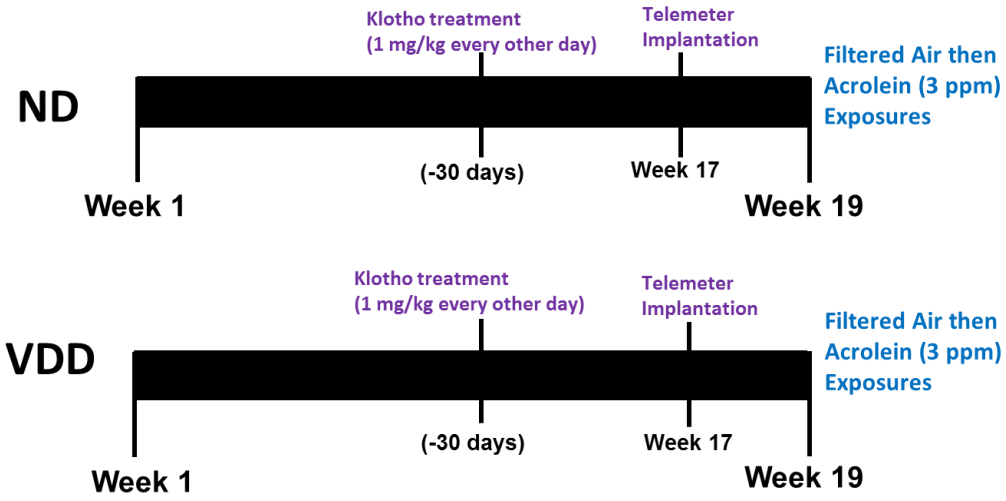


Figure 3-1. Experimental Design. ND and VDD mice were put on their respective diets for 19 weeks. A subset of mice was implanted with radiotelemeters and then exposed to filtered air and then acrolein (3ppm) for 3 hours. In a follow-up study, mice were treated with klotho every other day for a month before the same telemeter implantation and exposure scheme.

Surgical Implantation of Radiotelemeters - Animals were anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin, OH). Anesthesia was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic buprenorphrine (0.03 mg/kg, i.p. manufacturer). Briefly, using aseptic technique, each animal was implanted subcutaneously with a

radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed 7-10 days to recover from the surgery and reestablish circadian rhythms.(Kurhanewicz, McIntosh-Kastrinsky et al. 2014)

Radiotelemetery Data Acquistion - Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function by monitoring heart rate (HR) and ECG waveforms immediately following telemeter implantation and through exposure until the end of exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 15 minutes during the pre-exposure periods and continuously during exposure (baseline and hours 1-3); HR was automatically obtained from the waveforms (Dataquest ART Software, version 3.01, Data Sciences International, St. Paul, MN).

Electrocardiogram Analysis - ECGAuto software (EMKA Technologies USA, Falls Church, VA) was used to visualize individual ECG waveforms, analyze and quantify ECG segment durations and areas, as well as identify cardiac arrhythmias as previously described (Hazari, Haykal-Coates et al. 2009). Briefly, using ECGAuto, Pwave, QRS complex and T-wave were identified for individual ECG waveforms and compiled into a

library. Analysis of all experimental ECG waveforms was then based on established libraries. The following parameters were determined for each ECG waveform: PR interval (Pstart-R), QRS complex duration (Qstart-S), ST segment interval (S-Tend) and QT interval (Qstart-Tend). QT interval was corrected for HR using the correction formula for mice QTc = QT/(RR/100)1/2 (Mitchell, Jeron et al. 1998). Pre-exposure assessments were measured as the exposure time-matched four hours of data from 24 hours before exposure for each animal.

Heart Rate Variability Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RR intervals for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). HRV analysis was also conducted in the frequency domain using a Fast-Fourier transform. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions. The ratio of these two frequency domains (LF/HF) provides an estimate of the relative balance between sympathetic (LF) and vagal (HF) activity.

Whole-Body Plethysmography - Ventilatory function (e.g. enhanced pause, tidal volume and minute ventilation) was assessed in awake, unrestrained mice using a whole-body plethysmograph (Buxco, Wilmington, NC). Assessments were performed at

24 hours prior to the day of exposure and during exposure. The plethysmograph pressure was monitored using Biosystems XA software (Buxco Electronics Inc., Wilmington, NC). Using respiratory-induced fluctuations in ambient pressure, ventilatory parameters including tidal volume (VT), breathing frequency (f), inspiratory time (Ti), expiratory time (Te), minute volume (MV) and enhanced pause (Penh), which is a measure of ventilatory timing and can indicate airway irritation, were calculated and recorded on a breath-by-breath basis.

Tissue Collection and Analysis - Mice were euthanized using euthasol euthanasia solution immediately after exposure and blood was collected, processed and analyzed. Vitamin D concentrations were previously determined (Stratford, Haykal-Coates et al. 2018) and calicum and phosphorus were determined using (Sekisui Chemical, Japan).

Klotho – Klotho protein (R&D systems, Minneapolis, MN). Mice were intraperitoneally treated with 1 mg/kg every other day for the 28 days just before mice were exposed to FA or acrolein for a total of 14 doses. Klotho mRNA was assessed using klotho Taqman primer for mouse (ThermoFisher, Waltham, MA) and a qPCR was performed (ThermoFisher, Waltham, MA).

Acrolein Exposure – Acrolein exposures took place in whole-body plethysmography chambers (Model PLY3213, Buxco Electronics, Inc., Wilmington, NC). Acrolein gas was metered from a 1,000 ppm cylinder into a glass mixing chamber where the gas was mixed and diluted with dry filtered air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured once per hour using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN)

equipped with manual injection, a flame ionization detector and a DB-VRX capillary column.

Statistics - All data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) software. Mixed-model ANOVAs followed by Tukey's procedure for the post hoc comparisons were used to examine the statistical differences between exposure and diet. The statistical significance was set at $P < 0.05$.

Results

Klotho mRNA expression and serum calcium/phosphate levels – Klotho mRNA expression in the hearts of VDD mice was decreased compared to ND mice (Figure 3-2A). However, serum calcium (Figure 3-2B) and phosphate (Figure 3-2C) levels were not different between the diets.

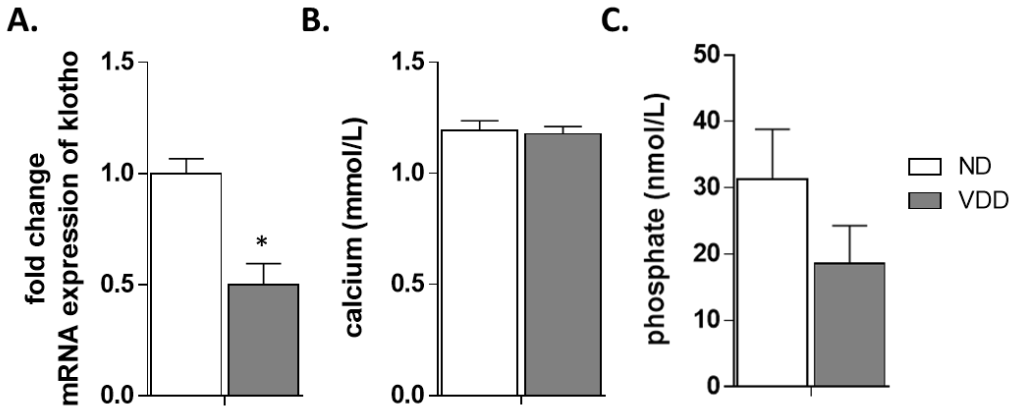


Figure 3-2. Klotho mRNA in the heart and serum calcium and phosphate levels. **(A.)** Klotho mRNA expression in the heart was significantly decreased in VDD mice. **(B.)** Serum calcium levels were not different between the diets. **(C.)** Serum phosphate levels were not different between the diets. *significantly different from ND ($p < 0.05$). Values represent means ± SEM. N=5-6

Heart rate and arrhythmias – Heart rate was measured before and during both filtered air and acrolein exposures. Heart rate was increased in VDD compared to ND mice pre-exposure and klotho blocked the response (Figure 3-3A). Acrolein exposure

increased HR in both ND and VDD mice, but during the first and third hours of exposure, VDD mice had significantly decreased HR compared to ND mice. There was no difference between the diets during the second hour of exposure. However, with klotho administration blocked the HR changes during exposure for both ND and VDD mice (Figure 3-3B). The number of arrhythmias observed in ND and VDD mice during FA exposure was similar to the first hour of acrolein exposure. Arrhythmias significantly increased during the third hour of acrolein exposure for both ND and VDD mice. Klotho further increased arrhythmias during the second and third hours of acrolein exposure in ND and VDD mice (Figure 3-3C).

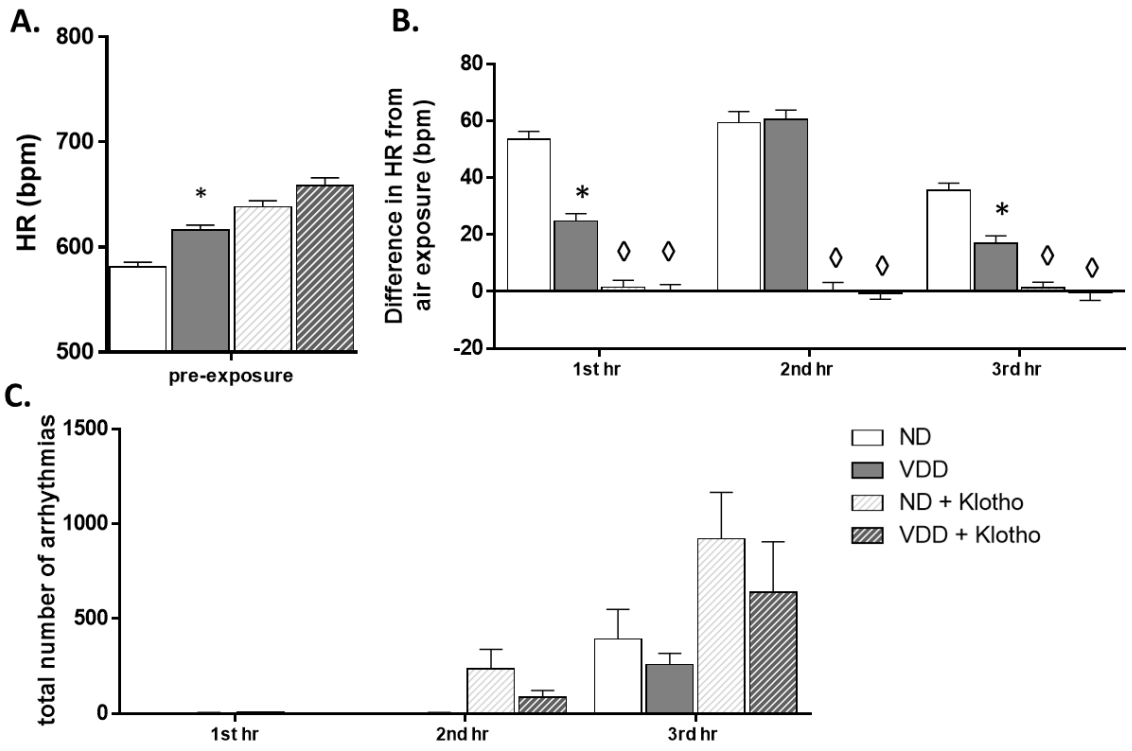


Figure 3-3. Klotho blocks acrolein-induced heart rate changes but worsens arrhythmias. (A.) VDD mice had higher baseline HR when compared to ND; although HR was higher for both diet groups with klotho, the difference between them was blocked by klotho. **(B.)** Acrolein increased HR in ND and VDD mice, however, HR was significantly less in the latter during the 1st and 3rd hours of exposure. The HR responses of both ND and VDD were completely blocked by klotho. **(C.)** There were no arrhythmias during FA or during the 1st and 2nd hours of acrolein exposure, however, arrhythmias increased during the 3rd hour of acrolein for both diet groups and klotho worsened the response. *significantly different from ND ($p < 0.05$). ◊significantly different from diet without klotho ($p < 0.05$). Values represent means ± SEM. N=7-9

Heart rate variability – Heart rate variability was measured before and during the FA and acrolein exposures. Prior to the exposures, VDD mice had significantly decreased SDNN and RMSSD when compared to ND, klotho decreased both SDNN and RMSSD in both diet groups but also blocked the differences between them (Figure 3-4A & B). The LF and HD showed a decreased trend in VDD mice (p=.055) when compared to ND before exposure, klotho significantly decreased these responses in

both diet groups (Figure 3-4C & D).

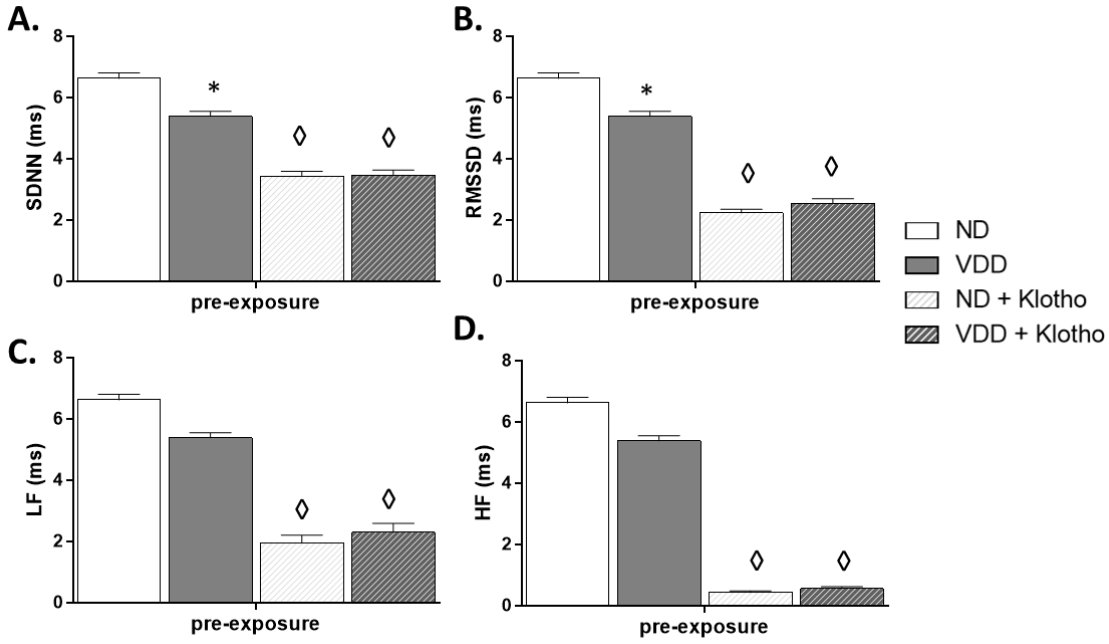


Figure 3-4. Klotho decreases baseline HRV. (A.) SDNN and RMSSD were significantly decreased in VDD mice during pre-exposure; although treatment with klotho decreased both parameters in both diet groups, it also blocked the differences between them **(A. and B.)** LF and HF were not different between the diet groups pre-exposure but klotho administration significantly decreased both in ND and VDD mice. **(C. and D.)** *significantly different from ND ($p < 0.05$). ◊ significantly different diet without klotho administration ($p < 0.05$). Values represent means ± SEM. N=7-9

Acrolein caused SDNN, RMSSD, LF and HF to increase during the second and third hours of exposure in both diet groups. However, overall cardiac autonomic function was significantly decreased in VDD mice compared to ND during that time period (Figure 3-5 A-D). Treatment with klotho increased virtually all of the HRV parameters during the 2nd hour of acrolein exposure in both diet groups. In contrast, klotho decreased all HRV parameters during the third hour of acrolein exposure in ND and VDD mice (Figure 3-5 A-D). Overall, treatment with klotho appeared to stabilize HRV

and prevent the drastic change normally observed with acrolein exposure, particularly the increase during the 3rd hour.(Kurhanewicz, McIntosh-Kastrinsky et al. 2017)

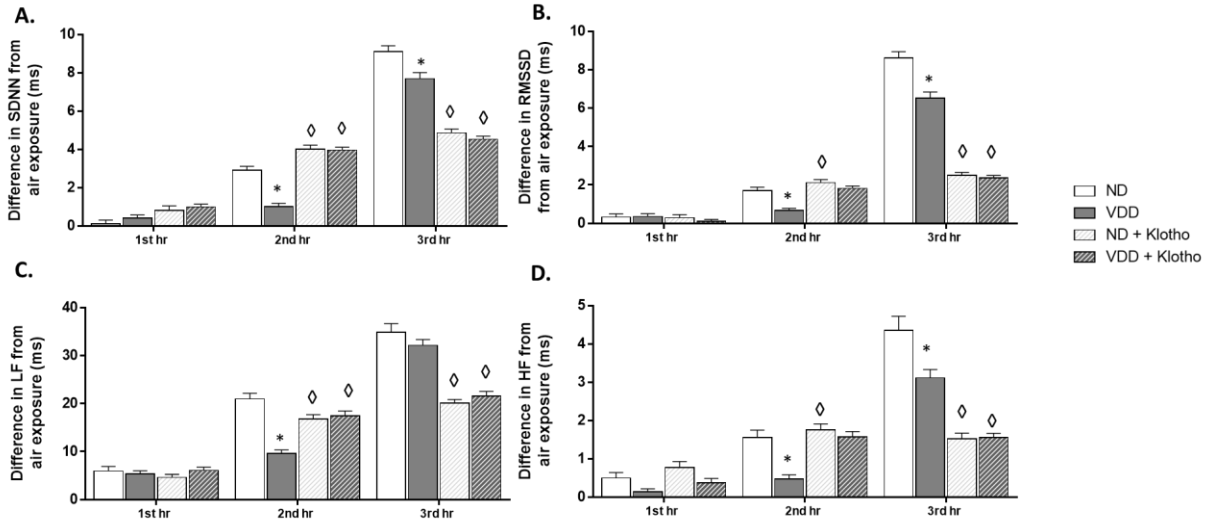


Figure 3-5. Acrolein-induced changes in HRV are modulated by klotho. Acrolein increased SDNN, RMSSD, LF and HF in ND and VDD mice, however, the parameters were significantly less for the latter during the 2nd and 3rd hours. Klotho increased these parameters during the 2nd hour but prevented them from increasing in the 3rd hour for both diets (A. – D.). *significantly different from ND ($p < 0.05$). ◊significantly different from diet without klotho ($p < 0.05$). Values represent means ± SEM. N=7-9

Ventilatory function – Ventilatory function was assessed before and during the FA and acrolein exposures. During air exposure, breathing frequency was only different between ND and VDD mice during the first hour while klotho further increased the response (Figure 3-6A).). Ventilatory timing was not different between ND and VDD mice during air exposure, however, klotho decreased it during the second and third hours for both diets (Figure 3-6B). Compared to FA, breathing frequency slightly decreased during acrolein exposure with ND mice having increased breathing frequency only during the second hour of exposure compared to VDD mice. Klotho significantly decreased breathing frequency with acrolein exposure especially during exposure compared to FA exposure (Figure 3-6C). Acrolein significantly increased

ventilatory timing compared to FA in both ND and VDD mice, this was significantly higher in VDD mice treated with klotho during the first hour. Klotho increased ventilatory timing in ND mice with acrolein exposure. (Figure 3-6D).

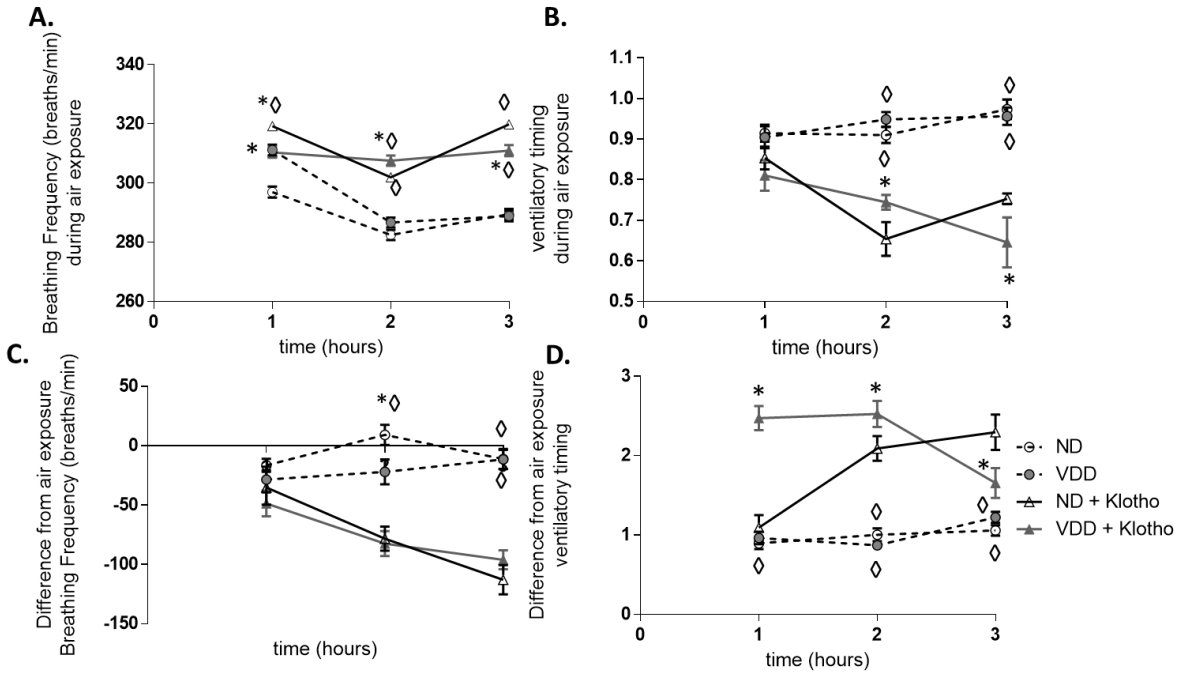


Figure 3-6. VDD-induced ventilatory function changes are modified by klotho during both air and acrolein exposure. (A.) During air exposure, breathing frequency was only different between ND and VDD mice during the first hour and klotho further increased the response. **(B.)** During air exposure, ventilatory timing was the same for ND and VDD mice; klotho decreased the response in both diet groups. **(C.)** Acrolein caused an initial decrease in breathing frequency in all groups. Although both ND and VDD animals increased their breathing rate thereafter, the latter had a greater increase. On the other hand, klotho significantly decreased breathing frequency in both diet groups. **(D.)** All animals experienced an increase in ventilatory timing during acrolein exposure and klotho administration further increased ventilatory timing in ND and VDD mice. *significantly different from ND ($p < 0.05$). ◊ significantly different from diet without klotho ($p < 0.05$). N=9

Discussion

This study demonstrates that VDD modifies the cardiopulmonary response of mice to acrolein and although klotho appears to block these effects of VDD, it also alters the cardiac function and response of ND mice. Basing the approach of this study on

commonly-accepted pharmacological principles, it was hypothesized that treatment with klotho would block the acrolein response only in deficient animals. This was due to klotho's low levels in VDD mice and primarily due to the fact that many of the effects of vitamin D are mediated by klotho.(Chen, Kuro et al. 2013, Berridge 2015, Navarro-Garcia, Fernandez-Velasco et al. 2018) We previously showed that acrolein has potent effects on the autonomic nervous system as indicated by HRV (Kurhanewicz, McIntosh-Kastrinsky et al. 2017) and we assumed that not only would vitamin D modify these effects but that klotho would block those effects much like a pharmacological drug. Vitamin D deficiency is known to cause potentially detrimental changes to the homeostatic mechanisms of the body, including autonomic imbalance, and it is believed that the effects may be due to a decrease in klotho.(Forman, Curhan et al. 2008, Weng, Sprague et al. 2013, Canpolat, Özcan et al. 2015, Chen, Sun et al. 2015) The data shown in this study demonstrates a role for klotho in cardioprotection of air pollution-induced cardiac responses irrespective of diet. Yet, klotho's role in vitamin D's cardiac effects show that it might protect against the deleterious effects of air pollution irrespective of diet.

Autonomic imbalance is characterized by inappropriate modulation of one of the branches of the autonomic nervous system leading to altered homeostatic responsiveness and in the case of the cardiovascular system, disease and ultimately increased mortality. This has been demonstrated with VDD and following exposures to air pollution.(Gold, Litonjua et al. 2000, Pope, Hansen et al. 2004, Brook, Rajagopalan et al. 2010, Carll, Lust et al. 2013, Canpolat, Özcan et al. 2015, Farraj, Walsh et al. 2015) HRV changes occur in susceptible populations such as diabetics, those with

hypertension and arrhythmias.(Thayer, Yamamoto et al. 2010) Although VDD has not traditionally been identified as increasing susceptibility to cardiovascular disease recent data has suggested a correlation, particularly with underlying chronic conditions.(Lee, O'Keefe et al. 2008) In this study, VDD mice had decreased HRV, particularly SDNN and RMSSD compared to normal mice, even before exposure. This decreased HRV response (i.e. relative to normal) was similarly observed during the second and third hours of acrolein exposure suggesting that VDD effects autonomic regulation after subsequent responses to air pollution. This indicates that VDD causes a shift in autonomic function towards greater sympathetic modulation. As stated above, klotho significantly decreased SDNN and RMSSD changes in both deficient and normal mice suggesting its efficacy is independent of vitamin D levels in the body.

