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Reduced SIRT3 contributes to large elastic artery stiffness with aging

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REDUCED SIRT3 CONTRIBUTES TO LARGE ELASTIC ARTERY STIFFNESS WITH AGING

By

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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To my parents Greg and Margaret, for believing in me even when I did not believe in myself.
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ABSTRACT

Age-related increases in arterial stiffness are mediated in part by mitochondrial dysfunction. Sirtuin 3 (SIRT3) is a mitochondrial NAD^+-dependent deacetylase that regulates mitochondrial function. SIRT3 deficiency contributes to physiological dysfunction in a variety of pathological conditions. Here, we tested the hypothesis that age-associated arterial stiffness, assessed by aortic pulse wave velocity (PWV), would be accompanied with decreased renal and aortic SIRT3 expression and activity due to decreased NAD^+ levels. We further tested whether boosting NAD^+ concentration with nicotinamide riboside (NR), a NAD^+ precursor, for 6 months would reverse the effects of aging. Old (~26 mo, n = 9) C57BL/6 male mice had higher PWV vs. young (6 mo, n = 10) (448 ± 14 vs 382 ± 13, $p < 0.005$), which was associated with reduced arterial SIRT3 protein (0.365 ± 0.088 AU's vs 1.000 ± 0.000); $p < 0.05$). Furthermore, SIRT3 deficient male mice demonstrated higher PWV compared to age-matched control mice (480 ± 21 n = 6 vs. 391 ±12 n = 7, $p < 0.005$). Aortic SIRT3 protein was negatively correlated with PWV (r=-0.7798, $p < 0.005$). Old mice also exhibited reduced kidney SIRT3 protein (0.73 ± 0.10 AU's) compared to young controls (1.00 ± 0.00; $p = 0.0192$) and reduced NAD^+ (918.6 ± 50.5 pmol/mg vs. young 1302.0 ± 56.6 pmol/mg, $p = 0.0036$). Old mice supplemented with NR had increased NAD^+ concentration in kidney tissue (1303.0 ± 90.2 pmol/mg) however, had no effect on normalizing age-associated arterial stiffness (402 ± 18 old with NR vs 418 ± 15 old; $p = 0.78$). Here we show for that SIRT3 protein correlates with aortic stiffness and may be required for the maintenance of healthy arteries and for the first time that supplementation with NR, a commercially available supplement, ameliorates age-associated decreases in renal NAD^+ demonstrating therapeutic potential in kidney disease.

PUBLIC ABSTRACT

During the 20th Century the United States saw a change in the leading causes of death, with a shift from infectious diseases such as tuberculosis, pneumonia, and influenza to chronic diseases associated with aging such as cardiovascular disease (Centers for Disease Control). Recent research has unveiled Sirtuin 3 (SIRT3), a protein that requires NAD^+ as a substrate, as a major player in age-related diseases throughout the body. The goal of this thesis was to measure SIRT3 protein and NAD^+ levels in young and old male mice, and to determine if aging causes changes that has meaningful impact on vascular function, such aortic stiffness, which is both a risk factor and predictor of cardiovascular diseases. Finally, we investigated whether increasing NAD^+ with a recently discovered form of vitamin B_3, nicotinamide riboside, could increase NAD^+ concentration in tissue and reverse age-related aortic stiffening.

We found that SIRT3 was associated with increased aortic stiffness perhaps due to age-related decreases in NAD^+. Furthermore, mice deficient in SIRT3 had increased aortic stiffness compared to their age-matched counterparts, indicating that SIRT3 plays a role in maintenance of healthy arteries. Long term NR supplementation increased NAD^+ concentration in kidney tissue in old mice, but did not have an effect on age associated arterial stiffness. We show here for the first time that supplementation with NR restores NAD^+ levels in the kidney with aging, indicating therapeutic potential in kidney disease.