The protective effect of klotho may be based on its role in calcium regulation, reduction of oxidative stress and inflammation, and/or the suppression of cardiac muscle pressure-load signaling. Even with normal vitamin D levels, klotho activates Na+, K+-ATPase to facilitate transepithelial calcium transport.(Imura, Tsuji et al. 2007, Razzaque 2008) Calcium regulation is critical not just in the cardiovascular system but throughout the body because overload is a significant risk factor for dysfunction.(Tang, Shen et al. 2018) and klotho helps maintain calcium homeostasis under any condition. Additionally, klotho inhibits reactive oxygen species production by increasing Nrf2(Zhu, Gao et al. 2017) and NF-kB(Guo, Zhuang et al. 2018) pro-inflammatory signaling pathways which promotes normal function of the heart. Interestingly, klotho also protects the brain against ischemic injuries, such as strokes, through similar mechanisms.(Zhou, Li et al. 2017) The brain is believed to be a target of klotho since it

is found in cerebrospinal fluid (Kuro-o, Matsumura et al. 1997) and studies suggest that the choroid plexus is responsible for its production due to the blood brain barrier.(Cararo-Lopes, Mazucanti et al. 2017) Klotho knockouts have neuronal deficits (e.g. diminished synapses and axon transport alterations) (Cararo-Lopes, Mazucanti et al. 2017) which could feasibly contribute to altered autonomic signaling. Moreover, biopsied cardiomyocytes from people with cardiovascular disease showed decreased klotho expression and increased FGF-23 signaling, oxidative stress, inflammation and fibrosis.(Corsetti, Pasini et al. 2016)

Although the klotho receptor has not been identified, it is a co-receptor to FGF-23 to (Dalton, Xie et al. 2017) which is expressed in cardiomyocytes and induced by during pressure overload conditions like hypertension.(Leifheit-Nestler, Kirchhoff et al. 2018) These data suggest that FGF-23 plays a role in pressure-overload-induced cardiac dysfunction and klotho counteracts the response. Although we showed that klotho expression in the heart of VDD mice was decreased, it is still unclear whether a similar decrease would be observed in normal mice undergoing cardiac stress or hypertrophy (e.g. isoproterenol or left anterior descending coronary artery ligation).

In addition, klotho deficient mice are characterized as having increased vascular calcifications suggesting klotho is needed to maintain normal structure and function within the vasculature.(Kuro-o, Matsumura et al. 1997) Previous intervention studies have shown that when klotho protein is administered intraperitoneally in mice for even a short period of time there is a reduction in vascular calcifications.(Chen, Kuro et al. 2013) A similar response was observed in mutagenic mice with a klotho deletion in distal/proximal tubules of the kidney.(Hu, Shi et al. 2011) Thus, the klotho effects on

vascular calcification are mediated by calcium and phosphate.(Mencke and Hillebrands 2017) This data suggests that regardless of diet, klotho can protect against vasculature damage due to calcium imbalance. This is pertinent to the findings of this study because many of the cardiac effects observed in air pollution studies are secondary to vascular or blood pressure changes.

Furthermore, proper levels of vitamin D and klotho regulate the RAAS pathway (by Wnt signaling), this is known to be cardioprotective due to its maintenance of normal blood pressure.(Mencke and Hillebrands 2017, Takenaka, Inoue et al. 2017, Eltablawy and Ashour 2018) In the RAAS pathway, renin is released by the kidneys (due to direct effects of sympathetic nerves, arterial pressure or mineral homeostasis), it then catalyzes the conversion of angiotensinogen to angiotensin I. Angiotensin I is then converted to angiotensin II by angiotensin converting enzyme (ACE) which is produced by the vascular endothelium. The formation of angiotensin II regulates mineral homeostasis by the kidneys, vascular constriction to increase pressure and stimulates release of aldosterone by the adrenal cortex. Aldosterone release increases sodium and fluid retention to restore blood volume or blood pressure.(Yang and Xu 2017) The result of these changes is an increase in blood pressure. klotho (as well as vitamin D) has been shown to be a negative regulator of RAAS (Li, Kong et al. 2002, Takenaka, Inoue et al. 2017) and directly inhibits aldosterone production by the adrenal glands, which protects against hypertension and eventually cardiac damage and dysfunction.(Yang and Xu 2017) Klotho inhibits RAAS by suppressing angiotensin II and activation of the Wnt/β-catenin pathway which in turn has been demonstrated to reduce cardiomyocyte damage.(Zhou, Mo et al. 2015, Yu, Meng et al. 2016) In addition, exogenous klotho

suppresses mRNA expression of chief RAAS proteins including angiotensinogen, renin and ACE to help with blood pressure control.(Zhou, Mo et al. 2015) Future studies should evaluate RAAS protein expression in ND and VDD mice and determine whether it plays a role in the acrolein-induced cardiac effects.

Our previous studies have showed that, the HR of mice increases during acrolein exposure, probably due to a combination of stress and sensory nerve irritation.(Farraj, Hazari et al. 2011, Hazari, Haykal-Coates et al. 2011, Carll, Lust et al. 2013) These data show that VDD mice were unable to increase their HR as much as ND mice during the first and third hours of acrolein exposure, which is consistent with Takeshita et. al, who demonstrated that klotho deficient mice were unable to increase their HR due to stress and actually had sinoatrial node dysfunction arrhythmias.(Takeshita, Fujimori et al. 2004) The inability of VDD mice to increase HR suggests that either the heart is unable to respond to normal autonomic signals or that abnormal hemodynamic properties (e.g. hypertension) have altered the cardiac function. In either case, klotho blocked these changes such that HR during acrolein was completely similar to FA.

Air pollution also increases cardiac arrhythmias in people and rodents.(Hazari, Haykal-Coates et al. 2009, Hazari, Haykal-Coates et al. 2011, Carll, Lust et al. 2013) Acrolein increased the total number of arrhythmias particularly during the third hour of exposure in both diet groups. Surprisingly, treatment with klotho made it worse. Arrhythmias as described here are most typically sinus block or “dropped beats” and not premature contractions of the atria or ventricles. We have rarely seen atrial or ventricular premature contraction arrhythmias in mice, probably due to their high heart rates. Regardless, the higher incidence of arrhythmia due to klotho may be a result of

the decreased HR, particularly as observed during the second and third hours of exposure. One of the limitations of mouse electrocardiogram is the ability to clearly identify a “dropped beat” because the methodology only uses a single lead. On the other hand, the dosing scheme for klotho could have possibly activated a divergent mechanism resulting in increased arrhythmias given that lower doses of klotho have been administered and shown to be efficacious in other studies.(Chen, Kuro et al. 2013) Optimization of the dosing scheme using a dose-response would be appropriate for future studies.

Our previous study demonstrated that the diet regimen described is adequate time for mice to become VDD.(Stratford, Haykal-Coates et al. 2018) Consistent with previous studies, serum calcium and phosphate levels are not different.(Girgis, Cha et al. 2015) This result may seem counter intuitive given the known physiological role of vitamin D to aid in calcium homeostasis. But serum calcium levels are tightly regulated by PTH so further studies should evaluate PTH and the tissue calcium levels, particularly in the heart. The heart has a high calcium demand due to its role in cardiomyocyte contraction. Therefore, the calcium levels in the heart may be negatively affected by VDD and those impairments may be mediated by decreased klotho expression.

As stated previously cardioprotection by klotho has been demonstrated previously (Xie, Cha et al. 2012, Song, Gao et al. 2013, Song and Si 2015) but this is the first study to demonstrate the cardiac effects of klotho in mice exposed to air pollution. Acrolein was used due to its ubiquitous presence in ambient air pollution and its known effects on the cardiac autonomic function. Acrolein also activates TRP

channels which have also been linked to klotho and VDD. (McNamara, Mandel-Brehm et al. 2007, Eke, Igwe et al. 2014) In fact, klotho supplementation reduces the activation of TRP channels in response to panful stimuli.(Eke, Igwe et al. 2014) There are still questions about whether klotho supplementation could directly target the receptors of the nose and lungs or even the heart and are the effects elicited due to upstream changes. Klotho is a transmembrane protein that when cleaved functions as a hormone; (Imura, Tsuji et al. 2007, Strewler 2007) therefore, it is not unreasonable to speculate that the benefits of klotho supplementation could be due to systemic effects including a regulatory role on irritant sensor TRP channels in the airways. Additionally, klotho may exert its effects by binding to monosialogangliosides in lipid rafts.(Dalton, An et al. 2017)

Finally, we previously showed that acrolein exposure alters ventilatory function in both mice and rats, which is not surprising given the high concentrations we used and irritating nature of the chemical. Although 3ppm of acrolein is not considered even remotely close to ambient, such levels can be measured in structural or wildfires. Rats exposed to similar concentrations of acrolein had lower breathing frequency and increased irritation.(Snow, McGee et al. 2017) Mice exposed to acrolein have similar ventilatory responses to acrolein as rats and we showed here that VDD actually further increases the rapid (shallow) breathing due to irritation. Klotho appeared to completely reverse this phenomenon because mice from both diet groups had significantly decreased breathing rates during acrolein. Once again it is not immediately known whether these are direct effects in the lungs or secondary to klotho's regulation of TRP channels or some other central neural process. It is worthwhile to note that these

ventilatory changes likely influence not only the HR but HRV as well, emphasizing once again a systemic shift due to VDD.

Conclusions

This data presented partially indicates that klotho may protect the heart during acrolein exposure regardless of whether a person is VDD or not. More importantly, VDD modifies the cardiac response to air pollution exposure by modulating the function of the ANS and altering breathing. Although these results do not necessarily point to a definitive increase in risk, it is still reasonable to conclude that such homeostatic shifts can be potentially harmful with other underlying disease or in the event of encountering a co-stressor. Regardless, klotho represents potential therapeutic target to protect against air pollution-induced adverse cardiovascular events.

REFERENCES

Bein, K. and G. D. Leikauf (2011). "Acrolein - a pulmonary hazard." Mol Nutr Food Res **55**(9): 1342-1360.

Ben-Jebria, A., R. Marthan, M. Rossetti, J. P. Savineau and J. S. Ultman (1994). "Human bronchial smooth muscle responsiveness after in vitro exposure to acrolein." Am J Respir Crit Care Med **149**(2 Pt 1): 382-386.

Bener, A., M. Al-Ali and G. F. Hoffmann (2009). "High prevalence of vitamin D deficiency in young children in a highly sunny humid country: a global health problem." Minerva pediatrica **61**(1): 15-22.

Berridge, M. J. (2015). "Vitamin D cell signalling in health and disease." Biochemical and Biophysical Research Communications **460**(1): 53-71.

Brook, R. D., S. Rajagopalan, C. A. Pope, J. R. Brook, A. Bhatnagar, A. V. Diez-Roux, F. Holguin, Y. Hong, R. V. Luepker, M. A. Mittleman, A. Peters, D. Siscovick, S. C. Smith, L. Whitsel, J. D. Kaufman, o. b. o. t. A. H. A. C. o. Epidemiology, C. o. t. K. i. C. D. Prevention, P. A. Council on Nutrition and Metabolism (2010). "Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement From the American Heart Association." Circulation **121**(21): 2331-2378.

Canpolat, U., F. Özcan, Ö. Özeke, O. Turak, Ç. Yayla, S. K. Açıkgöz, S. Çay, S. Topaloğlu, D. Aras and S. Aydoğdu (2015). "Impaired Cardiac Autonomic Functions in Apparently Healthy Subjects with Vitamin D Deficiency." Annals of Noninvasive Electrocardiology **20**(4): 378-385.

Cararo-Lopes, M. M., C. H. Y. Mazucanti, C. Scavone, E. M. Kawamoto and D. C. Berwick (2017). "The relevance of alpha-KLOTHO to the central nervous system: Some key questions." Ageing Res Rev **36**: 137-148.

Carll, A. P., R. M. Lust, M. S. Hazari, C. M. Perez, Q. T. Krantz, C. J. King, D. W. Winsett, W. E. Cascio, D. L. Costa and A. K. Farraj (2013). "Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats." Toxicol Sci **131**(2): 583-595.

Chen, S., Y. Sun and D. K. Agrawal (2015). "Vitamin D deficiency and essential hypertension." J Am Soc Hypertens **9**(11): 885-901.

Chen, T. H., O. M. Kuro, C. H. Chen, Y. M. Sue, Y. C. Chen, H. H. Wu and C. Y. Cheng (2013). "The secreted Klotho protein restores phosphate retention and suppresses accelerated aging in Klotho mutant mice." Eur J Pharmacol **698**(1-3): 67-73.

Conklin, D. J., P. Haberzettl, G. Jagatheesan, M. Kong and G. W. Hoyle (2017). "Role of TRPA1 in acute cardiopulmonary toxicity of inhaled acrolein." Toxicol Appl Pharmacol **324**: 61-72.

Corsetti, G., E. Pasini, T. M. Scarabelli, C. Romano, P. R. Agrawal, C. Chen-Scarabelli, R. Knight, L. Saravolatz, J. Narula, M. Ferrari-Vivaldi, V. Flati, D. Assanelli and F. S. Dioguardi (2016). "Decreased expression of Klotho in cardiac atria biopsy samples from patients at higher risk of atherosclerotic cardiovascular disease." J Geriatr Cardiol **13**(8): 701-711.

Dalton, G., S. W. An, S. I. Al-Juboori, N. Nischan, J. Yoon, E. Dobrinskikh, D. W. Hilgemann, J. Xie, K. Luby-Phelps, J. J. Kohler, L. Birnbaumer and C. L. Huang (2017). "Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling." Proc Natl Acad Sci U S A **114**(4): 752-757.

Dalton, G. D., J. Xie, S. W. An and C. L. Huang (2017). "New Insights into the Mechanism of Action of Soluble Klotho." Front Endocrinol (Lausanne) **8**: 323.
Ding, H.-Y. and H.-X. Ma (2015). "Significant roles of anti-aging protein klotho and fibroblast growth factor23 in cardiovascular disease." Journal of Geriatric Cardiology : JGC **12**(4): 439-447.

Eke, I. G., C. Igwe and A. O. Anaga (2014). "Vitamin D supplementation attenuated carageenan-induced paw edema and formalin-induced nociception in indomethacin-treated rats." Int J Basic Clin Pharmacol **3**(1): 206-210.

Eltablawy, N. and H. Ashour (2018). "Vitamin D protection from rat diabetic nephropathy is partly mediated through Klotho expression and renin-angiotensin inhibition." 1-7.
Esterbauer, H., R. J. Schaur and H. Zollner (1991). "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes." Free Radic Biol Med **11**(1): 81-128.

Farraj, A. K., M. S. Hazari, D. W. Winsett, A. Kulukulualani, A. P. Carll, N. Haykal-Coates, C. M. Lamb, E. Lappi, D. Terrell and W. E. Cascio (2011). "Overt and latent cardiac effects of ozone inhalation in rats: evidence for autonomic modulation and increased myocardial vulnerability." Environmental health perspectives **120**(3): 348-354.

Farraj, A. K., L. Walsh, N. Haykal-Coates, F. Malik, J. McGee, D. Winsett, R. Duvall, K. Kovalcik, W. E. Cascio, M. Higuchi and M. S. Hazari (2015). "Cardiac effects of seasonal ambient particulate matter and ozone co-exposure in rats." Part Fibre Toxicol **12**: 12.

Forman, J. P., G. C. Curhan and E. N. Taylor (2008). "Plasma 25-hydroxyvitamin D levels and risk of incident hypertension among young women." Hypertension **52**(5): 828-832.

Ghilarducci, D. P. and R. S. Tjeerdema (1995). "Fate and effects of acrolein." Rev Environ Contam Toxicol **144**: 95-146.

Girgis, C. M., K. M. Cha, P. J. Houweling, R. Rao, N. Mokbel, M. Lin, R. J. Clifton-Bligh and J. E. Gunton (2015). "Vitamin D Receptor Ablation and Vitamin D Deficiency Result in Reduced Grip Strength, Altered Muscle Fibers, and Increased Myostatin in Mice." Calcif Tissue Int **97**(6): 602-610.

Gold, D. R., A. Litonjua, J. Schwartz, E. Lovett, A. Larson, B. Nearing, G. Allen, M. Verrier, R. Cherry and R. Verrier (2000). "Ambient pollution and heart rate variability." Circulation **101**(11): 1267-1273.

Gordon, C. M., K. C. DePeter, H. A. Feldman, E. Grace and S. Emans (2004). "PRevalence of vitamin d deficiency among healthy adolescents." Archives of Pediatrics & Adolescent Medicine **158**(6): 531-537.

Gordon, C. M., H. A. Feldman, L. Sinclair and et al. (2008). "PRevalence of vitamin d deficiency among healthy infants and toddlers." Archives of Pediatrics & Adolescent Medicine **162**(6): 505-512.

Guo, Y., X. Zhuang, Z. Huang, J. Zou, D. Yang, X. Hu, Z. Du, L. Wang and X. Liao (2018). "Klotho protects the heart from hyperglycemia-induced injury by inactivating ROS and NF-kappaB-mediated inflammation both in vitro and in vivo." Biochim Biophys Acta **1864**(1): 238-251.

Hales, C. A., P. Barkin, W. Jung, D. Quinn, D. Lamborghini and J. Burke (1989). "Bronchial artery ligation modifies pulmonary edema after exposure to smoke with acrolein." J Appl Physiol (1985) **67**(3): 1001-1006.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, D. L. Costa and A. K. Farraj (2009). "A single exposure to particulate or gaseous air pollution increases the risk of aconitine-induced cardiac arrhythmia in hypertensive rats." Toxicol Sci **112**(2): 532-542.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, Q. T. Krantz, C. King, D. L. Costa and A. K. Farraj (2011). "TRPA1 and Sympathetic Activation Contribute to Increased Risk of Triggered Cardiac Arrhythmias in Hypertensive Rats Exposed to Diesel Exhaust." Environmental Health Perspectives **119**(7): 951-957.

Holick, M. F. (2007). "Vitamin D Deficiency." New England Journal of Medicine **357**(3): 266-281.

Hu, M. C., M. Shi, J. Zhang, H. Quinones, C. Griffith, M. Kuro-o and O. W. Moe (2011). "Klotho deficiency causes vascular calcification in chronic kidney disease." J Am Soc Nephrol **22**(1): 124-136.

Imura, A., Y. Tsuji, M. Murata, R. Maeda, K. Kubota, A. Iwano, C. Obuse, K. Togashi, M. Tominaga, N. Kita, K. Tomiyama, J. Iijima, Y. Nabeshima, M. Fujioka, R. Asato, S. Tanaka, K. Kojima, J. Ito, K. Nozaki, N. Hashimoto, T. Ito, T. Nishio, T. Uchiyama, T. Fujimori and Y. Nabeshima (2007). "alpha-Klotho as a regulator of calcium homeostasis." Science **316**(5831): 1615-1618.

Ismahil, M. A., T. Hamid, P. Haberzettl, Y. Gu, B. Chandrasekar, S. Srivastava, A. Bhatnagar and S. D. Prabhu (2011). "Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy." Am J Physiol Heart Circ Physiol **301**(5): H2050-2060.

Kachel, D. L. and W. J. Martin, 2nd (1994). "Cyclophosphamide-induced lung toxicity: mechanism of endothelial cell injury." J Pharmacol Exp Ther **268**(1): 42-46.
Kehrer, J. P. and S. S. Biswal (2000). "The molecular effects of acrolein." Toxicol Sci **57**(1): 6-15.

Kumar, J., P. Muntner, F. J. Kaskel, S. M. Hailpern and M. L. Melamed (2009). "Prevalence and Associations of 25-Hydroxyvitamin D Deficiency in US Children: NHANES 2001–2004." Pediatrics **124**(3): e362-e370.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, A. Ledbetter, L. Walsh, A. Farraj and M. Hazari (2017). "TRPA1 mediates changes in heart rate variability and cardiac mechanical function in mice exposed to acrolein." Toxicol Appl Pharmacol **324**: 51-60.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, L. Walsh, A. K. Farraj and M. S. Hazari (2014). "Ozone co-exposure modifies cardiac responses to fine and ultrafine ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses." Part Fibre Toxicol **11**: 54.

Kuro-o, M., Y. Matsumura, H. Aizawa, H. Kawaguchi, T. Suga, T. Utsugi, Y. Ohyama, M. Kurabayashi, T. Kaname, E. Kume, H. Iwasaki, A. Iida, T. Shiraki-Iida, S. Nishikawa, R. Nagai and Y. I. Nabeshima (1997). "Mutation of the mouse klotho gene leads to a syndrome resembling ageing." Nature **390**(6655): 45-51.

Kutzman, R. S., E. A. Popenoe, M. Schmaeler and R. T. Drew (1985). "Changes in rat lung structure and composition as a result of subchronic exposure to acrolein." Toxicology **34**(2): 139-151.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D deficiency an important, common, and easily treatable cardiovascular risk factor?" J Am Coll Cardiol **52**(24): 1949-1956.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D Deficiency: An Important, Common, and Easily Treatable Cardiovascular Risk Factor?" Journal of the American College of Cardiology **52**(24): 1949-1956.

Leifheit-Nestler, M., F. Kirchhoff, J. Nespor, B. Richter, B. Soetje, M. Klintschar, J. Heineke and D. Haffner (2018). "Fibroblast growth factor 23 is induced by an activated renin-angiotensin-aldosterone system in cardiac myocytes and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts." Nephrol Dial Transplant.

Leikauf, G. D., L. M. Leming, J. R. O'Donnell and C. A. Doupnik (1989). "Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein." J Appl Physiol (1985) **66**(1): 171-178.

Li, Y. C., J. Kong, M. Wei, Z. F. Chen, S. Q. Liu and L. P. Cao (2002). "1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system." J Clin Invest **110**(2): 229-238.

Mann, M., D. Exner, B. Hemmelgarn, D. Sola, T. Turin, L. Ellis and S. Ahmed (2013). "Vitamin D Levels Are Associated with Cardiac Autonomic Activity in Healthy Humans." Nutrients **5**(6): 2114-2127.

Martins, D., M. Wolf, D. Pan and et al. (2007). "Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin d in the united states: Data from the third national health and nutrition examination survey." Archives of Internal Medicine **167**(11): 1159-1165.

Masson, S., N. Agabiti, T. Vago, M. Miceli, F. Mayer, T. Letizia, U. Wienhues-Thelen, G. F. Mureddu, M. Davoli, A. Boccanelli, R. Latini and P. s. The Investigators of the (2014). "The fibroblast growth factor-23 and Vitamin D emerge as nontraditional risk factors and may affect cardiovascular risk." Journal of Internal Medicine: n/a-n/a.

McNamara, C. R., J. Mandel-Brehm, D. M. Bautista, J. Siemens, K. L. Deranian, M. Zhao, N. J. Hayward, J. A. Chong, D. Julius, M. M. Moran and C. M. Fanger (2007). "TRPA1 mediates formalin-induced pain." Proc Natl Acad Sci U S A **104**(33): 13525-13530.

Mencke, R. and J. L. Hillebrands (2017). "The role of the anti-ageing protein Klotho in vascular physiology and pathophysiology." Ageing Res Rev **35**: 124-146.

Navarro-Garcia, J. A., M. Fernandez-Velasco, C. Delgado, J. F. Delgado, O. M. Kuro, L. M. Ruilope and G. Ruiz-Hurtado (2018). "PTH, vitamin D, and the FGF-23-klotho axis and heart: going beyond the confines of nephrology." Eur J Clin Invest.

Pope, C. A., 3rd, M. L. Hansen, R. W. Long, K. R. Nielsen, N. L. Eatough, W. E. Wilson and D. J. Eatough (2004). "Ambient particulate air pollution, heart rate variability, and blood markers of inflammation in a panel of elderly subjects." Environ Health Perspect **112**(3): 339-345.

Razzaque, M. S. (2008). "Klotho and Na+,K+-ATPase activity: solving the calcium metabolism dilemma?" Nephrol Dial Transplant **23**(2): 459-461.

Sim, V. M. and R. E. Pattle (1957). "Effect of possible smog irritants on human subjects." J Am Med Assoc **165**(15): 1908-1913.

Snow, S. J., M. A. McGee, A. Henriquez, J. E. Richards, M. C. Schladweiler, A. D. Ledbetter and U. P. Kodavanti (2017). "Respiratory Effects and Systemic Stress Response Following Acute Acrolein Inhalation in Rats." Toxicol Sci **158**(2): 454-464.