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CHAPTER I

INTRODUCTION

Cardiovascular diseases (CVD) are the number one cause of death in the United States. CVD, or diseases affecting the heart and blood vessels of the body, were responsible for 30.8% of deaths in 2013 with an estimated 85.6 million Americans living with some form of CVD (Mozaffarian, et al., 2015). This incredibly high prevalence of CVD comes with an ever-increasing cost. The estimated total cost of CVD (including treatment and lost work) reached $656 billion in 2015 and is expected to reach $1.2 trillion by 2030. Importantly, of the 85.6 million Americans with CVD, 43.7 million are estimated to be older than 60 years of age, supporting age as an important factor in the progression of CVD (Mozaffarian, et al., 2015). This issue will become more widespread as the number of individuals 65 and over in the US is projected to more than double from 43.1 million to 83.7 million by the year 2050 (Ortman, Velkoff, & Hogan, 2014).

Changes in Arterial Stiffness with Aging

Although dyslipidemia, physical inactivity, diabetes, and tobacco use are important risk factors for CVD, the most predictive risk factor for CVD is age (North & Sinclair, 2012). Age-associated CVD risk is due, in part, to alterations within arteries such as the development of endothelial dysfunction and stiffening of the large elastic arteries (aorta and carotids) (Najjar, Scuteri, & Lakatta, 2005). Vascular dysfunction develops due to remodeling processes that occur with age, which contribute to changes in both the structure and function of blood vessels (Najjar, Scuteri, & Lakatta, 2005).

Blood vessels are comprised of three layers; the intima, media, and adventitia. These layers each undergo differing remodeling processes with aging. The outermost layer, the

adventitia, undergoes structural changes of proteins whereby collagen increases while elastin decreases (Dietz, 2007). Collagen is a stiffer protein than elastin, and this change in composition in the adventitia results in a net increase in overall arterial stiffness primarily impacting the large elastic arteries such as the aorta and carotid arteries (Dietz, 2007). Changes in the intima, the innermost layer of the vessel consisting of a monolayer of endothelial cells (i.e. endothelium) that produce the vascular-protective vasodilator molecule nitric oxide (NO). These changes can elicit changes observed in the media, the middle layer of the blood vessel consisting of vascular smooth muscle cells (VSMC) (Wilkinson, Franklin, & Cockcroft, 2004) (Schmitt, et al., 2005). After NO is produced by the endothelium it quickly diffuses to the VSCM activating cyclic guanosine monophosphate and causing vasodilation. With aging, NO bioavailability becomes impaired due to both decreased NO production via endothelial NO synthase (eNOS) and increased degradation of NO by superoxide (Mudau, Genis, Lochner, & Strijdom, 2012). The abundance of superoxide increases with aging due to increased production and decreased scavenging by antioxidant defense mechanisms such as superoxide dismutase (SOD) (Bratic & Larsson, 2013). This age-associated net increase in superoxide and decrease of bioavailable NO increases arterial tone (increased vasoconstriction) and has been demonstrated to increase arterial stiffness independent of changes in hemodynamics and heart rate (Wilkinson, Franklin, & Cockcroft, 2004) (Schmitt, et al., 2005). Furthermore, degradation of NO by superoxide produces the potent free radical peroxynitrite ($ONOO^-$) (Seals, Jablonski, & Donato, 2011) (Taddei, et al., 2001). $ONOO^-$ post-translationally modifies a variety of proteins rendering them dysfunctional, further promoting reactive oxygen species (ROS) production and vascular dysfunction (Murray, Taylor, Zhang, Ghosh, & Capaldi, 2003). Increases in nitrotyrosine abundance, a marker of cell damage resulting from $ONOO^-$, are associated with aging (Sindler 2011). Increased ROS also

contributes to the generation of advanced glycation end products (AGEs), which further exacerbate aortic stiffness by cross-linking with collagen in the arterial wall. (Goldin, Beckman, Schmidt, & Creager, 2006) (Wagenseil & Mecham, 2012) (Sindler, DeVan, Fleenor, & Seals, 2014).