Song, S., P. Gao, H. Xiao, Y. Xu and L. Y. Si (2013). "Klotho Suppresses Cardiomyocyte Apoptosis in Mice with Stress-Induced Cardiac Injury via Downregulation of Endoplasmic Reticulum Stress." PLoS ONE **8**(12): e82968.

Song, S. and L. Y. Si (2015). "Klotho ameliorated isoproterenol-induced pathological changes in cardiomyocytes via the regulation of oxidative stress." Life Sci **135**: 118-123.

Stratford, K., N. Haykal-Coates, L. Thompson, Q. T. Krantz, C. King and J. Krug (2018). "Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice."

Strewler, G. J. (2007). "Untangling klotho's role in calcium homeostasis." Cell Metab **6**(2): 93-95.

Takenaka, T., T. Inoue, T. Miyazaki, H. Kobori, A. Nishiyama, N. Ishii, M. Hayashi and H. Suzuki (2017). "Klotho suppresses the renin-angiotensin system in adriamycin nephropathy." Nephrol Dial Transplant **32**(5): 791-800.

Takeshita, K., T. Fujimori, Y. Kurotaki, H. Honjo, H. Tsujikawa, K. Yasui, J. K. Lee, K. Kamiya, K. Kitaichi, K. Yamamoto, M. Ito, T. Kondo, S. Iino, Y. Inden, M. Hirai, T. Murohara, I. Kodama and Y. Nabeshima (2004). "Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression." Circulation **109**(14): 1776-1782.

Tang, G., Y. Shen, P. Gao, S. S. Song and L. Y. Si (2018). "Klotho attenuates isoproterenol-induced hypertrophic response in H9C2 cells by activating Na(+)/K(+)-ATPase and inhibiting the reverse mode of Na(+)/Ca(2+)-exchanger."

Tare, M., S. J. Emmett, H. A. Coleman, C. Skordilis, D. W. Eyles, R. Morley and H. C. Parkington (2011). "Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats." J Physiol **589**(Pt 19): 4777-4786.

Thayer, J. F., S. S. Yamamoto and J. F. Brosschot (2010). "The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors." International Journal of Cardiology **141**(2): 122-131.

Toraason, M., M. E. Luken, M. Breitenstein, J. A. Krueger and R. E. Biagini (1989). "Comparative toxicity of allylamine and acrolein in cultured myocytes and fibroblasts from neonatal rat heart." Toxicology **56**(1): 107-117.

Weng, S., J. E. Sprague, J. Oh, A. E. Riek, K. Chin, M. Garcia and C. Bernal-Mizrachi (2013). "Vitamin D deficiency induces high blood pressure and accelerates atherosclerosis in mice." PLoS One **8**(1): e54625.

Xie, J., S. K. Cha, S. W. An, O. M. Kuro, L. Birnbaumer and C. L. Huang (2012). "Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart." Nat Commun **3**: 1238.

Yang, T. and C. Xu (2017). "Physiology and Pathophysiology of the Intrarenal Renin-Angiotensin System: An Update." J Am Soc Nephrol **28**(4): 1040-1049.

Yu, L., W. Meng, J. Ding and M. Cheng (2016). "Klotho inhibits angiotensin II-induced cardiomyocyte hypertrophy through suppression of the AT1R/beta catenin pathway." Biochem Biophys Res Commun **473**(2): 455-461.

Zhou, H. J., H. Li, M. Q. Shi, X. N. Mao, D. L. Liu, Y. R. Chang, Y. M. Gan, X. Kuang and J. R. Du (2017). "Protective Effect of Klotho against Ischemic Brain Injury Is Associated with Inhibition of RIG-I/NF-kappaB Signaling." Front Pharmacol **8**: 950.

Zhou, L., H. Mo, J. Miao, D. Zhou, R. J. Tan, F. F. Hou and Y. Liu (2015). "Klotho Ameliorates Kidney Injury and Fibrosis and Normalizes Blood Pressure by Targeting the Renin-Angiotensin System." Am J Pathol **185**(12): 3211-3223.

Zhu, H., Y. Gao, S. Zhu, Q. Cui and J. Du (2017). "Klotho Improves Cardiac Function by Suppressing Reactive Oxygen Species (ROS) Mediated Apoptosis by Modulating Mapks/Nrf2 Signaling in Doxorubicin-Induced Cardiotoxicity." Med Sci Monit **23**: 5283-5293.

Chapter 4: Vitamin D Deficiency-Induced Cardiovascular Dysfunction is Mediated by TRPC6

Introduction

Vitamin D is one of the only micronutrient molecules that is endogenously produced in the body and it has a critical homeostatic role across all of the organ systems.(Holick 2007, Wang, Pencina et al. 2008) It is a fat-soluble vitamin synthesized in the skin after ultraviolent B radiation exposure and acquired through the diet from a variety of foods like milk, fish, cheese and vitamin D fortified foods.(Lips 2006, Holick 2007) Vitamin D receptors are present on numerous tissues and cells throughout the body, including cardiomyocytes.(Holick 2007) Vitamin D exerts its effects through a steroid nuclear receptor that(Holick 2007, Norman and Powell 2014) upon activation forms a complex with the retinoid X receptor.(Fetahu, Höbaus et al. 2014, Saccone, Asani et al. 2015) Activated vitamin D receptors (VDR) bind to vitamin D response elements (VDRE) on DNA and influence expression of a wide variety of genes covering numerous functions (e.g. calcium homeostasis, antioxidant production, protein synthesis).(Sundar and Rahman 2011, Fetahu, Höbaus et al. 2014, Norman and Powell 2014, Saccone, Asani et al. 2015)

Cardiovascular disease is the leading cause of mortality and morbidity in the United States.(Mozaffarian, Benjamin et al. 2016) Although recent data suggests there is a link between VDD and cardiovascular impairment (Wang, Pencina et al. 2008, Anderson, May et al. 2010, Masson, Agabiti et al. 2014), the association with

cardiovascular disease development is still not firmly established nor is it clear whether it contributes to adverse responses due to stress (e.g. air pollution exposure, exercise, psychosocial stress). Minimal sun exposure, improper nutrition as well as a myriad of other factors result in vitamin D deficiency (VDD) (Holick 2007, Lee, O'Keefe et al. 2008), which has become a global public health concern affecting 8% of the pediatric population in the United States.(Kumar, Muntner et al. 2009) Early-life or childhood VDD can lead to vascular dysfunction, hypertension and other cardiac abnormalities(Carlton-Conway, Tulloh et al. 2004, Maiya, Sullivan et al. 2008, Tare, Emmett et al. 2011), yet it's precise role in electrocardiographic abnormalities and cardiac autonomic and mechanical changes has not been extensively characterized.

Numerous animal and epidemiological studies demonstrate that VDD is associated with hypertension.(Li, Qiao et al. 2004, Martins, Wolf et al. 2007, Dobnig, Pilz et al. 2008, Giovannucci, Liu et al. 2008, Wang, Pencina et al. 2008, Argacha, Egrise et al. 2011, Nigwekar and Thadhani 2013, Weng, Sprague et al. 2013) Vitamin D has been shown to be a negative regulator of the renin-angiotensin-aldosterone system (RAAS). Previous studies have demonstrated that VDR null mice have continuous increased plasma renin expression, which results in increased plasma angiotensin II production and hypertension.(Li, Kong et al. 2002) In a study that evaluated the effect of VDD on blood pressure in rats, increases in systolic blood pressure were observed with oxidative stress in the vasculature which leads to downregulation of genes involved in antioxidant defense and myocardial function.(Argacha, Egrise et al. 2011) Similarly, activation of RAAS has also been demonstrated in hypertensive and atherosclerotic mouse models fed a VDD.(Weng, Sprague et al. 2013)

Transient Receptor Potential Canonical 6 (TRPC6) channels, a subfamily of TRP channels, are non-selective cation channels that are expressed in cardiomyocytes and vascular smooth muscle cells, among other tissue types, and respond to neurohormonal and mechanical stressors.(Rowell, Koitabashi et al. 2010, Loga, Domes et al. 2013, Wang, Ma et al. 2016) Increased expression of TRPC6 has previously been linked to cardiovascular disease, hypertrophy and hypertension.(Rowell, Koitabashi et al. 2010, Watanabe, Iino et al. 2013, Seo, Rainer et al. 2014) For example, mice treated with isoproterenol to induce cardiac stress and hypertrophy, similar to a hypertensive state, have increased TRPC6 mRNA expression in the heart.(Xie, Cha et al. 2012) TRPC6 is also speculated to form a protein complex with other TRPC channels in the heart, thereby altering cation flux and muscular contraction.(Xie, Cha et al. 2012, Seo, Rainer et al. 2014) However, previous studies suggest that inhibition of TRPC6 alone is sufficient for cardioprotection and TRPC6 knockout mice are without obvious impairments in function.(Xie, Cha et al. 2012) This data suggests that the limited expression of TRPC6 in the heart is normal but it's presence under conditions of cardiac stress is potentially detrimental.(Xie, Cha et al. 2012)

The TRPC6 antagonist used in the study is a *Grammastola spatulata* toxin that inhibits cationic mechanosensitive ion channels such as TRPC6 and is most effective during pathological stress.(Wang, Ma et al. 2016) Previous *in* vivo studies using ischemia-reperfusion injury have demonstrated that TRPC6 antagonist reduced infarct area, improved cardiac output and decreased arrhythmias. In addition, cultured mouse cardiomyocytes exposed to normal and hypoxic conditions provided evidence that

TRPC6 antagonism improved contractility and blocked apoptotic signaling mechanisms in the long-term, which may improve cardiomyocyte survival.(Wang, Ma et al. 2016)

Therefore, the purpose of this study was to determine the role of TRPC6 in VDD-induced cardiovascular mechanical properties and responses in adult mice. We hypothesized that adult mice with persistent VDD would have increased blood pressure when compared to normal mice. Furthermore, VDD would cause baseline mechanical changes in the heart which are mediated by TRPC6. For the purposes of these experiments, dobutamine, which is typically used to mimic cardiac stress of exercise was used to increase heart rate and contractility, and thereby reveal underlying changes in cardiovascular compensatory mechanisms which may not be evident otherwise.

Materials and Methods

Animals - Three-week old female C57Bl/6 mice were used in this study (Jackson Laboratory, Raleigh, NC). Mice were housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22˚C and 50% relative humidity in an AAA-LAC-approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum during the quarantine period (3 days) after arrival. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering.

Diet - Three days after the quarantine period ended, mice were maintained ad libitum on either a vitamin D deficient (VDD) (D10073001-Research Diets Inc.) or normal diet (ND) (D10012G-Research Diets Inc.) for 19 weeks. The VDD diet had no

added vitamin D but had vitamin mix V10037. The ND has 1000 IU per 10 grams of vitamin D. The diets had equal levels of all other vitamins and minerals including calcium, which was at the concentration specified by the American Institute of Nutrition (Reeves, Nielsen et al. 1993). Water was provided ad libitum throughout the diet regimen.

Experimental Design and Groups – At the beginning of the study, mice were randomly assigned into a ND (n = 20-25) or VDD (n = 20-25) group and maintained on those diets for the extent of the study. Of those animals, 9 of the ND and 9 of the VDD mice were randomly chosen and implanted with radiotelemeters at 22 weeks of age. The remaining mice were used for intraventricular assessments. The design is depicted in 4-1.

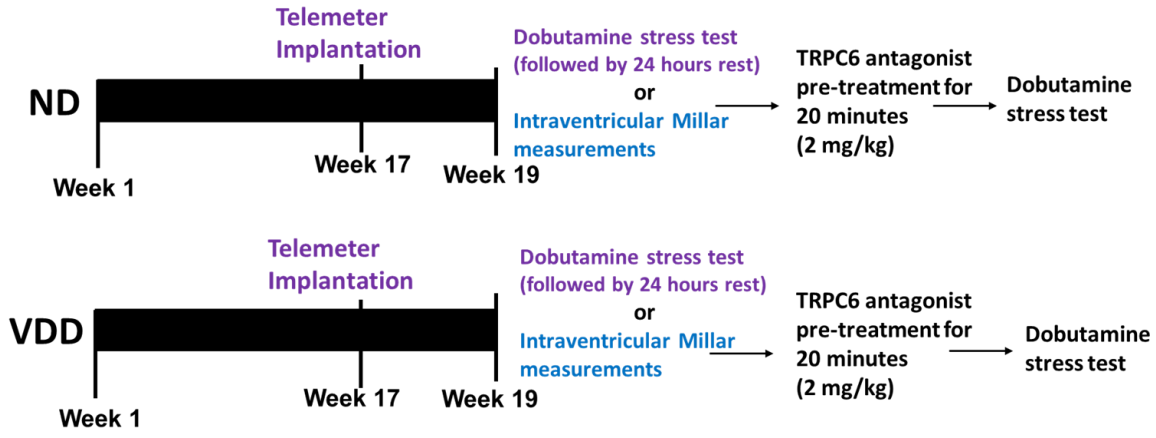


Figure 4-1. Experimental Design. ND and VDD mice were put on their respective diets for 19 weeks. A subset of mice, were implanted with radiotelemeters and allowed 7-10 days to recover and then underwent a dobutamine stress test with 2 doses (0.5 mg/kg (low) and 1.5 mg/kg (high). Twenty-four hours later the mice were pre-treated with a TRPC6 antagonist (2 mg/kg, i.p.) for 20 minutes prior to repeating the dobutamine stress test. The remaining mice were used for intraventricular measurements using a Millar pressure probe pre-treated with TRPC6 antagonist for 20 minutes prior to the dobutamine stress test. Upon confirming left ventricular placement of the pressure probe, baseline measurements were determined.

Drugs – GsMTx4 (2mg/kg, Tocris, Minneapolis, MN) was dissolved in saline. Two doses of freshly diluted dobutamine hydrochloride (Hospira, Lake Forest, IL) were administered at concentrations of 0.5 mg/kg (low dose) and 1.5 mg/kg (high dose). Previous studies describe increased HR after acute intraperitoneal administration of dobutamine.(Tyrankiewicz, Skorka et al. 2013, Puhl, Weeks et al. 2016)

Surgical Implantation of Radiotelemeters - Animals were anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin, OH). Anesthesia was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic buprenorphrine (0.03 mg/kg, i.p. manufacturer). Briefly, using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed 7-10 days to recover from the surgery and reestablish circadian rhythms (Kurhanewicz, McIntosh-Kastrinsky et al. 2014).

Radiotelemetery Data Acquistion - Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function

by monitoring heart rate (HR) and ECG waveforms immediately following telemeter implantation and through exposure until the end of exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 15 minutes during the 24 hours pre-experiment and immediately (imm.) before dobutamine stress test experiment periods and continuously during the experiment; HR was automatically obtained from the waveforms (Dataquest ART Software, version 4.0, Data Sciences International, St. Paul, MN). Baseline was defined as 4 hours before the start of dobutamine stress test.

Measurement of Intraventricular Pressure – Mice were anesthetized with urethane (1.5mg/kg intraperitoneally, Sigma) and then prepared for intraventricular measurements. While in a supine position and after achieving the appropriate anesthetic plane, the cervical region of the mouse was dissected exposing the right carotid artery. After isolation, the artery was catheterized with a 1.4 French transducer (SPR-671, ADInstruments, Colorado Springs, CO). The probe was connected via a Pressure Control Unit (PCU- 2000) to a receiver (Powerlab 8/30) and a computer acquiring data at 1000 Hz (LabChart Pro. 7.3.8) that is depicted in Figure 4-2. The probe was advanced while continuously monitoring the pressure trace until entry into the left ventricle was confirmed. Once in the left ventricle, a 4-min baseline was measured followed by dobutamine administration. Left ventricular pressure (LVP), dP/dTmax or the rate of left ventricular pressure rise and dP/dTmin or the rate of left ventricular pressure decrease were measured continuously during the baseline period,

dobutamine stress test and recovery. Adequate time was allowed for cardiac parameters to return to baseline between successive dobutamine doses.

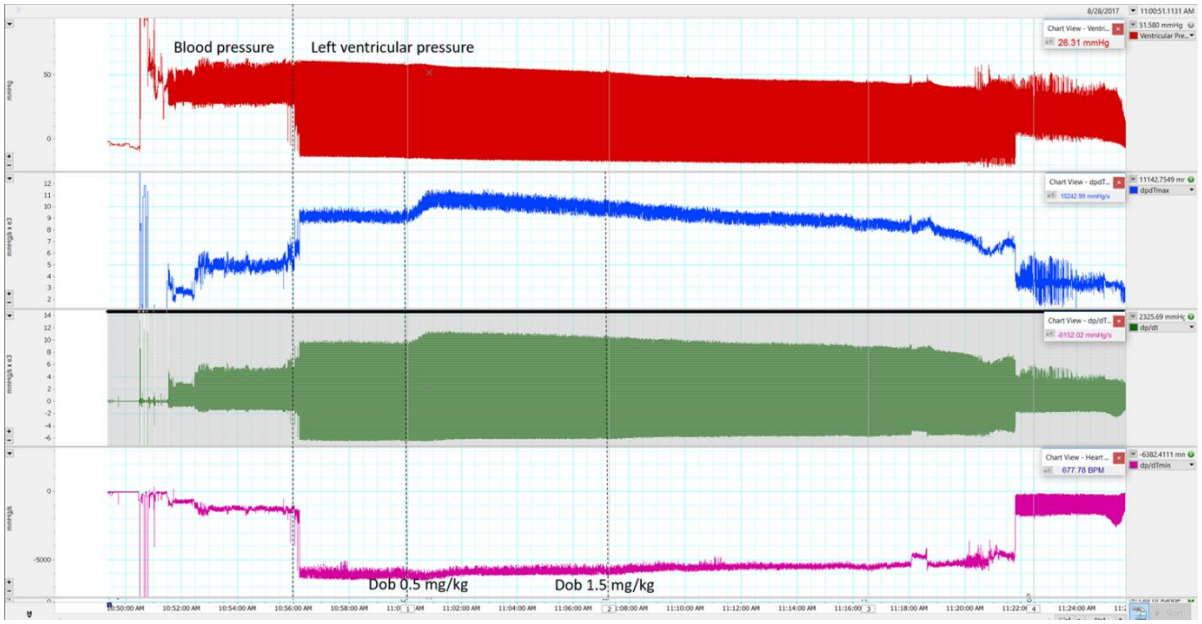


Figure 4-2. Representative example of intraventricular pressure measurements.

Heart Rate Variability Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RR intervals for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). HRV analysis was also conducted in the frequency domain using a Fast-Fourier transform. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions. The ratio of these two frequency

domains (LF/HF) provides an estimate of the relative balance between sympathetic (LF) and vagal (HF) activity.

Tissue Collection and Analysis – Radiotelemeter implanted mice were euthanized 24 hours after dobutamine stress test and blood collected, processed and analyzed. Vitamin D concentrations were determined in the serum spectro-photometrically using a Vitamin D EIA Kit (Cayman Chemical, Ann Arbor, Michigan) and previously published (Stratford, 2018).

Statistics - All data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) software. Mixed-model ANOVAs followed by Tukey's procedure for the post hoc comparisons were used to examine the statistical differences between TRPC6 antagonist and diet and repeated measures analysis was used as needed. The statistical significance was set at $P < 0.05$.

Results

Heart rate – Before the start of the dobutamine stress test, the baseline HR of ND and VDD mice was similar. Dobutamine caused an increase in the HR of ND mice, albeit not in a dose-dependent manner, and the response was significantly decreased in VDD mice. Treatment with the TRPC6 antagonist had no effect on the baseline HR of either diet group, however, it blocked the reduced response to dobutamine in VDD mice making it similar to ND. There was no effect of the drug on the dobutamine response of ND mice (Figure 4-3).

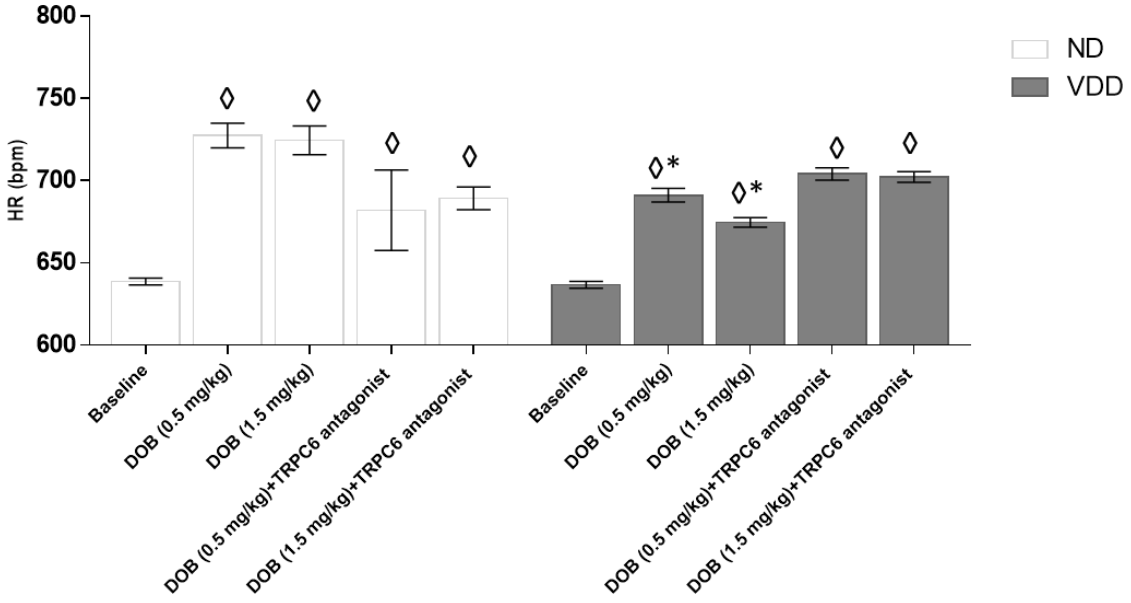


Figure 4-3. Impaired HR rate response to dobutamine in VDD mice is mediated by TRPC6. Dobutamine increased HR in ND and VDD mice from baseline but this response was significantly decreased in VDD mice. Pre-treatment with the TRPC6 antagonist restored the decreased response of VDD mice to dobutamine but had no effect in ND mice. *significantly different from ND ($p < 0.05$). ◊significantly different from baseline ($p < 0.05$) Values represent means ± SEM.

Heart rate variability – Before the dobutamine stress test, SDNN (which indicates overall autonomic function) and RMSSD, which indicates parasympathetic influence on the heart, as well as LF and HF which represent the balance between both autonomic branches and other inputs into the heart, were not significantly different between ND and VDD mice. Dobutamine caused a decrease in SDNN and LF only at the high dose in ND mice, but there were no effects on RMSSD or HF. In contrast, both low and high dobutamine caused a significant decrease in SDNN, RMSSD, LF and HF in VDD mice. Pretreatment of ND mice with the TRPC6 antagonist caused SDNN and LF to become further decreased during dobutamine challenge and caused RMSSD and HF to

decrease with the high dose. On the other hand, the TRPC6 antagonist had no effect on SDNN in VDD mice but blocked the decreases in LF and HF (Figure 4-4).