When the heart pumps blood, an elastic aorta buffers the pulsatile flow caused by left ventricular ejection allowing for more continuous flow to perfuse organs and tissues (Laurent, et al., 2006) (London & Pannier, 2010). Ejection from the left ventricle generates a pulse wave that travels down the aorta throughout the arterial tree. When the walls of large elastic arteries are stiffer, the velocity of the pulse wave is increased. The forward traveling wave is reflected at points of discontinuity in the tree, causing a reflected wave that travels back to the aorta and the heart (Laurent, et al., 2006) (London & Pannier, 2010). In healthy vessels, which are less stiff, the reflected pulse wave returns to the heart during diastole assisting with coronary perfusion (Davies, et al., 2010). With decreased compliance and increased arterial stiffness the reflected waves return to the heart faster, potentially arriving early enough to act in opposition to the heart (increasing cardiac afterload and failing to assist in coronary perfusion) (Barodka, Joshi, Berkowitz, Hogue, & Nyhan, 2011). This increases the workload of the heart, decreases myocardial oxygen supply, and contributes to left ventricular hypertrophy and fibrosis (Laurent, et al., 2006). Pulse wave velocity (PWV) is the gold standard measure of arterial stiffness where faster pulse wave velocities indicate stiffer vessels (Nichols, 2005). Increased pulse wave velocity is associated with end organ damage of sensitive organs such as the kidneys. For example, it has been documented that increased PWV contributes to impaired renal function measured by albuminuria and decreased glomerular filtration rate (Weber, et al., 2005) and higher indices of arterial stiffness are associated with a steeper decline in kidney function

(Sedaghat, et al., 2015). Importantly, PWV is a strong predictor of major cardiovascular events and death with advancing age and in individuals with kidney disease (Meaume, Benetos, Rudnichi, & Safar, 2001). Furthermore, individuals with chronic kidney disease (CKD) often die of CVD (Wright & Hutchison, 2009).

ROS, Mitochondria and Aging

In 1956 Dr. Denham Harman first proposed his Free Radical Theory of Aging, which suggested that the production of intracellular ROS is the major determinant of lifespan (Harman, 1956). There are several sources of ROS, including pro-oxidants such as, NADPH oxidase, eNOS uncoupling, xanthine oxidase, mitochondrial dysfunction, the oxidation of lipids by the peroxisome, and cyclooxygenases in the cytoplasm (Balaban, Nemoto, & Finkel, 2005). ROS generation is balanced by a collection of anti-oxidant enzymes including extracellular superoxide dismutase (ecSOD), copper zinc superoxide dismutase (CuZnSOD) manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase-1. Pathological conditions associated with aging are thought to be due to a dysregulated balance of endogenous pro- and anti-oxidants, resulting in excess ROS/oxidative stress (Dai, Rabinovitch, & Ungvari, 2012) (Wong & Ryan, 2015). With further study, the ROS produced during oxidative phosphorylation and ATP generation by the mitochondria was found to be an important contributor to overall oxidative stress. This lead to Harman expanding upon his Free Radical Theory in 1972, where he further postulated that the mitochondrial production of ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) was the main cause for age-related damage and physiological degeneration (Harman, 1972).

Recently, mitochondrial reactive oxygen species (mtROS) have been implicated as a possible cause of the age-associated changes in arterial function and structure (Gioscia-Ryan,

Battson, Cuevas, Zigler, & Sindler, 2016) (Dai, Rabinovitch, & Ungvari, 2012). As organisms age, the mitochondrial production of ROS increases in the vasculature because of a decline in overall mitochondrial function, declining activity of ROS scavenging enzymes, and mutations to mitochondrial DNA (Bratic & Larsson, 2013). Deficiency in mtROS scavenging proteins such as MnSOD severely exacerbates these phenotypes, resulting in impaired endothelial dependent dilation (Wenzel, et al., 2008). Mice lacking mitochondrial SOD2 (MnSOD) have stiffer aorta which is associated with increased collagen I and decreased elastin, compared to age-matched controls (Zhou, et al., 2011), indicating that loss of MnSOD (or increased mtROS) plays a key role in the pathogenesis of aortic stiffness. Markers of oxidative stress also correlate with increases in arterial stiffness in otherwise healthy humans (Patel, et al., 2011). Furthermore, increasing mitochondrial quality results in reduced oxidative stress and has been shown to decrease age-related arterial stiffness in mice (LaRocca, Hearon Jr., Henson, & Seals, 2014).

Similarly, mitochondria have recently become a therapeutic target for a variety of kidney pathologies including CKD and acute kidney injury (AKI) (Granata, Gassa, Tomei, Lupo, & Zaza, 2015). Renal function as determined by glomerular filtration rate (GFR) declines with advancing age due in part to impaired mitochondrial function within the kidneys. Furthermore, proteinuria, which is the presence of excessive proteins in the urine, is a diagnostic marker of CKD and has been associated with reduced mitochondrial function. In preclinical models of kidney disease, mitochondrial membrane potential, copy number of mtDNA, and ATP production all begin to decrease as the production of ROS increases (Che, Yuan, Huang, & Zhang, 2014) implicating mtROS as a probable cause of kidney dysfunction in CKD. In AKI, mitochondrial dysregulation causes tubular dysfunction leading to a rapid decrease in GFR as a result of increased mtROS (Pabla & Dong, 2008). Cisplatin, a widely used chemotherapy drug

with nephrotoxic effects, increases mitochondrial dysfunction (Morigi, et al., 2015) and dramatically increases ROS in the kidney (Pabla & Dong, 2008) increasing the need for anti-oxidant mechanisms.

NAD^+ and Sirtuin 3

Nicotinamide adenine dinucleotide (NAD) has emerged as a key regulator of ROS homeostasis in both individual cells and at the organism level (Houtkooper, Canto, Wanders, & Auwerx, 2010) (Nikiforov, Kulikova, & Ziegler, 2015). NAD^+ is an essential redox molecule that is responsible for carrying electrons in reduction/oxidation reactions, most notably in oxidative respiration in the mitochondria. More recently, NAD^+'s role in cellular homeostasis has come to light through the discovery of NAD^+-dependent proteins such as protein deacetylases (Sirtuins), poly(ADP-ribose) polymerases (PARPs), NADases such as CD38, and transcription factors that regulate metabolic functions (Houtkooper, Canto, Wanders, & Auwerx, 2010) (Imai & Guarente, 2014). Recently the role of NAD^+ as a substrate and important co-factor has gained biomedical interest with studies demonstrating that NAD^+ plays a major part in regulating energy metabolism, cell survival, and aging while simultaneously influencing the bioavailability of NAD^+ for redox reactions (Houtkooper, Canto, Wanders, & Auwerx, 2010).

Sirtuins (SIRT1-7) are a family of NAD^+-dependent enzymes that deacetylate key metabolic, inflammatory, and cardiovascular transcriptional regulators. Caloric restriction, exercise, and adenosine monophosphate-activated protein kinase (AMPK) all increase mitochondrial function and increase the expression of nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme in NAD^+ biosynthesis which is reduced with aging (Koltai, et al., 2010) (Winnik, Auwerx, Sinclair, & Matter, 2015). Increased expression of NAMPT leads to enhanced bioavailability of NAD^+, which can improve stimulation of SIRT1-7 (Winnik,