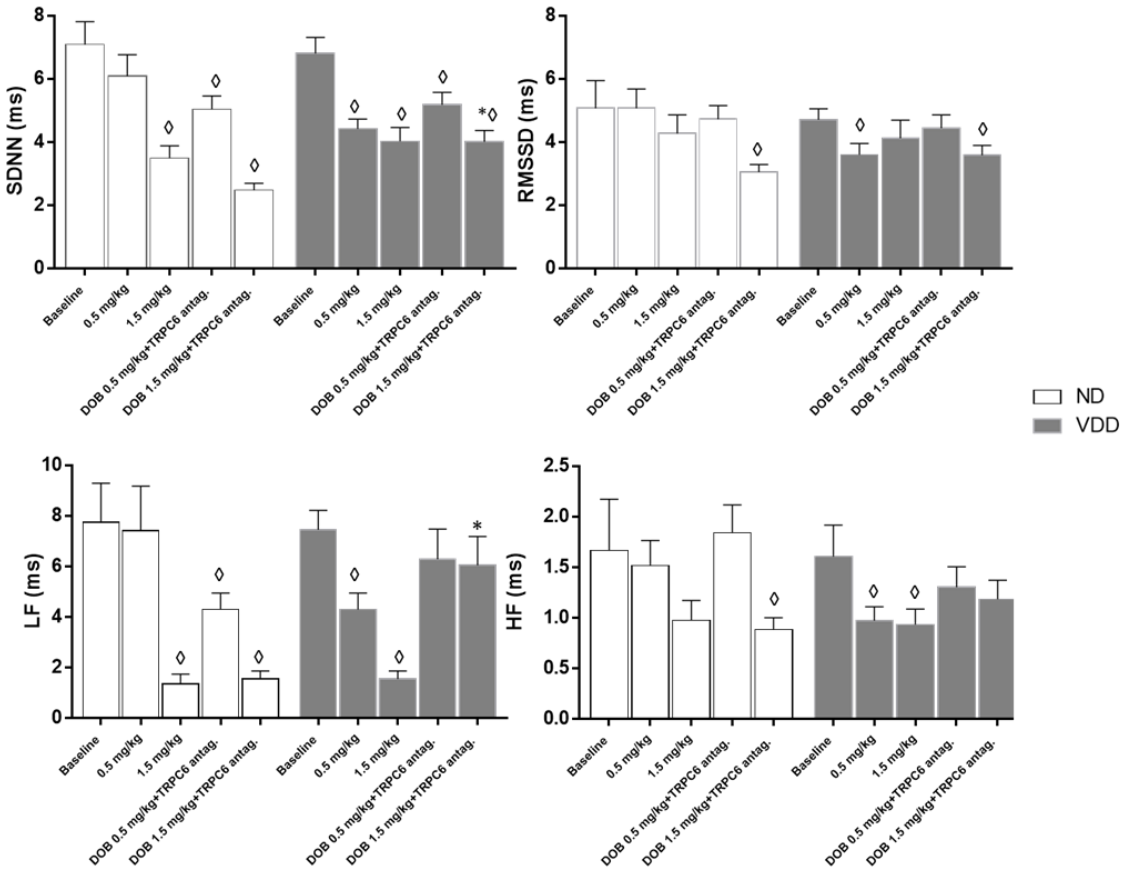


Figure 4-4. Vitamin D deficiency alters autonomic responses to dobutamine stress in mice. There were no differences in SDNN, RMSSD, LF or HF between ND and VDD mice at baseline. Dobutamine induced a significant decrease in only SDNN and LF at the high dose in ND mice, whereas, low and high dobutamine significant decreases in SDNN, RMSSD, LF and HF in VDD mice. Pretreatment of ND mice with TRPC6 antagonist induced SDNN and LF to further decrease during dobutamine stress test and RMSSD and HF significantly decreased with the high dose. On the other hand, TRPC6 antagonist had no effect on SDNN in VDD mice but blocked decrease in LF and HF. *significant change from ND ($p < 0.05$). ◊significantly different from baseline ($p < 0.05$) Values represent means ± SEM.

Blood pressure and baseline left ventricular function – There was no difference in the diastolic pressure between the diets (Figure 4-5A), however, VDD mice had

increased systolic pressure (Figure 4-5B) and mean arterial pressure (Figure 4-5C) when compared to ND. Similarly, baseline LVP was increased in VDD mice and the response was blocked by the TRPC6 antagonist (Figure 4-5D). The effect of the TRPC6 antagonist on blood pressure was not measured.

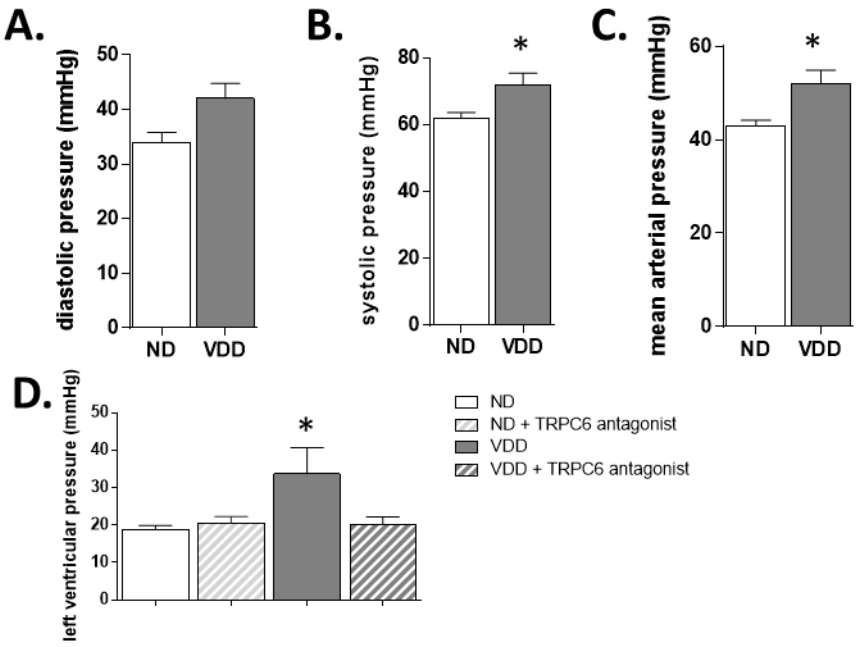


Figure 4-5. Vitamin D deficiency induces increased blood pressure and left ventricular pressure in mice. A.) There was no difference in the diastolic pressures of ND and VDD mice. However, systolic and mean arterial pressure were significantly increased in VDD when compared to controls **(B.** and **C.) D.)** Baseline LVP was significantly increased in VDD but blocked by TRPC6 antagonist. *significantly different from ND ($p < 0.05$). Values represent means ± SEM.

Left ventricular contractility and relaxation – Figure 4-6 shows the percent change in dP/dTmax and dP/dTmin from baseline during dobutamine challenge in ND and VDD mice. Low dobutamine increased dP/dTmax or contractility in ND mice, this was not significantly different for VDD mice nor did the TRPC6 antagonist have an effect on either diet (Figure 4-6A). High dobutamine also caused contractility to increase

in ND mice but not as much as the low dose. In contrast, VDD mice had a significantly decreased response to the high dose when compared to ND and this effect was blocked by the TRPC6 antagonist (Figure 4-6B). Although not statistically significant, VDD mice had a trend of decreased dP/dTmin or ventricular relaxation during the dobutamine challenge which was not apparent after TRPC6 blockade (Figure 4-6 C-D).

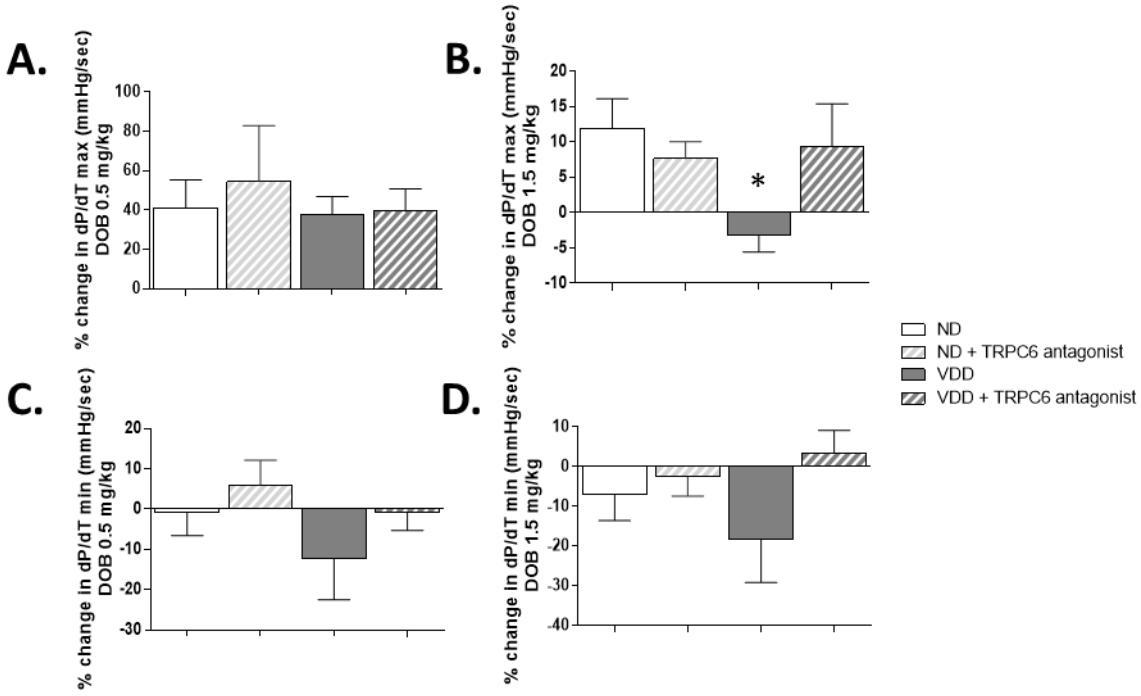


Figure 4-6. Vitamin D deficiency caused alterations in ventricular contractile function are mediated by TRPC6. A.) Low dobutamine caused increase in dP/dTmax in ND but there was no difference within VDD nor with TRPC6 pretreatment. **B.)** In contrast, VDD mice had significantly decreased responses to high dobutamine when compared to ND and the response was blocked by the TRPC6 antagonist. **C-D.)** VDD mice had a trend of decreased dP/dTmin during dobutamine stress test which was not apparent after TRPC6 blockade. *significantly different from ND ($p < 0.05$). Values represent means ± SEM.

Discussion

This study demonstrates that VDD-induced cardiovascular changes in mice are mediated by TRPC6. Persistent vitamin D deficiency can result in altered cardiovascular responses including autonomic imbalance and hypertension even in otherwise healthy adults.(Forman, Bischoff-Ferrari et al. 2005, Wang, Pencina et al. 2008, Canpolat, Özcan et al. 2015, Chen, Sun et al. 2015) Although chronic VDD was previously shown to increase the risk of hypertension, it was unknown whether there were any cardiac changes, either due to direct effects on the heart or as a result of the increased blood pressure. In this study, baseline cardiac electrical and mechanical function between normal and VDD mice was compared and a dobutamine stress test was used to uncover the presence of latent cardiac decrements due to VDD. Indeed, the results show that VDD causes increased blood pressure and altered cardiac contractile mechanics indicative of impairment and increased risk. Furthermore, this is the first study to demonstrate that VDD-induced mechanical changes in the heart are mediated by TRPC6, which is known to be involved in the worsening of cardiac hypertrophy.

Dobutamine is a β1-adrenergic agonist that causes increases in HR and contractility that can be compared to the cardiac effects of exercise.(Hazari, Callaway et al. 2012, Tyrankiewicz, Skorka et al. 2013, Puhl, Weeks et al. 2016) As such, dobutamine was used to unmask latent cardiac effects in mice due to VDD which otherwise may not be evident (i.e. during rest). Such tests are used clinically to evaluate the cardiac response to stress because an individual is suspected of increased risk of adverse cardiac events or having underlying cardiovascular disease, irrespective of the presence of symptoms or not.(Iyngkaran, Anavekar et al. 2017) Heart rate is an intrinsic and dynamic characteristic of the heart and therefore the degree to which it fluctuates

during sudden changes can be a profound indicator of cardiac distress or dysfunction. In this study, HR was not significantly different between normal and VDD mice at rest, however, the latter had decreased HR responses to both the low and high dose of dobutamine (i.e. VDD mice were unable to increase their HR as much as ND mice) when compared to normal mice. In a study of middle-aged men, blunted heart rate response to exercise was associated with increase cardiovascular mortality.(Savonen, Lakka et al. 2006) Furthermore, other pharmacological cardiac stress tests, for instance with dipyridamole, have shown the same heightened risk of cardiovascular complications when people had a blunted heart rate response,(Mathur, Shah et al. 2010) which in some cases may be due to under-recruitment of central neural pathways that regulate such responses.(Ginty, Gianaros et al. 2013) Chronotropic incompetence, which is the inability to raise heart rate commensurate with activity or exertion, can represent a serious deficit in the regulatory mechanisms that function to match heart rate with need for oxygen. Regardless, this condition likely occurs due to a change in the expression of β1-adrenergic receptors or autonomic balance.(Brubaker and Kitzman 2011) A study of myocardial cells demonstrated that normal *β*-adrenergic signal transduction may rely on the presence of vitamin D both locally (i.e. at the level of the heart) as well as centrally.(Santillan, Vazquez et al. 1999) The central influence of vitamin D is underscored by the high density of vitamin D receptors in the region of the brain where neurons of the autonomic nervous system are located.(Garcion, Wion-Barbot et al. 2002) In any case, the response observed here does not necessarily represent a toxicological effect in the heart, but it does signify that VDD impairs the heart's ability to respond normally to this stressor.

The blunted chronotropic effects of VDD were blocked by the TRPC6 antagonist given it restored responsive to dobutamine. Interestingly, the drug had no effect on resting heart rate in either normal or VDD mice. This suggests that the role of TRPC6 is related to neurohormonal or mechanical stress-induced cardiac changes,(Seo, Rainer et al. 2014) which do not necessarily occur under normal physiological circumstances but rather during pressure overload and oxidative stress. These latter conditions lead to increased influx of calcium into the myocyte and hence altered electrical-mechanical properties.(Koitabashi, Aiba et al. 2010) The fact that we did not see a dose-response to dobutamine or a more robust response, both with and without the drug, indicates that mouse sensitivity to this challenge is quite low.(Tyrankiewicz, Skorka et al. 2013) The TRPC6 antagonist used in this study (GsMTx4) prevents and raises the mechanical threshold for activation which appropriately explains the restoration of responsiveness to dobutamine.(Park, Kim et al. 2008, Xiao, Liu et al. 2017)

The chief physiological role of TRPC6 in the heart is calcium regulation, (Yamaguchi, Iribe et al. 2017) which contributes to pacemaker function (Ahmad, Streiff et al. 2017) and stretching of myocardium to allow for ejection of blood against mechanical stress(Yamaguchi, Iribe et al. 2017). An increase in TRPC6 expression even in the sinoatrial node and right atria(Ferdous, Qureshi et al. 2016) is associated with cardiovascular disease,(Rowell, Koitabashi et al. 2010, Loga, Domes et al. 2013, Watanabe, Iino et al. 2013) myocardial infarction(Hang, Zhao et al. 2015) and is the preliminary event of subsequent heart failure(Yamaguchi, Iribe et al. 2017) which contributes to its pathological role. Due to increased TRPC6, calcium and subsequent activation of calcineurin is also increased.(Tomilin, Mamenko et al. 2016) Calcineurin is

a calcium/calmodulin-dependent serine/threonine protein phosphatase(Bandyopadhyay, Lee et al. 2002) and is activated as a result of mechanical stress and sustained increased intracellular calcium levels(Yamaguchi, Iribe et al. 2017). Calcineurin activates NFAT (nuclear factor of activated T-cells) which is a transcription factor that further increases TRPC6 mRNA expression and calcium signaling.(Tomilin, Mamenko et al. 2016) In fact, HEK cells overexpressing TRPC6 inhibit Na+, K+-ATPase (membrane protein that facilitates transepithelial calcium transport) (Razzaque 2008) to decrease calcium signaling as a result of TRPC6 activation and reduce TRPC6 protein expression.(Chauvet, Boonen et al. 2015) Increased calcium handling and TRPC6 expression also increases calcium sensing receptor expression and consequently shown to be reversed by vitamin D supplementation.(Bernichtein, Pigat et al. 2017) As far as our model is concerned, it is unclear whether vitamin D supplementation would reverse the effect of increased TRPC6 mRNA expression on the electrical and mechanical function of the heart, whether through the pathway described above or otherwise. Calcium is the key mediator in VDD-induced cardiovascular dysfunction and increased TRPC6 expression. Future studies need to address the role of calcium signaling because it appears to be the key signaling ion in VDD-induced cardiovascular dysfunction and increased TRPC6 expression.

Assessment of heart rate variability also revealed some differences between ND and VDD mice. This is not surprising given HRV is influenced not only by HR but also blood pressure and respiratory patterns.(Tsuji, Larson et al. 1996) Our previous studies have shown that HRV decreased during dobutamine stress test in the rat.(Hazari, Callaway et al. 2012) The current data indicate that the same is true for mice,

regardless of whether they are normal or VDD, albeit with variable sensitivity to dobutamine. SDNN and LF, both of which indicate sympathetic modulation of the heart, appeared to be more decreased during dobutamine challenge in VDD mice than normal. A similar trend was observed with RMSSD and HF, which reflect parasympathetic modulation. It is not surprising that the VDD-induced LF and HF (i.e. frequency domain parameters) changes during dobutamine were blocked by the TRPC6 antagonist because they represent blood pressure regulation and cyclical breathing patterns, respectively(Shaffer and Ginsberg 2017), both of these parameters are known to be altered by VDD. Similar to the HR response, blockade of TRPC6 had no effect on any HRV parameter at rest, which supports the contention that physiological changes (i.e. blood pressure and breathing rates) during a stress challenge could mediate the decreases in LF and HF. Another possible explanation for the efficacy of the TRPC6 antagonist in blocking the LF and HF in VDD mice is the role TRPC6 plays in maintaining mechanical stress-induced vascular tone(Inoue, Jensen et al. 2009), which has a direct impact on blood pressure and therefore the variability in heart rate.

Experimental and epidemiological studies have linked VDD to hypertension in people(Li, Kong et al. 2002, Li, Qiao et al. 2004, Scragg, Sowers et al. 2007, Judd, Nanes et al. 2008, Carbone, Mach et al. 2014, Zhang, Xu et al. 2017) and based on this data the same is true for mice. The blood pressure and assessments were performed under anesthesia, thus, blood pressure was lower than the normal range of a conscious mouse. Low vitamin D levels impact systolic blood pressure in particular because vitamin D increases the activity of endothelial nitric oxide synthase, which leads to the synthesis of nitric oxide (NO) and subsequent vasodilation.(Talmor, Golan et al. 2008)

Another study showed that vitamin D inhibits the release of vasoconstrictor mediators which tend to increase in the absence of NO.(Wong, Delansorne et al. 2010) On the other hand, we cannot discount the effects of vitamin D on RAAS, which numerous studies have demonstrated is upregulated in the absence of vitamin D.(Tamez, Kalim et al. 2013) Yet, with respect to TRPC6, although the antagonist appeared to block the increased blood pressure in VDD mice (not shown), such a response is not conclusive because we had to make the measurement after dobutamine challenge and withdrawal of the probe from the left ventricle. It is conceivable that TRPC6 plays a role in the VDD-induced hypertension because its expression in vascular smooth muscle in humans and rodents is associated with higher blood pressure.(Zou, Xu et al. 2015) Additionally, it has been demonstrated that angiotensin II is activated in response to mechanical stress, and increased calcium and TRPC6 expression.(Santillan, Vazquez et al. 1999, Hang, Zhao et al. 2015) The relationship of VDD and hypertension likely involves the RAAS but further examination of the hormones involved in the pathway and in this paradigm is needed.

One of the many deleterious effects of high blood pressure is the impairment of left ventricular function. This can lead to altered contractile mechanics and a loss of compensatory capacity, particularly when the body is trying to match cardiac output with increased demand. To our knowledge, there is no previous study describing the effects of VDD on cardiac mechanical function, nor is there any indication whether decrements in the heart are due to intrinsic changes or a consequence of higher blood pressure. Indeed, VDD mice had increased LVP at rest which offers some insight into how the condition might contribute to the development of cardiovascular disease. As mentioned

above, these changes may be due to altered vascular tone because the heart needs to consistently develop a higher pressure in order to “push” blood out into the vasculature. Some evidence points to the uncoupling of NOS and the resulting increase in vascular smooth muscle contraction, particularly in the veins, as a cause of increased LVP.(Knuckles, Lund et al. 2008) Regardless, elevated baseline LVP can predispose the heart to arrhythmia and even failure in the long-term due to hypertrophy.(Lalande and Johnson 2010) It also alters the degree to which the heart can increase its contractility during periods of stress like the conditions created by dobutamine.

The fact that blocking TRPC6 attenuated the increased LVP in VDD mice points to a cardiac phenomenon in which the heart’s intrinsic contractile properties have changed. Xie et al. showed that stress-induced modification of pressure dynamics is mediated by TRPC6 and can be blocked by the putative anti-aging protein klotho, which consequently mediates the effects of vitamin D.(Xie, Cha et al. 2012) This might explain what we observed in the VDD mice because TRPC6 facilitates greater influx of calcium into cardiac myocytes during prolonged mechanical stress(Nishida, Watanabe et al. 2010), which is often a sequelae of hypertension.(Lin, Chang et al. 2014)

As a result, cardiac contractility was significantly decreased in VDD mice when challenged with high dobutamine and was restored by the TRPC6 antagonist, possibly due to the restoration of a normal LVP. In contrast, lusitropy was not significantly decreased in VDD mice during the challenge although there was a trend towards this and recovery with blockade of TRPC6. To determine whether hypertrophy played a role in these responses, heart weight was normalized to tibia length and determined to be the same for normal and VDD mice indicating that ventricular remodeling and

hypertrophy were not yet apparent (data not shown). However, it is unclear if VDD mice would eventually develop ventricular remodeling and hypertrophy following a much more extended diet regimen.

Conclusions

Increased left ventricular pressure was observed in VDD mice and blocked by the TRPC6 antagonist. Although diastolic pressure was not statistically significant there was a trend of VDD mice having elevated diastolic pressure. Therefore, taking into account increased left ventricular, systolic and mean arterial pressure it is certainly likely that VDD mice exhibit signs of essential hypertension.(Frohlich and Susic 2012) Hypertension is a risk factor for heart failure because it can cause pressure overload and when sustained can lead to ventricular remodeling and hypertrophy.(Frohlich and Susic 2012)

In any case, the HR, pressure and contractility changes due to VDD are independently significant indicators of increased cardiac risk but when combined demonstrate that VDD mice have impaired mechanical cardiac function.(Achinger and Ayus 2005, Frohlich and Susic 2012) A wide variety of TRP sub-family members respond to calcium influx and are modulated in cardiovascular disease.(Rowell, Koitabashi et al. 2010, Watanabe, Iino et al. 2013, Yue, Xie et al. 2015) Although more work is still needed to determine the complete mechanism behind VDD-induced cardiovascular dysfunction, this study points to the role TRPC6 as a potential mediator and therapeutic target of VDD-induced cardiovascular mechanical dysfunction. Therapeutic intervention of TRPC6 in renal fibrosis (Wu, Xie et al. 2017) and pulmonary hypertension (Malczyk, Erb et al. 2017) is currently being evaluated; thus, inhibition of TRPC6 expression could also be examined with respect to cardiovascular disease.

Further studies will need to focus on determining the role of calcium signaling in cardiac and vascular cells regulated by vitamin D. Regardless, these data contribute to our understanding of VDD, particularly if it persists from childhood to adulthood, and the specific areas of public health concern and intervention (e.g. hypertension versus cardiac dysfunction).

REFERENCES

Achinger, S. G. and J. C. Ayus (2005). "The role of vitamin D in left ventricular hypertrophy and cardiac function." Kidney International **67**: S37-S42.

Ahmad, A. A., M. Streiff, C. Hunter, Q. Hu and F. B. Sachse (2017). "Physiological and pathophysiological role of transient receptor potential canonical channels in cardiac myocytes." Prog Biophys Mol Biol **130**(Pt B): 254-263.

Anderson, J. L., H. T. May, B. D. Horne, T. L. Bair, N. L. Hall, J. F. Carlquist, D. L. Lappé and J. B. Muhlestein (2010). "Relation of Vitamin D Deficiency to Cardiovascular Risk Factors, Disease Status, and Incident Events in a General Healthcare Population." The American Journal of Cardiology **106**(7): 963-968.

Argacha, J. F., D. Egrise, S. Pochet, D. Fontaine, A. Lefort, F. Libert, S. Goldman, P. van de Borne, G. Berkenboom and R. Moreno-Reyes (2011). "Vitamin D deficiency-induced hypertension is associated with vascular oxidative stress and altered heart gene expression." J Cardiovasc Pharmacol **58**(1): 65-71.

Bandyopadhyay, J., J. Lee, J. Lee, J. I. Lee, J.-R. Yu, C. Jee, J.-H. Cho, S. Jung, M. H. Lee, S. Zannoni, A. Singson, D. H. Kim, H.-S. Koo and J. Ahnn (2002). "Calcineurin, a Calcium/Calmodulin-dependent Protein Phosphatase, Is Involved in Movement, Fertility, Egg Laying, and Growth in Caenorhabditis elegans." Molecular Biology of the Cell **13**(9): 3281-3293.

Bernichtein, S., N. Pigat, N. Barry Delongchamps, F. Boutillon, V. Verkarre, P. Camparo, E. Reyes-Gomez, A. Mejean, S. M. Oudard, E. M. Lepicard, M. Viltard, J. C. Souberbielle, G. Friedlander, T. Capiod and V. Goffin (2017). "Vitamin D3 Prevents Calcium-Induced Progression of Early-Stage Prostate Tumors by Counteracting TRPC6 and Calcium Sensing Receptor Upregulation." Cancer Res **77**(2): 355-365.

Brubaker, P. H. and D. W. Kitzman (2011). "Chronotropic incompetence: causes, consequences, and management." Circulation **123**(9): 1010-1020.

Canpolat, U., F. Özcan, Ö. Özeke, O. Turak, Ç. Yayla, S. K. Açıkgöz, S. Çay, S. Topaloğlu, D. Aras and S. Aydoğdu (2015). "Impaired Cardiac Autonomic Functions in Apparently Healthy Subjects with Vitamin D Deficiency." Annals of Noninvasive Electrocardiology **20**(4): 378-385.