Auwerx, Sinclair, & Matter, 2015). In pathological conditions, such as aging, NAD^+ bioavailability becomes impaired due to decreased synthesis and recycling and increased utilization by other NAD^+ consuming pathways such as PARPs (Gomes, et al., 2013) (Braidy, et al., 2014) and CD38 (Imai & Guarente, 2014). Studies investigating the effects of boosting NAD^+ levels when NAD^+ is insufficient have shown promise in improving physiological function. Increasing available NAD^+ has been shown to prevent the development of high-glucose induced mesangial hypertrophy in rat glomerular mesangial cells (Zhuo, et al., 2011). Supplementation with the NAD^+ precursors nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN) increase NAD^+ levels as they bypass the rate-limiting, age-reduced enzyme NAMPT (Trammell, et al., 2016). Supplementation with NR has been demonstrated to fully maintain NAD^+ (Grozio, et al., 2013), thereby improving cognitive function in mouse models of Alzheimer's disease (Gong, et al., 2013), improving respiratory chain defects and exercise intolerance in mouse models of mitochondrial disease (Cerutti, et al., 2014), and delaying the progression of mitochondrial myopathy (Khan, et al., 2014). The increase in NAD^+ levels associated with NR supplementation may be rate limited by nicotinamide riboside kinase (NRK1) (Trammell, et al., 2016). However, NR is of particular interest as it is a more stable, commercially available supplement shown increase NAD^+ in humans (Trammell, et al., 2016). Supplementation with NMN, another NAD^+ precursor, has been shown to ameliorate large elastic artery stiffening in male C57BL/6 mice (de Picciotto, et al., 2016). These benefits may be attributed to increased substrate for the Sirtuins and enhanced Sirtuin signaling (Cerutti, et al., 2014) but were not directly measured in (de Picciotto, et al., 2016).

Sirtuin 1 has been more extensively studied and is located in the nuclear and cytosolic compartments of the cell. However, Sirtuin 3 (SIRT3) is localized in the mitochondria and is

emerging as a novel protein contributing to mitochondrial dysfunction with age and in settings of kidney disease. SIRT3 is a mitochondrial NAD^+-dependent deacetylase that is highly prevalent in metabolic tissues such as the heart, liver, brain, kidneys, and brown adipose tissue (Lombard, et al., 2007). SIRT3 activity is induced in response to changes in energy balance caused by stimuli such as caloric restriction, exercise, or cold exposure (Giralt & Villarroya, 2012) (van de Ven, Santos, & Haigis, 2017). SIRT3 regulates a variety of pathways related to mitochondrial health including ROS detoxification, ATP generation, and mitochondrial dynamics leading to preservation of mitochondrial function. SIRT3 improves mitochondrial function through the regulation of mitochondrial protein lysine acetylation status by removing (deacetylating) acetyl groups from lysine residues resulting in more functional proteins such as key enzymes necessary for maintaining homeostasis and mitochondrial health. SIRT3 activity regulates the function of MnSOD, a mitochondrial superoxide dismutase responsible for the catalysis of superoxide molecules (Tao, Vassilopoulos, Parisadou, Yan, & Gius, 2014). In addition, SIRT3 regulates Forkhead Box O3 (FOXO3a), a transcription factor responsible for promoting mitochondrial biogenesis and protecting mitochondria from oxidative damage (Tseng, Shieh, & Wang, 2013). SIRT3 also deacetylates all of the electron transport chain complexes including ATP synthase, increasing the electron transport chain's efficiency resulting in increased ATP production and decreased ROS production (van de Ven, Santos, & Haigis, 2017). Increased acetylation of mitochondrial proteins is associated with decreased functionality, resulting in decreased mitochondrial quality and quantity (Baeza, Smallegan, & Denu, 2016).

Interestingly, mice lacking SIRT3 (SIRT3KO) or mice with reduced expression of SIRT3 exhibit accelerated development of several diseases of aging related to hyperacetylated mitochondrial proteins (Sundaresan, et al., 2009) (Paulin, et al., 2014) (Morigi, et al., 2015).