Carbone, F., F. Mach, N. Vuilleumier and F. Montecucco (2014). "Potential pathophysiological role for the vitamin D deficiency in essential hypertension." World J Cardiol **6**(5): 260-276.

Carlton-Conway, D., R. Tulloh, L. Wood and D. Kanabar (2004). "Vitamin D deficiency and cardiac failure in infancy." Journal of the Royal Society of Medicine **97**(5): 238-239.

Chauvet, S., M. Boonen, M. Chevallet, L. Jarvis, A. Abebe, M. Benharouga, P. Faller, M. Jadot and A. Bouron (2015). "The Na+/K+-ATPase and the amyloid-beta peptide abeta1-40 control the cellular distribution, abundance and activity of TRPC6 channels." Biochim Biophys Acta **1853**(11 Pt A): 2957-2965.

Chen, S., Y. Sun and D. K. Agrawal (2015). "Vitamin D deficiency and essential hypertension." J Am Soc Hypertens **9**(11): 885-901.

Dobnig, H., S. Pilz, H. Scharnagl, W. Renner, U. Seelhorst, B. Wellnitz, J. Kinkeldei, B. O. Boehm, G. Weihrauch and W. Maerz (2008). "Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality." Arch Intern Med **168**(12): 1340-1349.

Eke, I. G., C. Igwe and A. O. Anaga (2014). "Vitamin D supplementation attenuated carageenan-induced paw edema and formalin-induced nociception in indomethacin-treated rats." Int J Basic Clin Pharmacol **3**(1): 206-210.

Ferdous, Z., M. A. Qureshi, P. Jayaprakash, K. Parekh, A. John, M. Oz, H. Raza, H. Dobrzynski, T. E. Adrian and F. C. Howarth (2016). "Different Profile of mRNA Expression in Sinoatrial Node from Streptozotocin-Induced Diabetic Rat." PLoS One **11**(4): e0153934.

Fetahu, I. S., J. Höbaus and E. Kállay (2014). "Vitamin D and the epigenome." Frontiers in Physiology **5**: 164.

Forman, J. P., H. A. Bischoff-Ferrari, W. C. Willett, M. J. Stampfer and G. C. Curhan (2005). "Vitamin D intake and risk of incident hypertension: results from three large prospective cohort studies." Hypertension **46**(4): 676-682.

Frohlich, E. D. and D. Susic (2012). "Pressure overload." Heart Fail Clin **8**(1): 21-32.
Garcion, E., N. Wion-Barbot, C. N. Montero-Menei, F. Berger and D. Wion (2002). "New clues about vitamin D functions in the nervous system." Trends Endocrinol Metab **13**(3): 100-105.

Ginty, A. T., P. J. Gianaros, S. W. Derbyshire, A. C. Phillips and D. Carroll (2013). "Blunted cardiac stress reactivity relates to neural hypoactivation." Psychophysiology **50**(3): 219-229.

Giovannucci, E., Y. Liu, B. W. Hollis and E. B. Rimm (2008). "25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study." Arch Intern Med **168**(11): 1174-1180.

Hang, P., J. Zhao, B. Cai, S. Tian, W. Huang, J. Guo, C. Sun, Y. Li and Z. Du (2015). "Brain-derived neurotrophic factor regulates TRPC3/6 channels and protects against myocardial infarction in rodents." Int J Biol Sci **11**(5): 536-545.

Hazari, M. S., J. Callaway, D. W. Winsett, C. Lamb, N. Haykal-Coates, Q. Krantz, C. King, D. L. Costa and A. K. Farraj (2012). "Dobutamine" stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats." Environmental health perspectives **120**(8): 1088-1093.

Holick, M. F. (2007). "Vitamin D Deficiency." New England Journal of Medicine **357**(3): 266-281.

Inoue, R., L. J. Jensen, Z. Jian, J. Shi, L. Hai, A. I. Lurie, F. H. Henriksen, M. Salomonsson, H. Morita, Y. Kawarabayashi, M. Mori, Y. Mori and Y. Ito (2009). "Synergistic activation of vascular TRPC6 channel by receptor and mechanical stimulation via phospholipase C/diacylglycerol and phospholipase A2/omega-hydroxylase/20-HETE pathways." Circ Res **104**(12): 1399-1409.

Iyngkaran, P., N. S. Anavekar, C. Neil, L. Thomas and D. L. Hare (2017). "Shortness of breath in clinical practice: A case for left atrial function and exercise stress testing for a comprehensive diastolic heart failure workup." World J Methodol **7**(4): 117-128.

Judd, S. E., M. S. Nanes, T. R. Ziegler, P. W. Wilson and V. Tangpricha (2008). "Optimal vitamin D status attenuates the age-associated increase in systolic blood pressure in white Americans: results from the third National Health and Nutrition Examination Survey." Am J Clin Nutr **87**(1): 136-141.

Knuckles, T. L., A. K. Lund, S. N. Lucas and M. J. Campen (2008). "Diesel exhaust exposure enhances venoconstriction via uncoupling of eNOS." Toxicol Appl Pharmacol **230**(3): 346-351.

Koitabashi, N., T. Aiba, G. G. Hesketh, J. Rowell, M. Zhang, E. Takimoto, G. F. Tomaselli and D. A. Kass (2010). "Cyclic GMP/PKG-dependent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAT activation Novel mechanism of cardiac stress modulation by PDE5 inhibition." J Mol Cell Cardiol **48**(4): 713-724.

Kumar, J., P. Muntner, F. J. Kaskel, S. M. Hailpern and M. L. Melamed (2009). "Prevalence and Associations of 25-Hydroxyvitamin D Deficiency in US Children: NHANES 2001–2004." Pediatrics **124**(3): e362-e370.

Lalande, S. and B. D. Johnson (2010). "Breathing strategy to preserve exercising cardiac function in patients with heart failure." Med Hypotheses **74**(3): 416-421.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D Deficiency: An Important, Common, and Easily Treatable Cardiovascular Risk Factor?" Journal of the American College of Cardiology **52**(24): 1949-1956.

Li, Y. C., J. Kong, M. Wei, Z. F. Chen, S. Q. Liu and L. P. Cao (2002). "1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system." J Clin Invest **110**(2): 229-238.

Li, Y. C., G. Qiao, M. Uskokovic, W. Xiang, W. Zheng and J. Kong (2004). "Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure." J Steroid Biochem Mol Biol **89-90**(1-5): 387-392.

Lin, J. W., C. H. Chang, J. L. Caffrey, L. C. Wu and M. S. Lai (2014). "Examining the association of olmesartan and other angiotensin receptor blockers with overall and cause-specific mortality." Hypertension **63**(5): 968-976.

Lips, P. (2006). "Vitamin D physiology." Progress in biophysics and molecular biology **92**(1): 4-8.

Loga, F., K. Domes, M. Freichel, V. Flockerzi, A. Dietrich, L. Birnbaumer, F. Hofmann and J. W. Wegener (2013). "The role of cGMP/cGKI signalling and Trpc channels in regulation of vascular tone." Cardiovasc Res **100**(2): 280-287.

Maiya, S., I. Sullivan, J. Allgrove, R. Yates, M. Malone, C. Brain, N. Archer, Q. Mok, P. Daubeney, R. Tulloh and M. Burch (2008). "Hypocalcaemia and vitamin D deficiency: an important, but preventable, cause of life-threatening infant heart failure." Heart **94**(5): 581-584.

Malczyk, M., A. Erb, C. Veith, H. A. Ghofrani, R. T. Schermuly, T. Gudermann, A. Dietrich, N. Weissmann and A. Sydykov (2017). "The Role of Transient Receptor Potential Channel 6 Channels in the Pulmonary Vasculature." Front Immunol **8**: 707.

Martins, D., M. Wolf, D. Pan and et al. (2007). "Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin d in the united states: Data from the third national health and nutrition examination survey." Archives of Internal Medicine **167**(11): 1159-1165.

Masson, S., N. Agabiti, T. Vago, M. Miceli, F. Mayer, T. Letizia, U. Wienhues-Thelen, G. F. Mureddu, M. Davoli, A. Boccanelli, R. Latini and P. s. The Investigators of the (2014). "The fibroblast growth factor-23 and Vitamin D emerge as nontraditional risk factors and may affect cardiovascular risk." Journal of Internal Medicine: n/a-n/a.

Mathur, S., A. R. Shah, A. W. Ahlberg, D. M. Katten and G. V. Heller (2010). "Blunted heart rate response as a predictor of cardiac death in patients undergoing vasodilator stress technetium-99m sestamibi gated SPECT myocardial perfusion imaging." J Nucl Cardiol **17**(4): 617-624.

Mozaffarian, D., E. J. Benjamin, A. S. Go, D. K. Arnett, M. J. Blaha, M. Cushman, S. R. Das, S. de Ferranti, J. P. Despres, H. J. Fullerton, V. J. Howard, M. D. Huffman, C. R. Isasi, M. C. Jimenez, S. E. Judd, B. M. Kissela, J. H. Lichtman, L. D. Lisabeth, S. Liu, R.

H. Mackey, D. J. Magid, D. K. McGuire, E. R. Mohler, 3rd, C. S. Moy, P. Muntner, M. E. Mussolino, K. Nasir, R. W. Neumar, G. Nichol, L. Palaniappan, D. K. Pandey, M. J. Reeves, C. J. Rodriguez, W. Rosamond, P. D. Sorlie, J. Stein, A. Towfighi, T. N. Turan, S. S. Virani, D. Woo, R. W. Yeh and M. B. Turner (2016). "Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association." Circulation **133**(4): e38-360.

Nigwekar, S. U. and R. Thadhani (2013). "Vitamin D receptor activation: cardiovascular and renal implications." Kidney Int Suppl (2011) **3**(5): 427-430.

Nishida, M., K. Watanabe, M. Nakaya and H. Kurose (2010). "[Mechanism of cardiac hypertrophy via diacylglycerol-sensitive TRPC channels]." Yakugaku Zasshi **130**(3): 295-302.

Norman, P. E. and J. T. Powell (2014). "Vitamin D and Cardiovascular Disease." Circulation Research **114**(2): 379-393.

Park, S. P., B. M. Kim, J. Y. Koo, H. Cho, C. H. Lee, M. Kim, H. S. Na and U. Oh (2008). "A tarantula spider toxin, GsMTx4, reduces mechanical and neuropathic pain." Pain **137**(1): 208-217.

Puhl, S. L., K. L. Weeks, A. Ranieri and M. Avkiran (2016). "Assessing structural and functional responses of murine hearts to acute and sustained beta-adrenergic stimulation in vivo." J Pharmacol Toxicol Methods **79**: 60-71.

Razzaque, M. S. (2008). "Klotho and Na+,K+-ATPase activity: solving the calcium metabolism dilemma?" Nephrol Dial Transplant **23**(2): 459-461.

Rowell, J., N. Koitabashi and D. A. Kass (2010). "TRP-ing up heart and vessels: canonical transient receptor potential channels and cardiovascular disease." J Cardiovasc Transl Res **3**(5): 516-524.

Saccone, D., F. Asani and L. Bornman (2015). "Regulation of the vitamin D receptor gene by environment, genetics and epigenetics." Gene **561**(2): 171-180.

Santillan, G. E., G. Vazquez and R. L. Boland (1999). "Activation of a beta-adrenergic-sensitive signal transduction pathway by the secosteroid hormone 1,25-(OH)2-vitamin D3 in chick heart." J Mol Cell Cardiol **31**(5): 1095-1104.

Savonen, K. P., T. A. Lakka, J. A. Laukkanen, P. M. Halonen, T. H. Rauramaa, J. T. Salonen and R. Rauramaa (2006). "Heart rate response during exercise test and cardiovascular mortality in middle-aged men." Eur Heart J **27**(5): 582-588.

Scragg, R., M. Sowers and C. Bell (2007). "Serum 25-hydroxyvitamin D, ethnicity, and blood pressure in the Third National Health and Nutrition Examination Survey." Am J Hypertens **20**(7): 713-719.

Seo, K., P. P. Rainer, V. Shalkey Hahn, D. I. Lee, S. H. Jo, A. Andersen, T. Liu, X. Xu, R. N. Willette, J. J. Lepore, J. P. Marino, Jr., L. Birnbaumer, C. G. Schnackenberg and D. A. Kass (2014). "Combined TRPC3 and TRPC6 blockade by selective small-molecule or genetic deletion inhibits pathological cardiac hypertrophy." Proc Natl Acad Sci U S A **111**(4): 1551-1556.

Shaffer, F. and J. P. Ginsberg (2017). "An Overview of Heart Rate Variability Metrics and Norms." Front Public Health **5**: 258.

Sundar, I. K. and I. Rahman (2011). "Vitamin D and Susceptibility of Chronic Lung Diseases: Role of Epigenetics." Frontiers in Pharmacology **2**: 50.

Talmor, Y., E. Golan, S. Benchetrit, J. Bernheim, O. Klein, J. Green and G. Rashid (2008). "Calcitriol blunts the deleterious impact of advanced glycation end products on endothelial cells." Am J Physiol Renal Physiol **294**(5): F1059-1064.

Tamez, H., S. Kalim and R. I. Thadhani (2013). "Does vitamin D modulate blood pressure?" Curr Opin Nephrol Hypertens **22**(2): 204-209.

Tare, M., S. J. Emmett, H. A. Coleman, C. Skordilis, D. W. Eyles, R. Morley and H. C. Parkington (2011). "Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats." J Physiol **589**(Pt 19): 4777-4786.

Tomilin, V., M. Mamenko, O. Zaika and O. Pochynyuk (2016). "Role of renal TRP channels in physiology and pathology." Semin Immunopathol **38**(3): 371-383.

Tsuji, H., M. G. Larson, F. J. Venditti, Jr., E. S. Manders, J. C. Evans, C. L. Feldman and D. Levy (1996). "Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study." Circulation **94**(11): 2850-2855.

Tyrankiewicz, U., T. Skorka, M. Jablonska, P. Petkow-Dimitrow and S. Chlopicki (2013). "Characterization of the cardiac response to a low and high dose of dobutamine in the mouse model of dilated cardiomyopathy by MRI in vivo." J Magn Reson Imaging **37**(3): 669-677.

Wang, J., Y. Ma, F. Sachs, J. Li and T. M. Suchyna (2016). "GsMTx4-D is a cardioprotectant against myocardial infarction during ischemia and reperfusion." J Mol Cell Cardiol **98**: 83-94.

Wang, T. J., M. J. Pencina, S. L. Booth, P. F. Jacques, E. Ingelsson, K. Lanier, E. J. Benjamin, R. B. D'Agostino, M. Wolf and R. S. Vasan (2008). "Vitamin D deficiency and risk of cardiovascular disease." Circulation **117**(4): 503-511.

Watanabe, H., K. Iino, T. Ohba and H. Ito (2013). "Possible involvement of TRP channels in cardiac hypertrophy and arrhythmia." Curr Top Med Chem **13**(3): 283-294.

Weng, S., J. E. Sprague, J. Oh, A. E. Riek, K. Chin, M. Garcia and C. Bernal-Mizrachi (2013). "Vitamin D deficiency induces high blood pressure and accelerates atherosclerosis in mice." PLoS One **8**(1): e54625.

Wong, M. S., R. Delansorne, R. Y. Man, P. Svenningsen and P. M. Vanhoutte (2010). "Chronic treatment with vitamin D lowers arterial blood pressure and reduces endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat." Am J Physiol Heart Circ Physiol **299**(4): H1226-1234.

Wu, Y. L., J. Xie, S. W. An, N. Oliver, N. X. Barrezueta, M. H. Lin, L. Birnbaumer and C. L. Huang (2017). "Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho." Kidney Int **91**(4): 830-841.

Xiao, X., H. X. Liu, K. Shen, W. Cao and X. Q. Li (2017). "Canonical Transient Receptor Potential Channels and Their Link with Cardio/Cerebro-Vascular Diseases." Biomol Ther (Seoul) **25**(5): 471-481.

Xie, J., S. K. Cha, S. W. An, O. M. Kuro, L. Birnbaumer and C. L. Huang (2012). "Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart." Nat Commun **3**: 1238.

Yamaguchi, Y., G. Iribe, M. Nishida and K. Naruse (2017). "Role of TRPC3 and TRPC6 channels in the myocardial response to stretch: Linking physiology and pathophysiology." Prog Biophys Mol Biol **130**(Pt B): 264-272.

Yue, Z., J. Xie, A. S. Yu, J. Stock, J. Du and L. Yue (2015). "Role of TRP channels in the cardiovascular system." American Journal of Physiology - Heart and Circulatory Physiology **308**(3): H157-H182.

Zhang, M., X. Xu, H. Liu, H. Li, J. Zhang and M. Gao (2017). "Nocturnal diastolic blood pressure decline is associated with higher 25-hydroxyvitamin D level and standing plasma renin activity in a hypertensive population." Clinical and Experimental Hypertension **39**(8): 685-690.

Zou, Z. Q., J. Xu, L. Li and Y. S. Han (2015). "Down-regulation of SENCR promotes smooth muscle cells proliferation and migration in db/db mice through up-regulation of FoxO1 and TRPC6." Biomed Pharmacother **74**: 35-41.

Chapter 5: Significance, Conclusions, Future Directions and Implications

Significance

Ambient air pollution levels continue to be elevated in certain parts of the world and significantly contribute to increased hospitalizations and mortality. Fortunately, these levels have drastically decreased in developed countries like the U.S. in the past three decades. However, millions of Americans are still living in areas that meet or exceed federal regulatory standards for certain pollutants. Thus, air pollution is still an environmental public health concern despite the many improvements in technology, use of alternative fuels and individual awareness. In addition, susceptible populations and individuals with pre-existing cardiovascular conditions (e.g. elderly, hypertensive, metabolic-syndrome) have more recently been identified as especially sensitive to the adverse health effects of air pollution exposure. (Brook, Rajagopalan et al. 2010) This is particularly alarming because there are so many potential factors ranging from genetic predisposition to lifestyle to exposure to non-chemical environmental stressors, which can modify the development of disease and hence the body's ability to preserve normal function through changing conditions. In 2016, the AHA, in conjunction with several federal agencies, reported that heart disease is a significant national public health and economic concern and that nutrition was a considerable risk factor for early death or disability. In fact, 678,000 deaths, regardless of the cause of mortality, in 2010 were attributed to poor nutrition. (Mozaffarian, Benjamin et al. 2016)

Adverse health effects due to air pollution have been studied for well over half a century and these investigations are still relevant today because extreme episodes (e.g. wildfires) continue to occur each year. The initial study described in this dissertation examined whether persistent VDD in adult mice altered the cardiac response to atmospheric photochemical smog, which is a relevant multipollutant mixture similar in composition to the ambient air pollution found in many urban centers in the United States. The importance of this initial study is underscored by the fact that millions of people across the world suffer from VDD and many of them are routinely exposed to smog, increasing the risk of potential adverse cardiac responses. Our previous studies demonstrated that a smog atmosphere comprised of high ozone (and other gases) and low PM was more cardiotoxic to normal mice than one that had high PM and low ozone. (Hazari and Stratford 2018) Therefore, the goal of my work, as presented in chapter two, was to determine whether VDD further modified the cardiac effects due to PM-enriched smog such that the changes would be similar to ozone-enriched smog. Subsequent investigations (i.e. chapters 3) used a different approach and built on the findings of greater acute cardiac effects due to a predominantly gaseous smog atmosphere. These gases are extremely irritating to the airways and we have repeatedly demonstrated that they are responsible for potent cardiac effects through modulation of autonomic function. (Hazari, Griggs et al. 2014, Kurhanewicz, McIntosh-Kastrinsky et al. 2014, Conklin, Haberzettl et al. 2017) Therefore, acrolein, a ubiquitous reactive aldehyde and gaseous pollutant, was used to elicit acute cardiac effects because of its known ability to activate autonomic reflex arcs. This in turn was deemed

to be a suitable approach to assess whether the effects of VDD on homeostatic controls like the autonomic nervous system would predispose mice to modified responsiveness.

Proper function and regulation of the cardiovascular system depends on adequate levels of micronutrients like vitamin D. Vitamin D is required for calcium and phosphate homeostasis which are crucial for countless skeletal and non-skeletal bodily functions. Vitamin D deficiency affects approximately one billion people worldwide and is a global public health concern, however, the causes of VDD vary and the resulting deficits can range from high blood pressure to depression, rickets and even cancer. (Holick 2007) Vitamin D was chosen as an initial prototype micronutrient deficiency due to its known cardiovascular effects and immense public health relevance. Furthermore, the occurrence of VDD in people living in places where air pollution is a serious concern (e.g. New Delhi, India) is quite high and represents an important interaction that has yet to be properly characterized. Vitamin D deficiency also disproportionately affects certain nationalities and people residing in areas with longer winters, and for many of these people it begins early in life and can persist into adulthood. So, the work described in this dissertation attempted to understand early-life physiological changes due to VDD, and susceptibility to chronic disease during adulthood. Although these studies focused on post-weaning VDD, an interesting alternative paradigm could have been an investigation into the effects of maternal and in-utero VDD on the development of adverse cardiovascular responses.

The potential for adverse responses due to VDD are substantial given the vitamin's general activity throughout the body. As far as the cardiovascular system is concerned, most studies suggest that VDD leads to hypertension, however, with the

caveat that other factors clearly play a role as well. Blood pressure is increased even in individuals with VDD without hypertension (Forman, Bischoff-Ferrari et al. 2005) which confirms that the condition on its own may not be sufficient to cause pathological high blood pressure. In relation, VDD also affects signaling in the brain and downstream autonomic imbalance, which leads to impaired regulation of cardiac function (Canpolat, Özcan et al. 2015), demonstrating that vitamin D is centrally neuroactive (Wadhwania 2017). In fact, a study reported that individuals with VDD had impaired autonomic balance at rest and those with the lowest levels of serum 1,25-dihydroxy vitamin D had had “unfavorable” cardiac activity during a stress challenge. (Mann, Exner et al. 2013) It is not surprising that the mechanisms underlying the VDD-induced adverse physiological changes are quite varied because the autonomic nervous system is itself regulated by numerous central signals and innervates virtually every tissue type in the body. Almost 15 years ago the AHA recognized that vitamin D inhibits oxidative stress and inflammation and recommended dietary supplementation in people at risk for cardiovascular disease. (Kris-Etherton, Akabas et al. 2014) Therefore, the findings presented in this dissertation confirm the cardiac effects of VDD and begin to elucidate how it might be involved in altered responsiveness to air pollution exposure.

The human body experiences numerous stressors during the course of a day that usually will not cause adverse responses. Persistent deficits in the nutritional state of the body and/or repeated insults that prevent the body from recovery can significantly hamper the ability to maintain homeostasis. Indeed, even with repeated insults, proper nutritional intake can mitigate the development of dysfunction. As such, VDD has been shown to play a role in aging disorders due to subtle changes in calcium signaling

subsequently leading to calcium deposits that contribute to the formation of reactive oxygen species, cognitive impairment and adverse cardiovascular impacts including toxicity. (Berridge 2016) On the other hand, vitamin D repletion could stimulate cardiac cell differentiation and improve recovery from infarct injury. (Hlaing, Garcia et al. 2014)

Yet, when the body is both nutritionally deprived and exposed to potentially damaging stimuli then the risk of disease increases significantly. The work described in this dissertation is the first to demonstrate that early-life persistent VDD alters the cardiovascular response to air pollution and potentially predisposes the heart to dysfunction due to a mismatch in the availability of vitamin D and the requirements of a particular organ. Several studies have demonstrated that individual vitamin D status determines the response to stress and critical illness. (Lucidarme, Messai et al. 2010, Kestenbaum, Katz et al. 2011, Lee 2011) However, under certain circumstances it is not necessarily the absolute level of vitamin D in the body that determines if a stress-induced adverse response occurs or not, but rather whether enough active vitamin D is made available to suffice a given tissue's need at that moment. (Quraishi and Camargo 2012) Furthermore, hypocalcemia is common under conditions of stress or critical illness and can cause a rise in PTH, which would increase conversion of inactive vitamin D to active vitamin D to maintain calcium homeostasis. In this setting, the rapid consumption of inactive vitamin D due to secondary hyper-parathyroidism would further exacerbate VDD. This is supported by studies that have showed that acutely stressed or ill patients have secondary hyperparathyroidism with hypocalcemia and VDD. (Lucidarme, Messai et al. 2010, Flynn, Zimmerman et al. 2012)

Therefore, three-week-old mice were put on a normal or vitamin D deficient diet for 16-19 weeks and cardiopulmonary function was evaluated after a subsequent stressor (e.g. air pollution exposure or dobutamine stress test). The initial study objective was to characterize the cardiopulmonary response of VDD after a complex, realistic, multipollutant exposure. A major component of the multipollutant was then chosen as a single-pollutant exposure to determine the cardiopulmonary response in the context of a potential vitamin D mediator called klotho. Lastly, the impact of early-life VDD on mechanical cardiac function was determined in adult mice and the role of TRPC6, which is a cation channel expressed in the heart, was assessed with respect to it. These data describe mechanisms of underlying cardiovascular dysfunction due to VDD that are modified by a subsequent stressor. Taken together, these results highlight the influence of nutritional factors in determining murine responses to air pollution. If present in people, these effect modifications could have significant relevance to public health.