Mice lacking SIRT3 exhibit signs of cardiac hypertrophy and interstitial fibrosis as young as eight weeks of age without being exposed to a hypertrophic stimulus (Sundaresan, et al., 2009). SIRT3 deficiency has also been linked to the development of pulmonary arterial hypertension (PAH) in rats, mice, and humans (Paulin, et al., 2014). Reduced SIRT3 protein also contributes to exacerbated renal damage and even death in response to cisplatin-induced AKI in mice (Morigi, et al., 2015). Normalizing SIRT3 activity with adenovirus gene therapy, induction of AMPK with AICAR, or the biphenol honokiol reverses or protects the dysfunction observed when SIRT3 is lacking in PAH (Paulin, et al., 2014), AKI (Morigi, et al., 2015), and cardiac hypertrophy (Pillai, et al., 2015). The severity of PAH in mice also has a dose-response relationship with SIRT3 (Paulin, et al., 2014). SIRT3 overexpressing transgenic mice are protected from cardiac hypertrophic stimuli (Sundaresan, et al., 2009). Furthermore, mice with overexpressed SIRT3 have enhanced SIRT3 signaling and less acetylated proteins contributing to increased mitochondrial function and decreased oxidative stress (Sundaresan, et al., 2009). Taken together, these data suggest that impaired SIRT3 activity contributes to mitochondrial dysfunction in a variety of tissues (including the kidneys), cell types, and pathological conditions such as aging and may be a key regulator involved in the development of age-associated vascular dysfunction (Kendrick, et al., 2011) (Winnik, et al., 2016).

Present Study

Although enhancing SIRT3 expression (or activity) has recently generated interest as a potential therapeutic target for a variety of diseases, an association between aortic SIRT3 and PWV has not yet been determined. Furthermore, the effects of activating SIRT3 on arterial stiffness are currently unknown, and no current translational therapy has been identified to safely enhance SIRT3 protein or activity. To address this, SIRT3 protein expression and its relation to

arterial stiffness was evaluated in young and old male C57 mice. We also evaluated the total collagen in the aorta and renal NAD^+ of young and old mice. Additionally, arterial stiffness was assessed in mice with reduced SIRT3. We also investigated the effects of supplementation with NR on NAD^+ levels and arterial stiffness.

Here we tested the hypothesis that age-related stiffness would be accompanied with decreased SIRT3 protein expression due to the age-related loss of NAD^+. Additionally, we tested the hypothesis that SIRT3 deficient mice would have increased aortic stiffness. Finally, we tested the hypothesis that supplementation with NR for six months would boost NAD^+ and normalize age-related aortic stiffness.

Aims

Specific Aim 1a: Determine aortic stiffness as assessed by PWV, aortic SIRT3 protein and collagen, and renal SIRT3 protein and NAD^+ in young (~6 months old) and old (~26 months old) C57BL/6 male mice.

Specific Aim 1b: Determine PWV in C57Bl/6 SIRT3 deficient mice (10 months old) and age matched control mice.

Specific Aim 2: Determine renal NAD^+ and aortic stiffness after 6 months of supplementation with NR (3g/kg in food) in old (~26-month-old) mice.

CHAPTER II

METHODS

Animals

Experiments were performed on young (~6 months old) & old (~26 months old) C57BL/6 male mice and C57BL/6 SIRT3 deficient & control male mice (10 months old). Mice were housed in clear plastic cages in rooms with temperature and light cycle (12-hour light-dark cycle, lights on between 6:00AM and 6:00PM). TEKLAD 2920X global soy protein free chow and tap water were provided ad-libitum. NR-treated animals consumed 2920X chow with 3g NR per kilogram chow for six months. Food intake was assessed over one week by Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments) operated by the Metabolic Phenotyping Core at the University of Iowa. Experimental protocols were approved by the Institutional Animal Care and Use Review Committee of the University of Iowa.

Aortic Pulse Wave Velocity

Aortic pulse wave velocity (PWV) was measured non-invasively using Doppler probes (Doppler Flow Velocity System, Indus Instruments, Webster, TX). Briefly, mice were anesthetized using 1.5-2% isoflourane and placed supine on a warming ECG monitoring plate set to 37°C (MouseMonitor S, Indus Instruments, Webster, TX). Electrode cream was placed on all four limbs, which were then secured to the ECG plates with medical tape. The first Doppler probe was placed at the transverse aortic arch, and the other placed on a segment of the abdominal aorta. The distance between the two probes was recorded and a snapshot was taken of the Doppler waveforms for later data analysis. Pre-ejection time was determined by measuring the time between the R-wave of the ECG to the foot of the Doppler signal. PWV was calculated

by dividing the distance between the transverse and abdominal probes by the difference in the thoracic and abdominal pre-ejection times.

Blood Pressure

Mice were placed in tube restraints on a warming platform and allowed 5 minutes to acclimate. An occlusion cuff was then placed as close to the base of the tail as possible with a sensor cuff placed within 2mm of the occlusion cuff (Kent Scientific CODA High Throughput). Blood pressure measures were taken using the volume pressure recording method utilizing zero acclimatization cycles, 20 regular cycles, and a deflation time of 20 seconds. Data was recorded utilizing Kent Scientific software.

Tissue Collection

C57BL/6 Mice were euthanized using isoflurane followed by vital tissue harvest. Kidney samples for NAD^+ were immediately removed and freeze-clamped at liquid nitrogen temperature upon euthanasia. The second kidney was removed, flash frozen with liquid nitrogen, pulverized and stored at -80°C for Western blot analysis. The aorta was dissected, cleaned of perivascular fat and divided for separate uses. One 1.5-2mm section of proximal aorta was placed in OCT gel and frozen for histology. The rest of the aorta was flash frozen with liquid nitrogen, pulverized and stored at -80°C for Western blot analysis.

Western Blotting

Tissue samples were prepared in lysis buffer (Sigma R0278 RIPA Buffer and Roche cOmplete protease cocktail) and protein content was quantified using BCA assay (Thermo-Scientific Pierce BCA Protein Assay Kit). Protein was loaded and run out on SDS-Page (BIO-RAD Mini-Protean TGX stain free, 4-20%,15 well, 15 µl gel) and transferred to nitrocellulose membrane. Membranes were blocked with 5% skim milk in Tris-buffered saline with Tween

(TBST) for 60 minutes, then washed 4x5 minutes with TBST. Membranes were then incubated in primary antibody overnight (Cell Signaling SIRT3 (D22A3) Rabbit mAb at 1:10,000 for kidney, 1:1000 for aorta; Cell signaling GAPDH (14C10) Rabbit mAb 1:10,000 for kidney, 1:1000 for aorta). The membranes were then washed with TBST 4x5 minutes, and incubated in secondary antibody (abcam ab6721 Goat Anti Rabbit IgG H&L (HRP) at 1:10,000) for 60 minutes. The membranes were then washed 4x5 minutes with TBST, wet with chemiluminescent detection substrate (BIO-RAD Clarity Western ECL Substrate) and imaged with X-Ray film (Research Products International, base blue, high sensitivity). Developed images were then analyzed using ImageJ software for densitometry. SIRT3 protein measures were normalized to GAPDH, and to young controls as 1.0.

NAD^+ Levels

Measurements of NAD^+ were taken using liquid chromatography-mass spectrometry (LC-MS). Measurements were performed using Waters Acquity LC interfaced with a Waters TQD MS operated in positive ion multiple reaction monitoring mode. Frozen samples were sonicated in dry ice-chilled, deoxygenated, alkaline buffered, aqueous methanol. Extracts were heated with shaking and centrifuged. Supernatants were placed into 2 ml centrifuge tubes and placed in dry ice. Pellets were extracted twice with the supernatants combined after each step. Samples were dried with N_2 gas then reconstituted in 0.1 ml of 50 mM ammonium acetate pH 9 immediately before analysis and placed in a Waters H class autosampler at 8°C. Analytes were detected using a Waters TQD operated in single ion monitoring, negative ion mode.

Aorta Collagen Content

OCT prepared aorta samples were cut into 7-micron sections via cryostat, placed on slides and stained with Picrosirius Red stain for total collagen. Stained aorta sections were

imaged at 10x with bright field illumination using a slide scanner (Leica Aperio Ariol Automated slide scanner and analysis system, NIH Shared Instrumentation Grant # 1S10 OD014165-01A1) and analyzed using threshold analysis in ImageJ software measuring for total collagen area.