Principal Conclusions

Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice.

The objective of this study was to determine the effect of early-life persistent VDD on cardiopulmonary responses to a photochemical smog composed of high PM and low pollutant gases like ozone. The results demonstrate that VDD modulates the adverse cardiovascular response of adult mice to a single air pollution exposure. Although body weight was similar between normal and VDD mice, VDD mice had decreased HR, increased HRV and decreased tidal volume when compared to controls. In addition, VDD appeared to blunt the normal increase in tidal volume from five to

fifteen weeks of age. Smog had variable effects on normal and deficient mice. Heart rate decreased during air exposure, this response was blunted by smog in ND mice and to a lesser degree in VDD (i.e. HR remained more elevated in VDD). Smog exposure also further potentiated the increased HRV in VDD mice and increased cardiac arrhythmias only in ND mice. The findings of this study show that VDD modifies the cardiopulmonary effects of smog exposure; thus, highlighting the possible impact of nutritional factors on the responsiveness of the body to environmental stressors. Although the effects described in this study do not necessarily represent a clinical disease or pathological processes, they do represent a shift in the homeostatic control mechanisms of the body such that there is a potential for impaired compensation and maintenance of equilibrium. This is important to consider because the absence of significant symptoms and clinical presentation does not necessarily mean the absence of risk.

Klotho blocks heart rate and heart rate variability changes in early-life persistent vitamin D deficient mice during acrolein exposure

Acrolein is a ubiquitous gas found in complex air pollution mixtures like smog and it is known to contribute to adverse cardiovascular health effects. Yet, those effects in the presence of underlying nutritional deficiency, as well as the mechanisms underlying them, are still unknown. Acrolein elicits its cardiovascular effects in part through the activation of airway sensory nerves and subsequent modulation of autonomic function. Adequate levels of essential micronutrients like vitamin D are necessary to maintain autonomic balance and proper regulation of cardiac function. Studies have revealed that vitamin D exerts some of its effects in the body through klotho, which is an anti-aging

transmembrane protein that is cleaved into a soluble form and is secreted into the blood and cerebrospinal fluid. Consequently, klotho effects numerous cell tissues including the heart and is reduced in VDD. Therefore, the goal of this study was to determine the role of klotho in early-life VDD- induced adverse cardiac responses to acrolein in adult mice. Prior to exposure, HR was increased and HRV was decreased in VDD mice when compared to ND mice. When compared to FA exposure, HR and HRV significantly increased during acrolein in both ND and VDD mice. However, HR and HRV were significantly lower in VDD mice when compared to ND during acrolein exposure. Interestingly, klotho blocked the acrolein-induced HR response in both ND and VDD mice. Acrolein also increased arrhythmias, decreased breathing frequency and increased ventilatory irritation in both ND and VDD mice. Klotho administration unexpectedly caused the arrhythmias to increase in both diet groups and altered the breathing response. Therefore, VDD modifies the cardiac response of mice to acrolein through a mechanism involving klotho, however, contrary to what we expected, treatment with klotho appears to “benefit” normal mice during acrolein exposure as well. Although additional studies are needed to verify these findings, these data suggest klotho treatment could potentially lessen the effects of air pollution in people in general, but particularly in those with VDD.

Cardiac mechanical dysfunction due to vitamin D deficiency is mediated by TRPC6

Vitamin D modulates transient receptor potential (TRP) channel expression and function, and both have significant implications in cardiac physiology. However, it is still unknown whether VDD induces any cardiac contractile effects or if TRP channels are

involved. As such, it is believed that the impact of VDD on the heart occurs due to hypertension and pressure overload on the heart. Transient Receptor Potential C6 (TRPC6) is a mechanosensitive ion channel found in the heart and its overexpression has been linked to certain cardiovascular diseases. Blockade of TRPC6 improves cardiac output and contractility *in vivo* and *in vitro*, respectively, following ischemia/reperfusion cardiac injury. Yet, the role of TRPC6 on cardiac mechanical responses of VDD mice is unknown. Compared to ND mice, VDD mice had higher blood pressure and a blunted HR response during dobutamine stress test, which was restored when they were treated with the TRPC6 antagonist. Increased left ventricular pressure and decreased contractility in VDD mice were also restored by TRPC6 antagonist, whereas there was no change in normal mice. Heart rate variability was decreased in VDD mice during dobutamine stress testing, but TRPC6 antagonism had no effect. Thus, TRPC6 mediates VDD-induced mechanical cardiovascular dysfunction and altered responsiveness to an exercise-like stressor, but future studies with a prolonged diet regimen are needed to evaluate whether TRPC6 contributes to ventricular remodeling and hypertrophy in VDD.

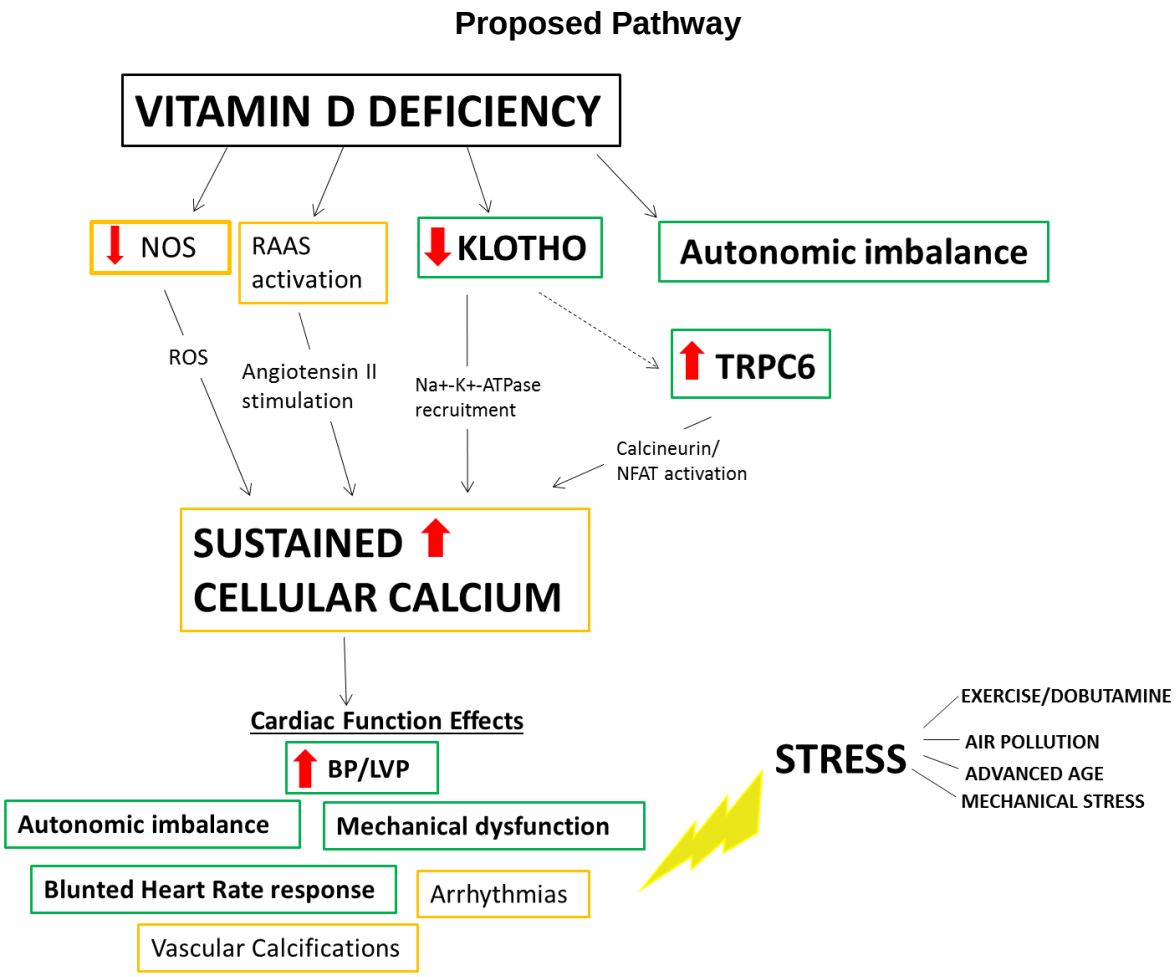


Figure 5.1. Potential pathway of the cardiac effects due to VDD.

A proposed pathway for the cardiac effects due to VDD is demonstrated in Figure 5-1. Modifiable factors such as diet, can influence systemic responses to everyday stressors like exercise, air pollution exposure or even non-chemical insults like noise. The studies presented in this dissertation, clearly demonstrate that VDD represents a potential modifiable factor that under certain conditions primes the cardiovascular system to an adverse response. Although VDD mice have increased blood pressure, altered autonomic balance and cardiac mechanical changes it is only during a stressful challenge (e.g. exercise/dobutamine or air pollution) that these latent effects are

revealed. Some of these effects appear to be mediated by klotho and TRPC6, which depend on adequate levels of vitamin D for proper physiological function. Previous data on TRPC6 and klotho conclusively demonstrate that sustained increased calcium is a direct effect of TRPC6 and klotho actions on the heart. Although this dissertation did not directly examine the role of calcium, it is likely to be a key mediator in this response given the actions of TRPC6, klotho and vitamin D and future studies should be performed to further evaluate its role. Changes in the activity of nitric oxide synthase (NOS) and RAAS activation have also been demonstrated to affect calcium signaling, particularly in the vasculature, and certainly represent an area that needs further investigation.

When increased calcium is sustained, adverse cardiac effects are experienced including autonomic imbalance, mechanical dysfunction (e.g. decrements in contractility and lusitropy) and hypertension. This dissertation does not explicitly examine vascular calcification but previous studies suggest that these effects result from increased calcium as well and may contribute to the overall syndrome. The data presented in this dissertation cannot fully explain the increase in arrhythmias due to VDD or klotho treatment, which may involve modulation of other channels involved in the cardiac action potential (e.g. Na+ channels). Finally, stress is ubiquitous and likely involves other regulatory pathways in the body and these could also alter the cardiac effects induced by VDD.

Future Directions

The body constantly monitors blood calcium levels to ensure adequate levels for the maintenance of normal physiology. Calcium dysregulation and inadequacy results in

aberrant processes in the body which cannot be sustained. The serum calcium and phosphate findings presented in this dissertation are consistent with previous studies (Girgis, Cha et al. 2015) but serum levels are tightly regulated and may not reflect deficits in intracellular calcium signaling. Future studies should evaluate PTH levels to get a better understanding of overall calcium homeostasis and regulation attributed to VDD. In persistent and severe VDD, PTH and calcium levels would also be decreased. Furthermore, the kidney, brain and heart are tissues with increased calcium demand and can be negatively affected by VDD. Understanding the differential effect of VDD and calcium availability in each of these tissues would help to determine calcium's contribution to cardiovascular disease.

Increased intracellular calcium levels may result from modulation of TRPC6 and klotho. As depicted in Figure 5-1, increased TRPC6 and decreased klotho result in increased calcium through activation of calcineurin/NFAT pathway and increased Na+-K+-ATPase recruitment to the plasma membrane respectively. Determining the activity of the calcineurin/NFAT pathway in this model would demonstrate that increased TRPC6 causes increased intracellular calcium signaling. As such, demonstrating increased protein expression of Na+-K+-ATPase will also establish a mechanism of increased calcium through klotho resulting in adverse cardiac responses. Additionally, electrophysiology experiments using TRPC6 inhibitors (McPate, Bhalay et al. 2014) or even calcium channel blockers would determine the role of cation channels in VDD-induced cardiac dysfunction. The role of calcium through TRPC6 and klotho in this paradigm is currently unresolved but is the latent central mediator in the cardiovascular responses observed.

Clinically, vitamin D supplementation is regularly recommended in order to restore VDD to normal levels likely due to the known bone effects especially in children. A myriad of studies examining vitamin D supplementation in relation to heart disease have been conducted and mostly inconclusive unfortunately. (Muscogiuri, Annweiler et al. 2017) For example, studies have demonstrated that vitamin D supplementation has no effect on inflammation in heart failure patents (Mousa, Naderpoor et al. 2017, Rodriguez, Mousa et al. 2018) or insulin sensitivity in obesity (Mousa, Naderpoor et al. 2017), even with a wide variety of dosing regimens. Vitamin D supplementation had no effect on blood pressure (Arora, Song et al. 2015), hospital admissions after ischemic injury (Harvey, D'Angelo et al. 2018) and prevention of heart failure (D'Amore, Marsico et al. 2017) or cardiovascular disease (Scragg, Stewart et al. 2017). However, current clinical trials are being conducted in a more focused manner to determine the effect of vitamin D supplementation (with or without omega-3 fatty acids) in the prevention of cardiovascular disease. (Pradhan and Manson 2016)

On the other hand, previous studies have intraperitoneally administered klotho and found that klotho diminished calcium accumulations in the kidneys and aorta, which may protect mice against disorders (i.e. vascular calcifications) associated with age. (Chen, Kuro et al. 2013) However, it is not currently known if and how cardiomyocytes uptake exogenous klotho to subsequently exert cardioprotective effects. A klotho receptor has not been identified but may be involved in its downstream cardioprotective effects through lipid rafts. (Dalton, An et al. 2017, Mencke and Hillebrands 2017) Dose optimization of klotho is also needed and may vary per diet regimen.

Increased TRPC6 mRNA expression in the heart was suggested in this work and it has been published previously and linked to cardiovascular disease. (Watanabe, Murakami et al. 2009, Xie, Cha et al. 2012, Watanabe, Iino et al. 2013, Yamaguchi, Iribe et al. 2017) Despite the increased TRPC6 in the heart with VDD, numerous questions remain, including: 1) are the increased channels functionally active, 2) is the threshold for their activation altered due to changes in hemodynamics and 3) how is calcium handling modulated and contributing to adverse cardiac responses.

As such, vitamin D and klotho are both negative regulators of RAAS contributing to anti-hypertensive and cardioprotective effects. (Li, Kong et al. 2002, Takenaka, Inoue et al. 2017) In fact, renin production is blocked by vitamin D to regulate blood pressure. (Trehan, Afonso et al. 2017) Therefore, an important step in determining the impact of VDD on cardiovascular disease development would be to evaluate the components of RAAS (e.g. angiotensinogen, renin, angiotensin 1 and 2 and angiotensin converting enzyme) and how they contribute to dysfunction.

Finally, the work presented here can be taken a step further would to examine a heart failure murine model such as ischemia-reperfusion injury, isoproterenol-stress or even left ascending coronary artery ligation to assess how early-life persistent VDD modifies the cardiovascular response to these stressors. Each of these procedures represent a common cardiac injury, which are also present in humans and known to impair overall cardiac function. Using the regimen described here, normal and VDD mice could undergo one of these cardiac injury procedures to determine the extent of the response and thereafter the response to air pollution. In all of the studies presented in this dissertation, the mice were healthy when exposed to the stressor and therefore,

further understanding of the influence of micronutrient deficiency on cardiac response to stress after an injury (e.g. myocardial infarct) is critical.

Implications

Studies have used klotho and TRPC6 as biomarkers or therapeutics and the work presented here provides further evidence for the utilization of klotho and TRPC6 as potential therapeutic targets. Additional studies are needed to validate klotho as a potential nutritional supplement irrespective of diet and TRPC6 antagonism in preventing cardiovascular disease. This body of work is the first to demonstrate that nutritional modifiable factors should be evaluated in the context of air pollution health effects research.

REFERENCES

Abdelmalik, P. A., R. D. Stevens, S. Singh, J. Skinner, J. R. Carhuapoma, S. Noel, R. Johns and R. J. Fuchs (2018). "Anti-aging factor, serum alpha-Klotho, as a marker of acute physiological stress, and a predictor of ICU mortality, in patients with septic shock." J Crit Care **44**: 323-330.

Achinger, S. G. and J. C. Ayus (2005). "The role of vitamin D in left ventricular hypertrophy and cardiac function." Kidney International **67**: S37-S42.

Ahmad, A. A., M. Streiff, C. Hunter, Q. Hu and F. B. Sachse (2017). "Physiological and pathophysiological role of transient receptor potential canonical channels in cardiac myocytes." Prog Biophys Mol Biol **130**(Pt B): 254-263.

Al-Khalidi, B., S. M. Kimball, M. A. Rotondi and C. I. Ardern (2017). "Standardized serum 25-hydroxyvitamin D concentrations are inversely associated with cardiometabolic disease in U.S. adults: a cross-sectional analysis of NHANES, 2001-2010." Nutr J **16**(1): 16.

Anderson, J. L., H. T. May, B. D. Horne, T. L. Bair, N. L. Hall, J. F. Carlquist, D. L. Lappé and J. B. Muhlestein (2010). "Relation of Vitamin D Deficiency to Cardiovascular Risk Factors, Disease Status, and Incident Events in a General Healthcare Population." The American Journal of Cardiology **106**(7): 963-968.

Argacha, J. F., D. Egrise, S. Pochet, D. Fontaine, A. Lefort, F. Libert, S. Goldman, P. van de Borne, G. Berkenboom and R. Moreno-Reyes (2011). "Vitamin D deficiency-induced hypertension is associated with vascular oxidative stress and altered heart gene expression." J Cardiovasc Pharmacol **58**(1): 65-71.

Arora, P., Y. Song, J. Dusek, G. Plotnikoff, M. S. Sabatine, S. Cheng, A. Valcour, H. Swales, B. Taylor, E. Carney, D. Guanaga, J. R. Young, C. Karol, M. Torre, A. Azzahir, S. M. Strachan, D. C. O'Neill, M. Wolf, F. Harrell, C. Newton-Cheh and T. J. Wang (2015). "Vitamin D therapy in individuals with prehypertension or hypertension: the DAYLIGHT trial." Circulation **131**(3): 254-262.

Artaza, J. N., R. Mehrotra and K. C. Norris (2009). "Vitamin D and the cardiovascular system." Clin J Am Soc Nephrol **4**(9): 1515-1522.

Baksi, S. N. "Deficiency in dietary vitamin D, not calcium, alters noradrenergic responsiveness in rat atria in vitro." Journal of molecular and cellular cardiology **18**(7): 653-656.

Bandyopadhyay, J., J. Lee, J. Lee, J. I. Lee, J.-R. Yu, C. Jee, J.-H. Cho, S. Jung, M. H. Lee, S. Zannoni, A. Singson, D. H. Kim, H.-S. Koo and J. Ahnn (2002). "Calcineurin, a Calcium/Calmodulin-dependent Protein Phosphatase, Is Involved in Movement, Fertility, Egg Laying, and Growth in Caenorhabditis elegans." Molecular Biology of the Cell **13**(9): 3281-3293.

Bein, K. and G. D. Leikauf (2011). "Acrolein - a pulmonary hazard." Mol Nutr Food Res **55**(9): 1342-1360.

Ben-Jebria, A., R. Marthan, M. Rossetti, J. P. Savineau and J. S. Ultman (1994). "Human bronchial smooth muscle responsiveness after in vitro exposure to acrolein." Am J Respir Crit Care Med **149**(2 Pt 1): 382-386.

Bener, A., M. Al-Ali and G. F. Hoffmann (2009). "High prevalence of vitamin D deficiency in young children in a highly sunny humid country: a global health problem." Minerva pediatrica **61**(1): 15-22.

Bernichtein, S., N. Pigat, N. Barry Delongchamps, F. Boutillon, V. Verkarre, P. Camparo, E. Reyes-Gomez, A. Mejean, S. M. Oudard, E. M. Lepicard, M. Viltard, J. C. Souberbielle, G. Friedlander, T. Capiod and V. Goffin (2017). "Vitamin D3 Prevents Calcium-Induced Progression of Early-Stage Prostate Tumors by Counteracting TRPC6 and Calcium Sensing Receptor Upregulation." Cancer Res **77**(2): 355-365.

Berridge, M. J. (2015). "Vitamin D cell signalling in health and disease." Biochemical and Biophysical Research Communications **460**(1): 53-71.

Berridge, M. J. (2016). "Vitamin D, reactive oxygen species and calcium signalling in ageing and disease." Philos Trans R Soc Lond B Biol Sci **371**(1700).

Bharadwaj, P., J. G. Zivin and J. T. Mullins (2016). "Early-Life Exposure to the Great Smog of 1952 and the Development of Asthma." **194**(12): 1475-1482.

Binkley, P. F., E. Nunziata, G. J. Haas, S. D. Nelson and R. J. Cody (1991). "Parasympathetic withdrawal is an integral component of autonomic imbalance in congestive heart failure: demonstration in human subjects and verification in a paced canine model of ventricular failure." Journal of the American College of Cardiology **18**(2): 464-472.

Brook, R. D. and S. Julius (2000). "Autonomic imbalance, hypertension, and cardiovascular risk." American journal of hypertension **13**(S4): 112S-122S.

Brook, R. D., S. Rajagopalan, C. A. Pope, J. R. Brook, A. Bhatnagar, A. V. Diez-Roux, F. Holguin, Y. Hong, R. V. Luepker, M. A. Mittleman, A. Peters, D. Siscovick, S. C. Smith, L. Whitsel, J. D. Kaufman, o. b. o. t. A. H. A. C. o. Epidemiology, C. o. t. K. i. C. D. Prevention, P. A. Council on Nutrition and Metabolism (2010). "Particulate Matter Air Pollution and Cardiovascular Disease: An Update to the Scientific Statement From the American Heart Association." Circulation **121**(21): 2331-2378.

Brubaker, P. H. and D. W. Kitzman (2011). "Chronotropic incompetence: causes, consequences, and management." Circulation **123**(9): 1010-1020.

Canpolat, U., F. Özcan, Ö. Özeke, O. Turak, Ç. Yayla, S. K. Açıkgöz, S. Çay, S. Topaloğlu, D. Aras and S. Aydoğdu (2015). "Impaired Cardiac Autonomic Functions in Apparently Healthy Subjects with Vitamin D Deficiency." Annals of Noninvasive Electrocardiology **20**(4): 378-385.

Cararo-Lopes, M. M., C. H. Y. Mazucanti, C. Scavone, E. M. Kawamoto and D. C. Berwick (2017). "The relevance of alpha-KLOTHO to the central nervous system: Some key questions." Ageing Res Rev **36**: 137-148.

Carbone, F., F. Mach, N. Vuilleumier and F. Montecucco (2014). "Potential pathophysiological role for the vitamin D deficiency in essential hypertension." World J Cardiol **6**(5): 260-276.

Carll, A. P., S. M. Crespo, M. S. Filho, D. H. Zati, B. A. Coull, E. A. Diaz, R. D. Raimundo, T. N. G. Jaeger, A. L. Ricci-Vitor, V. Papapostolou, J. E. Lawrence, D. M. Garner, B. S. Perry, J. R. Harkema and J. J. Godleski (2017). "Inhaled ambient-level traffic-derived particulates decrease cardiac vagal influence and baroreflexes and increase arrhythmia in a rat model of metabolic syndrome." Part Fibre Toxicol **14**(1): 16.

Carll, A. P., R. M. Lust, M. S. Hazari, C. M. Perez, Q. T. Krantz, C. J. King, D. W. Winsett, W. E. Cascio, D. L. Costa and A. K. Farraj (2013). "Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats." Toxicol Sci **131**(2): 583-595.

Carlton-Conway, D., R. Tulloh, L. Wood and D. Kanabar (2004). "Vitamin D deficiency and cardiac failure in infancy." Journal of the Royal Society of Medicine **97**(5): 238-239.

Chauvet, S., M. Boonen, M. Chevallet, L. Jarvis, A. Abebe, M. Benharouga, P. Faller, M. Jadot and A. Bouron (2015). "The Na+/K+-ATPase and the amyloid-beta peptide abeta1-40 control the cellular distribution, abundance and activity of TRPC6 channels." Biochim Biophys Acta **1853**(11 Pt A): 2957-2965.

Chen, L., R. Wilson, E. Bennett and G. R. Zosky (2016). "Identification of vitamin D sensitive pathways during lung development." Respir Res **17**: 47.

Chen, S., Y. Sun and D. K. Agrawal (2015). "Vitamin D deficiency and essential hypertension." J Am Soc Hypertens **9**(11): 885-901.

Chen, T. H., O. M. Kuro, C. H. Chen, Y. M. Sue, Y. C. Chen, H. H. Wu and C. Y. Cheng (2013). "The secreted Klotho protein restores phosphate retention and suppresses accelerated aging in Klotho mutant mice." Eur J Pharmacol **698**(1-3): 67-73.

Chiu, H. F., Y. H. Weng, Y. W. Chiu and C. Y. Yang (2017). "Short-term effects of ozone air pollution on hospital admissions for myocardial infarction: A time-stratified case-crossover study in Taipei." J Toxicol Environ Health A **80**(5): 251-257.

Conklin, D. J., P. Haberzettl, G. Jagatheesan, M. Kong and G. W. Hoyle (2017). "Role of TRPA1 in acute cardiopulmonary toxicity of inhaled acrolein." Toxicol Appl Pharmacol **324**: 61-72.

Coogan, P. F., L. F. White, J. Yu, R. D. Brook, R. T. Burnett, J. D. Marshall, T. N. Bethea, L. Rosenberg and M. Jerrett (2017). "Long-Term Exposure to NO2 and Ozone and Hypertension Incidence in the Black Women's Health Study." Am J Hypertens **30**(4): 367-372.

Corsetti, G., E. Pasini, T. M. Scarabelli, C. Romano, P. R. Agrawal, C. Chen-Scarabelli, R. Knight, L. Saravolatz, J. Narula, M. Ferrari-Vivaldi, V. Flati, D. Assanelli and F. S. Dioguardi (2016). "Decreased expression of Klotho in cardiac atria biopsy samples from patients at higher risk of atherosclerotic cardiovascular disease." J Geriatr Cardiol **13**(8): 701-711.

Costa, A. F., G. Hoek, B. Brunekreef and A. C. M. Ponce de Leon (2017). "Effects of NO2 exposure on daily mortality in Sao Paulo, Brazil." Environ Res **159**: 539-544.

D'Amore, C., F. Marsico, A. Parente, S. Paolillo, F. De Martino, P. Gargiulo, F. Ferrazzano, A. M. De Roberto, L. La Mura, C. Marciano, S. Dellegrottaglie, B. Trimarco and P. Perrone Filardi (2017). "Vitamin D deficiency and clinical outcome in patients with chronic heart failure: A review." Nutr Metab Cardiovasc Dis **27**(10): 837-849.

Dalton, G., S. W. An, S. I. Al-Juboori, N. Nischan, J. Yoon, E. Dobrinskikh, D. W. Hilgemann, J. Xie, K. Luby-Phelps, J. J. Kohler, L. Birnbaumer and C. L. Huang (2017). "Soluble klotho binds monosialoganglioside to regulate membrane microdomains and growth factor signaling." Proc Natl Acad Sci U S A **114**(4): 752-757.

Dalton, G. D., J. Xie, S. W. An and C. L. Huang (2017). "New Insights into the Mechanism of Action of Soluble Klotho." Front Endocrinol (Lausanne) **8**: 323.

Davies, P. F. (2009). "Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology." Nat Clin Pract Cardiovasc Med **6**(1): 16-26.

Davoodi, G., A. Y. Sharif, A. Kazemisaeid, S. Sadeghian, A. V. Farahani, M. Sheikhvatan and M. Pashang (2010). "Comparison of heart rate variability and cardiac arrhythmias in polluted and clean air episodes in healthy individuals." Environ Health Prev Med **15**(4): 217-221.

Day, D. B., J. Xiang, J. Mo, F. Li, M. Chung, J. Gong, C. J. Weschler, P. A. Ohman-Strickland, J. Sundell, W. Weng, Y. Zhang and J. J. Zhang (2017). "Association of Ozone Exposure With Cardiorespiratory Pathophysiologic Mechanisms in Healthy Adults." JAMA Intern Med **177**(9): 1344-1353.

de Bruyne, M. C., J. A. Kors, A. W. Hoes, P. Klootwijk, J. M. Dekker, A. Hofman, J. H. van Bemmel and D. E. Grobbee (1999). "Both decreased and increased heart rate

variability on the standard 10-second electrocardiogram predict cardiac mortality in the elderly: the Rotterdam Study." Am J Epidemiol **150**(12): 1282-1288.

DeLuca, G. C., S. M. Kimball, J. Kolasinski, S. V. Ramagopalan and G. C. Ebers (2013). "Review: the role of vitamin D in nervous system health and disease." Neuropathol Appl Neurobiol **39**(5): 458-484.

Dietrich, A., D. Steinritz and T. Gudermann (2017). "Transient receptor potential (TRP) channels as molecular targets in lung toxicology and associated diseases." Cell Calcium **67**: 123-137.

Dimova, R., T. Tankova and N. Chakarova (2017). "Vitamin D in the Spectrum of Prediabetes and Cardiovascular Autonomic Dysfunction." The Journal of Nutrition **147**(9): 1607-1615.

Ding, H.-Y. and H.-X. Ma (2015). "Significant roles of anti-aging protein klotho and fibroblast growth factor23 in cardiovascular disease." Journal of Geriatric Cardiology : JGC **12**(4): 439-447.

Dobnig, H., S. Pilz, H. Scharnagl, W. Renner, U. Seelhorst, B. Wellnitz, J. Kinkeldei, B. O. Boehm, G. Weihrauch and W. Maerz (2008). "Independent association of low serum 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d levels with all-cause and cardiovascular mortality." Arch Intern Med **168**(12): 1340-1349.

Dooley, E. E. (2002). "Fifty years later: clearing the air over the London smog." Environ Health Perspect **110**(12): A748.

Dusso, A. S. (2011). "Kidney disease and vitamin D levels: 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and VDR activation." Kidney International Supplements **1**(4): 136-141.

Eguchi, K., J. E. Schwartz, T. G. Pickering, S. Hoshide, J. Ishikawa, K. Shimada and K. Kario (2010). "Increased heart rate variability during sleep is a predictor for future cardiovascular events in patients with type 2 diabetes." Hypertens Res **33**(7): 737-742.

Eke, I. G., C. Igwe and A. O. Anaga (2014). "Vitamin D supplementation attenuated carageenan-induced paw edema and formalin-induced nociception in indomethacin-treated rats." Int J Basic Clin Pharmacol **3**(1): 206-210.

Eltablawy, N. and H. Ashour (2018). "Vitamin D protection from rat diabetic nephropathy is partly mediated through Klotho expression and renin-angiotensin inhibition." 1-7.
Esterbauer, H., R. J. Schaur and H. Zollner (1991). "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes." Free Radic Biol Med **11**(1): 81-128.

Farraj, A. K., M. S. Hazari, D. W. Winsett, A. Kulukulualani, A. P. Carll, N. Haykal-Coates, C. M. Lamb, E. Lappi, D. Terrell and W. E. Cascio (2011). "Overt and latent cardiac effects of ozone inhalation in rats: evidence for autonomic modulation and increased myocardial vulnerability." Environmental health perspectives **120**(3): 348-354.

Farraj, A. K., L. Walsh, N. Haykal-Coates, F. Malik, J. McGee, D. Winsett, R. Duvall, K. Kovalcik, W. E. Cascio, M. Higuchi and M. S. Hazari (2015). "Cardiac effects of seasonal ambient particulate matter and ozone co-exposure in rats." Part Fibre Toxicol **12**: 12.

Ferdous, Z., M. A. Qureshi, P. Jayaprakash, K. Parekh, A. John, M. Oz, H. Raza, H. Dobrzynski, T. E. Adrian and F. C. Howarth (2016). "Different Profile of mRNA Expression in Sinoatrial Node from Streptozotocin-Induced Diabetic Rat." PLoS One **11**(4): e0153934.

Fetahu, I. S., J. Höbaus and E. Kállay (2014). "Vitamin D and the epigenome." Frontiers in Physiology **5**: 164.

Flynn, L., L. H. Zimmerman, K. McNorton, M. Dolman, J. Tyburski, A. Baylor, R. Wilson and H. Dolman (2012). "Effects of vitamin D deficiency in critically ill surgical patients." Am J Surg **203**(3): 379-382; discussion 382.

Foong, R. E., A. Bosco, A. C. Jones, A. Gout, S. Gorman, P. H. Hart and G. R. Zosky (2015). "The effects of in utero vitamin D deficiency on airway smooth muscle mass and lung function." Am J Respir Cell Mol Biol **53**(5): 664-675.

Forman, J. P., H. A. Bischoff-Ferrari, W. C. Willett, M. J. Stampfer and G. C. Curhan (2005). "Vitamin D intake and risk of incident hypertension: results from three large prospective cohort studies." Hypertension **46**(4): 676-682.

Forman, J. P., G. C. Curhan and E. N. Taylor (2008). "Plasma 25-hydroxyvitamin D levels and risk of incident hypertension among young women." Hypertension **52**(5): 828-832.

Forrest, K. Y. and W. L. Stuhldreher (2011). "Prevalence and correlates of vitamin D deficiency in US adults." Nutr Res **31**(1): 48-54.

Frohlich, E. D. and D. Susic (2012). "Pressure overload." Heart Fail Clin **8**(1): 21-32.
Garcion, E., N. Wion-Barbot, C. N. Montero-Menei, F. Berger and D. Wion (2002). "New clues about vitamin D functions in the nervous system." Trends Endocrinol Metab **13**(3): 100-105.

Gehrmann, J., P. E. Hammer, C. T. Maguire, H. Wakimoto, J. K. Triedman and C. I. Berul (2000). "Phenotypic screening for heart rate variability in the mouse." American Journal of Physiology - Heart and Circulatory Physiology **279**(2): H733-H740.

Ghilarducci, D. P. and R. S. Tjeerdema (1995). "Fate and effects of acrolein." Rev Environ Contam Toxicol **144**: 95-146.

Ginty, A. T., P. J. Gianaros, S. W. Derbyshire, A. C. Phillips and D. Carroll (2013). "Blunted cardiac stress reactivity relates to neural hypoactivation." Psychophysiology **50**(3): 219-229.

Giovannucci, E., Y. Liu, B. W. Hollis and E. B. Rimm (2008). "25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study." Arch Intern Med **168**(11): 1174-1180.

Girgis, C. M., K. M. Cha, P. J. Houweling, R. Rao, N. Mokbel, M. Lin, R. J. Clifton-Bligh and J. E. Gunton (2015). "Vitamin D Receptor Ablation and Vitamin D Deficiency Result in Reduced Grip Strength, Altered Muscle Fibers, and Increased Myostatin in Mice." Calcif Tissue Int **97**(6): 602-610.

Gold, D. R., A. Litonjua, J. Schwartz, E. Lovett, A. Larson, B. Nearing, G. Allen, M. Verrier, R. Cherry and R. Verrier (2000). "Ambient pollution and heart rate variability." Circulation **101**(11): 1267-1273.

Gordon, C. M., K. C. DePeter, H. A. Feldman, E. Grace and S. Emans (2004). "PRevalence of vitamin d deficiency among healthy adolescents." Archives of Pediatrics & Adolescent Medicine **158**(6): 531-537.

Gordon, C. M., H. A. Feldman, L. Sinclair and et al. (2008). "PRevalence of vitamin d deficiency among healthy infants and toddlers." Archives of Pediatrics & Adolescent Medicine **162**(6): 505-512.

Gradinaru, D., C. Borsa, C. Ionescu and D. Margina (2013). "Advanced oxidative and glycoxidative protein damage markers in the elderly with type 2 diabetes." J Proteomics **92**: 313-322.

Guo, Y., X. Zhuang, Z. Huang, J. Zou, D. Yang, X. Hu, Z. Du, L. Wang and X. Liao (2018). "Klotho protects the heart from hyperglycemia-induced injury by inactivating ROS and NF-kappaB-mediated inflammation both in vitro and in vivo." Biochim Biophys Acta **1864**(1): 238-251.

Haagen-Smit, A. J. (1952). "Chemistry and physiology of Los Angeles smog." Industrial & Engineering Chemistry **44**(6): 1342-1346.

Hales, C. A., P. Barkin, W. Jung, D. Quinn, D. Lamborghini and J. Burke (1989). "Bronchial artery ligation modifies pulmonary edema after exposure to smoke with acrolein." J Appl Physiol (1985) **67**(3): 1001-1006.

Hang, P., J. Zhao, B. Cai, S. Tian, W. Huang, J. Guo, C. Sun, Y. Li and Z. Du (2015). "Brain-derived neurotrophic factor regulates TRPC3/6 channels and protects against myocardial infarction in rodents." Int J Biol Sci **11**(5): 536-545.

Harvey, N. C., S. D'Angelo, J. Paccou, E. M. Curtis, M. Edwards, Z. Raisi-Estabragh, K. Walker-Bone, S. E. Petersen and C. Cooper (2018). "Calcium and Vitamin D Supplementation Are Not Associated With Risk of Incident Ischemic Cardiac Events or Death: Findings From the UK Biobank Cohort." J Bone Miner Res.

Hazari, M., J. Griggs, D. Winsett, N. Haykal-Coates, A. Ledbetter, D. Costa and A. Farraj (2014). "A Single Exposure to Acrolein Desensitizes Baroreflex Responsiveness and Increases Cardiac Arrhythmias in Normotensive and Hypertensive Rats." Cardiovascular Toxicology **14**(1): 52-63.

Hazari, M. S., J. Callaway, D. W. Winsett, C. Lamb, N. Haykal-Coates, Q. Krantz, C. King, D. L. Costa and A. K. Farraj (2012). "Dobutamine" stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats." Environmental health perspectives **120**(8): 1088-1093.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, D. L. Costa and A. K. Farraj (2009). "A single exposure to particulate or gaseous air pollution increases the risk of aconitine-induced cardiac arrhythmia in hypertensive rats." Toxicol Sci **112**(2): 532-542.

Hazari, M. S., N. Haykal-Coates, D. W. Winsett, Q. T. Krantz, C. King, D. L. Costa and A. K. Farraj (2011). "TRPA1 and Sympathetic Activation Contribute to Increased Risk of Triggered Cardiac Arrhythmias in Hypertensive Rats Exposed to Diesel Exhaust." Environmental Health Perspectives **119**(7): 951-957.

Hazari, M. S. and K. M. Stratford (2018). "Comparative Cardiopulmonary Effects of Particulate Matter- And Ozone-Enhanced Smog Atmospheres in Mice."
Heusser, K., J. Tank, F. C. Luft and J. Jordan (2005). "Baroreflex Failure." Hypertension **45**(5): 834.

Hlaing, S. M., L. A. Garcia, J. R. Contreras, K. C. Norris, M. G. Ferrini and J. N. Artaza (2014). "1,25-Vitamin D3 promotes cardiac differentiation through modulation of the WNT signaling pathway." J Mol Endocrinol **53**(3): 303-317.

Holick, M. F. (2007). "Vitamin D Deficiency." New England Journal of Medicine **357**(3): 266-281.

Hu, M. C., M. Shi, J. Zhang, H. Quinones, C. Griffith, M. Kuro-o and O. W. Moe (2011). "Klotho deficiency causes vascular calcification in chronic kidney disease." J Am Soc Nephrol **22**(1): 124-136.

Hunt, A., J. L. Abraham, B. Judson and C. L. Berry (2003). "Toxicologic and epidemiologic clues from the characterization of the 1952 London smog fine particulate matter in archival autopsy lung tissues." Environ Health Perspect **111**(9): 1209-1214.

Imura, A., Y. Tsuji, M. Murata, R. Maeda, K. Kubota, A. Iwano, C. Obuse, K. Togashi, M. Tominaga, N. Kita, K. Tomiyama, J. Iijima, Y. Nabeshima, M. Fujioka, R. Asato, S. Tanaka, K. Kojima, J. Ito, K. Nozaki, N. Hashimoto, T. Ito, T. Nishio, T. Uchiyama, T. Fujimori and Y. Nabeshima (2007). "alpha-Klotho as a regulator of calcium homeostasis." Science **316**(5831): 1615-1618.

Inoue, R., L. J. Jensen, Z. Jian, J. Shi, L. Hai, A. I. Lurie, F. H. Henriksen, M. Salomonsson, H. Morita, Y. Kawarabayashi, M. Mori, Y. Mori and Y. Ito (2009). "Synergistic activation of vascular TRPC6 channel by receptor and mechanical stimulation via phospholipase C/diacylglycerol and phospholipase A2/omega-hydroxylase/20-HETE pathways." Circ Res **104**(12): 1399-1409.

Ismahil, M. A., T. Hamid, P. Haberzettl, Y. Gu, B. Chandrasekar, S. Srivastava, A. Bhatnagar and S. D. Prabhu (2011). "Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy." Am J Physiol Heart Circ Physiol **301**(5): H2050-2060.

Iyngkaran, P., N. S. Anavekar, C. Neil, L. Thomas and D. L. Hare (2017). "Shortness of breath in clinical practice: A case for left atrial function and exercise stress testing for a comprehensive diastolic heart failure workup." World J Methodol **7**(4): 117-128.

Judd, S. E., M. S. Nanes, T. R. Ziegler, P. W. Wilson and V. Tangpricha (2008). "Optimal vitamin D status attenuates the age-associated increase in systolic blood pressure in white Americans: results from the third National Health and Nutrition Examination Survey." Am J Clin Nutr **87**(1): 136-141.

Jung, C. H., S. H. Jung, K. J. Kim, B. Y. Kim, C. H. Kim, S. K. Kang and J. O. Mok (2015). "The relationship between vitamin D status and cardiac autonomic neuropathy in patients with type 2 diabetes mellitus." Diab Vasc Dis Res **12**(5): 342-351.

Just, A., J. Faulhaber and H. Ehmke (2000). "Autonomic cardiovascular control in conscious mice." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **279**(6): R2214-R2221.

Kachel, D. L. and W. J. Martin, 2nd (1994). "Cyclophosphamide-induced lung toxicity: mechanism of endothelial cell injury." J Pharmacol Exp Ther **268**(1): 42-46.

Kara, T., K. Narkiewicz and V. K. Somers (2003). "Chemoreflexes--physiology and clinical implications." Acta Physiol Scand **177**(3): 377-384.

Kehrer, J. P. and S. S. Biswal (2000). "The molecular effects of acrolein." Toxicol Sci **57**(1): 6-15.

Kestenbaum, B., R. Katz, I. de Boer, A. Hoofnagle, M. J. Sarnak, M. G. Shlipak, N. S. Jenny and D. S. Siscovick (2011). "Vitamin D, parathyroid hormone, and cardiovascular events among older adults." J Am Coll Cardiol **58**(14): 1433-1441.

Kieu, A., A. Shaikh, M. Kaeppler, R. J. Miles and M. E. Widlansky (2017). "Patients with hypertensive responses to exercise or dobutamine stress testing differ in resting hypertensive phenotype." J Am Soc Hypertens.

Knuckles, T. L., A. K. Lund, S. N. Lucas and M. J. Campen (2008). "Diesel exhaust exposure enhances venoconstriction via uncoupling of eNOS." Toxicol Appl Pharmacol **230**(3): 346-351.

Koitabashi, N., T. Aiba, G. G. Hesketh, J. Rowell, M. Zhang, E. Takimoto, G. F. Tomaselli and D. A. Kass (2010). "Cyclic GMP/PKG-dependent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAT activation Novel mechanism of cardiac stress modulation by PDE5 inhibition." J Mol Cell Cardiol **48**(4): 713-724.

Koyner, J. L. and L. S. Chawla (2017). "Use of stress tests in evaluating kidney disease." Curr Opin Nephrol Hypertens **26**(1): 31-35.

Kris-Etherton, P. M., S. R. Akabas, C. W. Bales, B. Bistrian, L. Braun, M. S. Edwards, C. Laur, C. M. Lenders, M. D. Levy, C. A. Palmer, C. A. Pratt, S. Ray, C. L. Rock, E. Saltzman, D. L. Seidner and L. Van Horn (2014). "The need to advance nutrition education in the training of health care professionals and recommended research to evaluate implementation and effectiveness." Am J Clin Nutr **99**(5 Suppl): 1153s-1166s.

Krug, J. D. (2018). "Photochemical Conversion of Surrogate Emissions for Use in Toxicological Studies: Role of Particulate- and Gas-Phase Products." **52**(5): 3037-3044.

Kumar, J., P. Muntner, F. J. Kaskel, S. M. Hailpern and M. L. Melamed (2009). "Prevalence and Associations of 25-Hydroxyvitamin D Deficiency in US Children: NHANES 2001–2004." Pediatrics **124**(3): e362-e370.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, A. Ledbetter, L. Walsh, A. Farraj and M. Hazari (2017). "TRPA1 mediates changes in heart rate variability and cardiac mechanical function in mice exposed to acrolein." Toxicol Appl Pharmacol **324**: 51-60.

Kurhanewicz, N., R. McIntosh-Kastrinsky, H. Tong, L. Walsh, A. K. Farraj and M. S. Hazari (2014). "Ozone co-exposure modifies cardiac responses to fine and ultrafine ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses." Part Fibre Toxicol **11**: 54.

Kuro-o, M., Y. Matsumura, H. Aizawa, H. Kawaguchi, T. Suga, T. Utsugi, Y. Ohyama, M. Kurabayashi, T. Kaname, E. Kume, H. Iwasaki, A. Iida, T. Shiraki-Iida, S. Nishikawa,

R. Nagai and Y. I. Nabeshima (1997). "Mutation of the mouse klotho gene leads to a syndrome resembling ageing." Nature **390**(6655): 45-51.

Kutzman, R. S., E. A. Popenoe, M. Schmaeler and R. T. Drew (1985). "Changes in rat lung structure and composition as a result of subchronic exposure to acrolein." Toxicology **34**(2): 139-151.

La Rovere, M. T., G. D. Pinna, S. H. Hohnloser, F. I. Marcus, A. Mortara, R. Nohara, J. T. Bigger, Jr., A. J. Camm and P. J. Schwartz (2001). "Baroreflex sensitivity and heart rate variability in the identification of patients at risk for life-threatening arrhythmias: implications for clinical trials." Circulation **103**(16): 2072-2077.

Lalande, S. and B. D. Johnson (2010). "Breathing strategy to preserve exercising cardiac function in patients with heart failure." Med Hypotheses **74**(3): 416-421.

Lee, J. E. (2011). "Circulating levels of vitamin D, vitamin D receptor polymorphisms, and colorectal adenoma: a meta-analysis." Nutr Res Pract **5**(5): 464-470.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D deficiency an important, common, and easily treatable cardiovascular risk factor?" J Am Coll Cardiol **52**(24): 1949-1956.

Lee, J. H., J. H. O'Keefe, D. Bell, D. D. Hensrud and M. F. Holick (2008). "Vitamin D Deficiency: An Important, Common, and Easily Treatable Cardiovascular Risk Factor?" Journal of the American College of Cardiology **52**(24): 1949-1956.

Leifheit-Nestler, M., F. Kirchhoff, J. Nespor, B. Richter, B. Soetje, M. Klintschar, J. Heineke and D. Haffner (2018). "Fibroblast growth factor 23 is induced by an activated renin-angiotensin-aldosterone system in cardiac myocytes and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts." Nephrol Dial Transplant.

Leikauf, G. D., L. M. Leming, J. R. O'Donnell and C. A. Doupnik (1989). "Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein." J Appl Physiol (1985) **66**(1): 171-178.

Li, Y. C., J. Kong, M. Wei, Z. F. Chen, S. Q. Liu and L. P. Cao (2002). "1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system." J Clin Invest **110**(2): 229-238.

Li, Y. C., G. Qiao, M. Uskokovic, W. Xiang, W. Zheng and J. Kong (2004). "Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure." J Steroid Biochem Mol Biol **89-90**(1-5): 387-392.

Liao, D., J. Creason, C. Shy, R. Williams, R. Watts and R. Zweidinger (1999). "Daily variation of particulate air pollution and poor cardiac autonomic control in the elderly." Environmental Health Perspectives **107**(7): 521.

Lin, J. W., C. H. Chang, J. L. Caffrey, L. C. Wu and M. S. Lai (2014). "Examining the association of olmesartan and other angiotensin receptor blockers with overall and cause-specific mortality." Hypertension **63**(5): 968-976.

Lips, P. (2006). "Vitamin D physiology." Progress in biophysics and molecular biology **92**(1): 4-8.

Loga, F., K. Domes, M. Freichel, V. Flockerzi, A. Dietrich, L. Birnbaumer, F. Hofmann and J. W. Wegener (2013). "The role of cGMP/cGKI signalling and Trpc channels in regulation of vascular tone." Cardiovasc Res **100**(2): 280-287.

Lucidarme, O., E. Messai, T. Mazzoni, M. Arcade and D. du Cheyron (2010). "Incidence and risk factors of vitamin D deficiency in critically ill patients: results from a prospective observational study." Intensive Care Med **36**(9): 1609-1611.

Lynne Page, S. (1994). ""The Death-Dealing Smog over Donora, Pennsylvania": Industrial Air Pollution, Public Health Policy, and the Politics of Expertise, 1948-1949." Environmental History Review **18**(1): 117-139.

Maiya, S., I. Sullivan, J. Allgrove, R. Yates, M. Malone, C. Brain, N. Archer, Q. Mok, P. Daubeney, R. Tulloh and M. Burch (2008). "Hypocalcaemia and vitamin D deficiency: an important, but preventable, cause of life-threatening infant heart failure." Heart **94**(5): 581-584.

Malczyk, M., A. Erb, C. Veith, H. A. Ghofrani, R. T. Schermuly, T. Gudermann, A. Dietrich, N. Weissmann and A. Sydykov (2017). "The Role of Transient Receptor Potential Channel 6 Channels in the Pulmonary Vasculature." Front Immunol **8**: 707.
Mann, M., D. Exner, B. Hemmelgarn, D. Sola, T. Turin, L. Ellis and S. Ahmed (2013). "Vitamin D Levels Are Associated with Cardiac Autonomic Activity in Healthy Humans." Nutrients **5**(6): 2114-2127.

Mann, M. C., M. D. Hollenberg, D. A. Hanley and S. B. Ahmed (2015). "Vitamin D, the autonomic nervous system, and cardiovascular risk." Physiol Rep **3**(4).
Martins, D., M. Wolf, D. Pan and et al. (2007). "Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin d in the united states: Data from the third national health and nutrition examination survey." Archives of Internal Medicine **167**(11): 1159-1165.

Masson, S., N. Agabiti, T. Vago, M. Miceli, F. Mayer, T. Letizia, U. Wienhues-Thelen, G. F. Mureddu, M. Davoli, A. Boccanelli, R. Latini and P. s. The Investigators of the (2014). "The fibroblast growth factor-23 and Vitamin D emerge as nontraditional risk factors and may affect cardiovascular risk." Journal of Internal Medicine: n/a-n/a.

Mathur, S., A. R. Shah, A. W. Ahlberg, D. M. Katten and G. V. Heller (2010). "Blunted heart rate response as a predictor of cardiac death in patients undergoing vasodilator

stress technetium-99m sestamibi gated SPECT myocardial perfusion imaging." J Nucl Cardiol **17**(4): 617-624.

Matsumura, Y., H. Aizawa, T. Shiraki-Iida, R. Nagai, M. Kuro-o and Y. Nabeshima (1998). "Identification of the human klotho gene and its two transcripts encoding membrane and secreted klotho protein." Biochem Biophys Res Commun **242**(3): 626-630.

McNamara, C. R., J. Mandel-Brehm, D. M. Bautista, J. Siemens, K. L. Deranian, M. Zhao, N. J. Hayward, J. A. Chong, D. Julius, M. M. Moran and C. M. Fanger (2007). "TRPA1 mediates formalin-induced pain." Proc Natl Acad Sci U S A **104**(33): 13525-13530.

McPate, M., G. Bhalay, M. Beckett, S. Fairbrother, M. Gosling, P. J. Groot-Kormelink, R. Lane, T. Kent, M. T. Van Diepen, P. Tranter and J. M. Verkuyl (2014). "The development of automated patch clamp assays for canonical transient receptor potential channels TRPC3, 6, and 7." Assay Drug Dev Technol **12**(5): 282-292.

Mencke, R. and J. L. Hillebrands (2017). "The role of the anti-ageing protein Klotho in vascular physiology and pathophysiology." Ageing Res Rev **35**: 124-146.

Mirowsky, J. E., M. S. Carraway, R. Dhingra, H. Tong, L. Neas, D. Diaz-Sanchez, W. Cascio, M. Case, J. Crooks, E. R. Hauser, Z. Elaine Dowdy, W. E. Kraus and R. B. Devlin (2017). "Ozone exposure is associated with acute changes in inflammation, fibrinolysis, and endothelial cell function in coronary artery disease patients." Environ Health **16**(1): 126.

Mousa, A., N. Naderpoor and M. P. de Courten (2017). "Vitamin D supplementation has no effect on insulin sensitivity or secretion in vitamin D-deficient, overweight or obese adults: a randomized placebo-controlled trial." **105**(6): 1372-1381.

Mousa, A., N. Naderpoor and J. Johnson (2017). "Effect of vitamin D supplementation on inflammation and nuclear factor kappa-B activity in overweight/obese adults: a randomized placebo-controlled trial." **7**(1): 15154.

Mozaffarian, D., E. J. Benjamin, A. S. Go, D. K. Arnett, M. J. Blaha, M. Cushman, S. R. Das, S. de Ferranti, J. P. Despres, H. J. Fullerton, V. J. Howard, M. D. Huffman, C. R. Isasi, M. C. Jimenez, S. E. Judd, B. M. Kissela, J. H. Lichtman, L. D. Lisabeth, S. Liu, R. H. Mackey, D. J. Magid, D. K. McGuire, E. R. Mohler, 3rd, C. S. Moy, P. Muntner, M. E. Mussolino, K. Nasir, R. W. Neumar, G. Nichol, L. Palaniappan, D. K. Pandey, M. J. Reeves, C. J. Rodriguez, W. Rosamond, P. D. Sorlie, J. Stein, A. Towfighi, T. N. Turan, S. S. Virani, D. Woo, R. W. Yeh and M. B. Turner (2016). "Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association." Circulation **133**(4): e38-360.

Muscogiuri, G., C. Annweiler, G. Duval, S. Karras, G. Tirabassi, G. Salvio, G. Balercia, S. Kimball, K. Kotsa, L. Mascitelli, H. P. Bhattoa and A. Colao (2017). "Vitamin D and cardiovascular disease: From atherosclerosis to myocardial infarction and stroke." Int J Cardiol **230**: 577-584.

Navarro-Garcia, J. A., M. Fernandez-Velasco, C. Delgado, J. F. Delgado, O. M. Kuro, L. M. Ruilope and G. Ruiz-Hurtado (2018). "PTH, vitamin D, and the FGF-23-klotho axis and heart: going beyond the confines of nephrology." Eur J Clin Invest.

Nigwekar, S. U. and R. Thadhani (2013). "Vitamin D receptor activation: cardiovascular and renal implications." Kidney Int Suppl (2011) **3**(5): 427-430.

Nishida, M., K. Watanabe, M. Nakaya and H. Kurose (2010). "[Mechanism of cardiac hypertrophy via diacylglycerol-sensitive TRPC channels]." Yakugaku Zasshi **130**(3): 295-302.

Norman, P. E. and J. T. Powell (2014). "Vitamin D and Cardiovascular Disease." Circulation Research **114**(2): 379-393.

Ogard, C. G., S. B. Sondergaard, H. Vestergaard, H. Jakobsen and S. L. Nielsen (2005). "Myocardial perfusion defects and the left ventricular ejection fraction disclosed by scintigraphy in patients with primary hyperparathyroidism." World J Surg **29**(7): 914-916.

Ozcan, O. U., A. Gurlek, E. Gursoy, D. M. Gerede and C. Erol (2015). "Relation of vitamin D deficiency and new-onset atrial fibrillation among hypertensive patients." J Am Soc Hypertens **9**(4): 307-312.

Park, S. P., B. M. Kim, J. Y. Koo, H. Cho, C. H. Lee, M. Kim, H. S. Na and U. Oh (2008). "A tarantula spider toxin, GsMTx4, reduces mechanical and neuropathic pain." Pain **137**(1): 208-217.

Pham, H., A. C. Bonham, K. E. Pinkerton and C.-Y. Chen (2009). "Central neuroplasticity and decreased heart rate variability after particulate matter exposure in mice." Environmental health perspectives **117**(9): 1448.

Pilz, S., W. März, B. Wellnitz, U. Seelhorst, A. Fahrleitner-Pammer, H. P. Dimai, B. O. Boehm and H. Dobnig (2008). "Association of Vitamin D Deficiency with Heart Failure and Sudden Cardiac Death in a Large Cross-Sectional Study of Patients Referred for Coronary Angiography." The Journal of Clinical Endocrinology & Metabolism **93**(10): 3927-3935.

Pope, C. A., 3rd, M. L. Hansen, R. W. Long, K. R. Nielsen, N. L. Eatough, W. E. Wilson and D. J. Eatough (2004). "Ambient particulate air pollution, heart rate variability, and blood markers of inflammation in a panel of elderly subjects." Environ Health Perspect **112**(3): 339-345.

Pradhan, A. D. and J. E. Manson (2016). "Update on the Vitamin D and OmegA-3 trial (VITAL)." J Steroid Biochem Mol Biol **155**(Pt B): 252-256.

Puhl, S. L., K. L. Weeks, A. Ranieri and M. Avkiran (2016). "Assessing structural and functional responses of murine hearts to acute and sustained beta-adrenergic stimulation in vivo." J Pharmacol Toxicol Methods **79**: 60-71.

Quraishi, S. A. and C. A. Camargo, Jr. (2012). "Vitamin D in acute stress and critical illness." Curr Opin Clin Nutr Metab Care **15**(6): 625-634.

Rai, V. and D. K. Agrawal (2017). "Role of Vitamin D in Cardiovascular Diseases." Endocrinol Metab Clin North Am **46**(4): 1039-1059.

Rajagopalan, S. and R. D. Brook (2012). "Air Pollution and Type 2 Diabetes: Mechanistic Insights." Diabetes **61**(12): 3037-3045.

Razzaque, M. S. (2008). "Klotho and Na+,K+-ATPase activity: solving the calcium metabolism dilemma?" Nephrol Dial Transplant **23**(2): 459-461.

Reeves, P. G., F. H. Nielsen and G. C. Fahey (1993). "AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet." The Journal of Nutrition **123**(11): 1939-1951.

Requia, W. J., M. D. Adams, A. Arain, S. Papatheodorou, P. Koutrakis and M. Mahmoud (2017). "Global Association of Air Pollution and Cardiorespiratory Diseases: A Systematic Review, Meta-Analysis, and Investigation of Modifier Variables." Am J Public Health: e1-e8.

Rodriguez, A. J., A. Mousa, P. R. Ebeling and D. Scott (2018). "Effects of vitamin D supplementation on inflammatory markers in heart failure: a systematic review and meta-analysis of randomized controlled trials." **8**(1): 1169.

Rowan, W. H., 3rd, M. J. Campen, L. B. Wichers and W. P. Watkinson (2007). "Heart rate variability in rodents: uses and caveats in toxicological studies." Cardiovasc Toxicol **7**(1): 28-51.

Rowell, J., N. Koitabashi and D. A. Kass (2010). "TRP-ing up heart and vessels: canonical transient receptor potential channels and cardiovascular disease." J Cardiovasc Transl Res **3**(5): 516-524.

Saccone, D., F. Asani and L. Bornman (2015). "Regulation of the vitamin D receptor gene by environment, genetics and epigenetics." Gene **561**(2): 171-180.

Santillan, G. E., G. Vazquez and R. L. Boland (1999). "Activation of a beta-adrenergic-sensitive signal transduction pathway by the secosteroid hormone 1,25-(OH)2-vitamin D3 in chick heart." J Mol Cell Cardiol **31**(5): 1095-1104.

Savonen, K. P., T. A. Lakka, J. A. Laukkanen, P. M. Halonen, T. H. Rauramaa, J. T. Salonen and R. Rauramaa (2006). "Heart rate response during exercise test and cardiovascular mortality in middle-aged men." Eur Heart J **27**(5): 582-588.

Sawamura, S., H. Shirakawa, T. Nakagawa, Y. Mori and S. Kaneko (2017). Frontiers in Neuroscience

TRP Channels in the Brain: What Are They There For? Neurobiology of TRP Channels. nd and T. L. R. Emir. Boca Raton (FL), CRC Press/Taylor & Francis
(c) 2018 by Taylor & Francis Group, LLC.**:** 295-322.

Scragg, R., M. Sowers and C. Bell (2007). "Serum 25-hydroxyvitamin D, ethnicity, and blood pressure in the Third National Health and Nutrition Examination Survey." Am J Hypertens **20**(7): 713-719.

Scragg, R., A. W. Stewart, D. Waayer, C. M. M. Lawes, L. Toop, J. Sluyter, J. Murphy, K. T. Khaw and C. A. Camargo, Jr. (2017). "Effect of Monthly High-Dose Vitamin D Supplementation on Cardiovascular Disease in the Vitamin D Assessment Study : A Randomized Clinical Trial." JAMA Cardiol **2**(6): 608-616.

Seo, K., P. P. Rainer, V. Shalkey Hahn, D. I. Lee, S. H. Jo, A. Andersen, T. Liu, X. Xu, R. N. Willette, J. J. Lepore, J. P. Marino, Jr., L. Birnbaumer, C. G. Schnackenberg and D. A. Kass (2014). "Combined TRPC3 and TRPC6 blockade by selective small-molecule or genetic deletion inhibits pathological cardiac hypertrophy." Proc Natl Acad Sci U S A **111**(4): 1551-1556.

Shaffer, F. and J. P. Ginsberg (2017). "An Overview of Heart Rate Variability Metrics and Norms." Front Public Health **5**: 258.

Shen, M. J. and D. P. Zipes (2014). "Role of the autonomic nervous system in modulating cardiac arrhythmias." Circ Res **114**(6): 1004-1021.

Sim, V. M. and R. E. Pattle (1957). "Effect of possible smog irritants on human subjects." J Am Med Assoc **165**(15): 1908-1913.

Snow, S. J., M. A. McGee, A. Henriquez, J. E. Richards, M. C. Schladweiler, A. D. Ledbetter and U. P. Kodavanti (2017). "Respiratory Effects and Systemic Stress Response Following Acute Acrolein Inhalation in Rats." Toxicol Sci **158**(2): 454-464.

Song, S., P. Gao, H. Xiao, Y. Xu and L. Y. Si (2013). "Klotho Suppresses Cardiomyocyte Apoptosis in Mice with Stress-Induced Cardiac Injury via Downregulation of Endoplasmic Reticulum Stress." PLoS ONE **8**(12): e82968.

Song, S. and L. Y. Si (2015). "Klotho ameliorated isoproterenol-induced pathological changes in cardiomyocytes via the regulation of oxidative stress." Life Sci **135**: 118-123.

Stein, P. K., P. P. Domitrovich, N. Hui, P. Rautaharju and J. Gottdiener (2005). "Sometimes higher heart rate variability is not better heart rate variability: results of graphical and nonlinear analyses." J Cardiovasc Electrophysiol **16**(9): 954-959.

Stratford, K., N. Haykal-Coates, L. Thompson, Q. T. Krantz, C. King and J. Krug (2018). "Early-Life Persistent Vitamin D Deficiency Alters Cardiopulmonary Responses to Particulate Matter-Enhanced Atmospheric Smog in Adult Mice."

Strewler, G. J. (2007). "Untangling klotho's role in calcium homeostasis." Cell Metab **6**(2): 93-95.

Sundar, I. K. and I. Rahman (2011). "Vitamin D and Susceptibility of Chronic Lung Diseases: Role of Epigenetics." Frontiers in Pharmacology **2**: 50.

Takenaka, T., T. Inoue, T. Miyazaki, H. Kobori, A. Nishiyama, N. Ishii, M. Hayashi and H. Suzuki (2017). "Klotho suppresses the renin-angiotensin system in adriamycin nephropathy." Nephrol Dial Transplant **32**(5): 791-800.

Takeshita, K., T. Fujimori, Y. Kurotaki, H. Honjo, H. Tsujikawa, K. Yasui, J. K. Lee, K. Kamiya, K. Kitaichi, K. Yamamoto, M. Ito, T. Kondo, S. Iino, Y. Inden, M. Hirai, T. Murohara, I. Kodama and Y. Nabeshima (2004). "Sinoatrial node dysfunction and early unexpected death of mice with a defect of klotho gene expression." Circulation **109**(14): 1776-1782.

Talmor, Y., E. Golan, S. Benchetrit, J. Bernheim, O. Klein, J. Green and G. Rashid (2008). "Calcitriol blunts the deleterious impact of advanced glycation end products on endothelial cells." Am J Physiol Renal Physiol **294**(5): F1059-1064.

Tamez, H., S. Kalim and R. I. Thadhani (2013). "Does vitamin D modulate blood pressure?" Curr Opin Nephrol Hypertens **22**(2): 204-209.

Tang, G., Y. Shen, P. Gao, S. S. Song and L. Y. Si (2018). "Klotho attenuates isoproterenol-induced hypertrophic response in H9C2 cells by activating Na(+)/K(+)-ATPase and inhibiting the reverse mode of Na(+)/Ca(2+)-exchanger."

Tare, M., S. J. Emmett, H. A. Coleman, C. Skordilis, D. W. Eyles, R. Morley and H. C. Parkington (2011). "Vitamin D insufficiency is associated with impaired vascular endothelial and smooth muscle function and hypertension in young rats." J Physiol **589**(Pt 19): 4777-4786.

Thayer, J. F., S. S. Yamamoto and J. F. Brosschot (2010). "The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors." International Journal of Cardiology **141**(2): 122-131.

Tishkoff, D. X., K. A. Nibbelink, K. H. Holmberg, L. Dandu and R. U. Simpson (2008). "Functional vitamin D receptor (VDR) in the t-tubules of cardiac myocytes: VDR knockout cardiomyocyte contractility." Endocrinology **149**(2): 558-564.

Tomilin, V., M. Mamenko, O. Zaika and O. Pochynyuk (2016). "Role of renal TRP channels in physiology and pathology." Semin Immunopathol **38**(3): 371-383.

Toraason, M., M. E. Luken, M. Breitenstein, J. A. Krueger and R. E. Biagini (1989). "Comparative toxicity of allylamine and acrolein in cultured myocytes and fibroblasts from neonatal rat heart." Toxicology **56**(1): 107-117.

Trehan, N., L. Afonso, D. L. Levine and P. D. Levy (2017). "Vitamin D Deficiency, Supplementation, and Cardiovascular Health." Crit Pathw Cardiol **16**(3): 109-118.

Tsuji, H., M. G. Larson, F. J. Venditti, Jr., E. S. Manders, J. C. Evans, C. L. Feldman and D. Levy (1996). "Impact of reduced heart rate variability on risk for cardiac events. The Framingham Heart Study." Circulation **94**(11): 2850-2855.

Tyrankiewicz, U., T. Skorka, M. Jablonska, P. Petkow-Dimitrow and S. Chlopicki (2013). "Characterization of the cardiac response to a low and high dose of dobutamine in the mouse model of dilated cardiomyopathy by MRI in vivo." J Magn Reson Imaging **37**(3): 669-677.

Wadhwania, R. (2017). "Is Vitamin D Deficiency Implicated in Autonomic Dysfunction?" J Pediatr Neurosci **12**(2): 119-123.

Walters, M. R. "1,25-Dihydroxyvitamin D3 receptors identified in the rat heart." Journal of molecular and cellular cardiology **18**(1): 67-72.

Wang, J., Y. Ma, F. Sachs, J. Li and T. M. Suchyna (2016). "GsMTx4-D is a cardioprotectant against myocardial infarction during ischemia and reperfusion." J Mol Cell Cardiol **98**: 83-94.

Wang, T. J., M. J. Pencina, S. L. Booth, P. F. Jacques, E. Ingelsson, K. Lanier, E. J. Benjamin, R. B. D'Agostino, M. Wolf and R. S. Vasan (2008). "Vitamin D deficiency and risk of cardiovascular disease." Circulation **117**(4): 503-511.

Watanabe, H., K. Iino, T. Ohba and H. Ito (2013). "Possible involvement of TRP channels in cardiac hypertrophy and arrhythmia." Curr Top Med Chem **13**(3): 283-294.

Watanabe, H., M. Murakami, T. Ohba, K. Ono and H. Ito (2009). "The pathological role of transient receptor potential channels in heart disease." Circ J **73**(3): 419-427.

Weng, S., J. E. Sprague, J. Oh, A. E. Riek, K. Chin, M. Garcia and C. Bernal-Mizrachi (2013). "Vitamin D deficiency induces high blood pressure and accelerates atherosclerosis in mice." PLoS One **8**(1): e54625.

Widdicombe, J. and L. Y. Lee (2001). "Airway reflexes, autonomic function, and cardiovascular responses." Environ Health Perspect **109 Suppl 4**: 579-584.

Wilkins, E. T. (1954). "Air Pollution and the London Fog of December, 1952." The Journal of the Royal Society for the Promotion of Health **74**(1): 1-21.

Wong, M. S., R. Delansorne, R. Y. Man, P. Svenningsen and P. M. Vanhoutte (2010). "Chronic treatment with vitamin D lowers arterial blood pressure and reduces endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat." Am J Physiol Heart Circ Physiol **299**(4): H1226-1234.

Wu, Y. L., J. Xie, S. W. An, N. Oliver, N. X. Barrezueta, M. H. Lin, L. Birnbaumer and C. L. Huang (2017). "Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho." Kidney Int **91**(4): 830-841.

Xiao, X., H. X. Liu, K. Shen, W. Cao and X. Q. Li (2017). "Canonical Transient Receptor Potential Channels and Their Link with Cardio/Cerebro-Vascular Diseases." Biomol Ther (Seoul) **25**(5): 471-481.

Xie, J., S. K. Cha, S. W. An, O. M. Kuro, L. Birnbaumer and C. L. Huang (2012). "Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart." Nat Commun **3**: 1238.

Yamaguchi, Y., G. Iribe, M. Nishida and K. Naruse (2017). "Role of TRPC3 and TRPC6 channels in the myocardial response to stretch: Linking physiology and pathophysiology." Prog Biophys Mol Biol **130**(Pt B): 264-272.

Yang, B. Y., Z. M. Qian, M. G. Vaughn, E. J. Nelson, S. C. Dharmage, J. Heinrich, S. Lin, W. R. Lawrence, H. Ma, D. H. Chen, L. W. Hu, X. W. Zeng, S. L. Xu, C. Zhang and G. H. Dong (2017). "Is prehypertension more strongly associated with long-term ambient air pollution exposure than hypertension? Findings from the 33 Communities Chinese Health Study." Environ Pollut **229**: 696-704.

Yang, K., C. Wang, L. Nie, X. Zhao, J. Gu, X. Guan, S. Wang, T. Xiao, X. Xu, T. He, X. Xia, J. Wang and J. Zhao (2015). "Klotho Protects Against Indoxyl Sulphate-Induced Myocardial Hypertrophy." J Am Soc Nephrol **26**(10): 2434-2446.

Yang, T. and C. Xu (2017). "Physiology and Pathophysiology of the Intrarenal Renin-Angiotensin System: An Update." J Am Soc Nephrol **28**(4): 1040-1049.

Yu, L., W. Meng, J. Ding and M. Cheng (2016). "Klotho inhibits angiotensin II-induced cardiomyocyte hypertrophy through suppression of the AT1R/beta catenin pathway." Biochem Biophys Res Commun **473**(2): 455-461.

Yue, Z., J. Xie, A. S. Yu, J. Stock, J. Du and L. Yue (2015). "Role of TRP channels in the cardiovascular system." American Journal of Physiology - Heart and Circulatory Physiology **308**(3): H157-H182.

Zhang, M., X. Xu, H. Liu, H. Li, J. Zhang and M. Gao (2017). "Nocturnal diastolic blood pressure decline is associated with higher 25-hydroxyvitamin D level and standing plasma renin activity in a hypertensive population." Clinical and Experimental Hypertension **39**(8): 685-690.

Zhou, H. J., H. Li, M. Q. Shi, X. N. Mao, D. L. Liu, Y. R. Chang, Y. M. Gan, X. Kuang and J. R. Du (2017). "Protective Effect of Klotho against Ischemic Brain Injury Is Associated with Inhibition of RIG-I/NF-kappaB Signaling." Front Pharmacol **8**: 950.

Zhou, L., H. Mo, J. Miao, D. Zhou, R. J. Tan, F. F. Hou and Y. Liu (2015). "Klotho Ameliorates Kidney Injury and Fibrosis and Normalizes Blood Pressure by Targeting the Renin-Angiotensin System." Am J Pathol **185**(12): 3211-3223.

Zhou, L., H. Mo, J. Miao, D. Zhou, R. J. Tan, F. F. Hou and Y. Liu (2015). "Klotho Ameliorates Kidney Injury and Fibrosis and Normalizes Blood Pressure by Targeting the Renin-Angiotensin System." The American Journal of Pathology **185**(12): 3211-3223.

Zhu, H., Y. Gao, S. Zhu, Q. Cui and J. Du (2017). "Klotho Improves Cardiac Function by Suppressing Reactive Oxygen Species (ROS) Mediated Apoptosis by Modulating Mapks/Nrf2 Signaling in Doxorubicin-Induced Cardiotoxicity." Med Sci Monit **23**: 5283-5293.

Zittermann, A. (2006). "Vitamin D and disease prevention with special reference to cardiovascular disease." Prog Biophys Mol Biol **92**(1): 39-48.

Zosky, G. R., P. H. Hart, A. J. Whitehouse, M. M. Kusel, W. Ang, R. E. Foong, L. Chen, P. G. Holt, P. D. Sly and G. L. Hall (2014). "Vitamin D deficiency at 16 to 20 weeks' gestation is associated with impaired lung function and asthma at 6 years of age." Ann Am Thorac Soc **11**(4): 571-577.

Zou, Z. Q., J. Xu, L. Li and Y. S. Han (2015). "Down-regulation of SENCR promotes smooth muscle cells proliferation and migration in db/db mice through up-regulation of FoxO1 and TRPC6." Biomed Pharmacother **74**: 35-41.