Statistical Analyses

Data are presented as mean ± SEM in text, figures, and tables. All analyses were performed with Graphpad Prism. Student's *t*-test was used to compare pulse wave velocity, aortic collagen, aortic SIRT3 protein, renal SIRT3 protein, and renal NAD^+ in young and old mice. Student's *t*-test was also used to compare PWV of SIRT3KO mice and controls. A Pearson product-moment correlation coefficient was used to determine relationship between aortic SIRT3 protein and PWV. One-way ANOVA with Sidak's multiple comparisons test was used to analyze NAD^+ levels and PWV in young, old, and old mice supplemented with NR. One-way ANOVA with Tukey's post-hoc test was used to analyze animal characteristics in young, old, and old mice supplemented with NR.

CHAPTER III

RESULTS

Aim 1 Results

Old control mice demonstrated increased PWV (448 ± 14 cm/sec) compared to young controls (382 ± 13 cm/sec; $p = 0.0031$) (Figure B.1). Aortic collagen was increased in old mice (192669 ± 16930 μm^2) compared to young mice (134244 ± 6955 μm^2; $p = 0.028$) (Figure B.2). Aortic SIRT3 expression was reduced in old mice (0.365 ± 0.088 AU's) compared to young controls (1.000; $p = 0.0152$) (Figure B.4). Increases in PWV were correlated with decreases in aorta SIRT3 expression in young and old mice ($r = -0.7798$, $n = 12$, $p = 0.0028$) (Figure B.4). Old mice exhibited reduced kidney SIRT3 (0.73 ± 0.10 AU's) compared to young controls (1.00 ± 0.00; $p = 0.0192$) (Figure B.5). Old mice also demonstrated decreased NAD^+ (918.6 ± 50.5 pmol/mg) compared to young controls (1302 ± 56.62 pmol/mg; $p = 0.001$) (Figure B.6). Furthermore, 10-month-old SIRT3KO mice exhibited increased PWV (480 ± 21 cm/sec) compared to age matched controls (391 ± 12 cm/sec; $p = 0.0029$) (Figure B.7). However, the genotyping of these animals is not complete and we cannot be certain that all of the animals are complete knockouts, and preliminary data suggests they might be a mixed population of homozygous and heterozygous knockouts (Supplemental Figure 1).

Aim 2 Results

Animal Characteristics

Animal characteristics are shown in Table A.1. At sacrifice, old mice had significantly less body mass (31.1 ± 2.0g) than young (40.0 ± 1.3 g; $p = 0.0038$). There were no differences in kidney weight between young (204 ± 10 mg), old (223 ± 18 mg; $p = 0.742$ vs young), and old mice supplemented with NR (242 ± 15 mg; $p = 0.69$ vs old). Decreases in muscle mass were observed in soleus muscle between young (9.0 ± 0.5 mg) and old mice (6.5 ± 0.2 mg; $p = 0.005$). No differences were observed between old mice and old mice treated with NR (6.5 ± 0.2 mg vs 7.0 ± 0.4 mg; p = 0.77). Decreased muscle mass was also observed in the gastrocnemius of old mice (134 ± 6 mg) when compared to young mice (175 ± 9 mg; $p = 0.042$) and a trend of decreased gastrocnemius mass in old mice with NR (141 ± 9 mg; $p = 0.065$ vs young). There were no differences observed in the length of the tibia and fibula between young (2.0 ± 0.0 cm), old (2.1 ± 0.0 cm; $p = 0.36$ vs young), and old mice supplemented with NR (2.1 ± 0.0 cm; $p = 0.995$ vs old). There were no differences in food consumption between young (2.99 ± 0.19 g) and old mice (3.76 ± 0.41 g; $p = 0.445$), and old controls vs old mice supplemented with NR (3.33 ± 0.54 g; $p = 0.765$).

NAD^+ Levels

Kidney NAD^+ levels in old mice (918.6 ± 50.5 pmol/mg) were lower than in young controls (1302.0 ± 56.6 pmol/mg, $p = 0.0036$), which NR normalized (1303.0 ± 90.2 pmol/mg; $p = 0.754$ vs old) (Figure B.8).

Large Elastic Artery Stiffness

PWV in old mice (417 ± 15 cm/sec) was significantly higher than young controls (292 ± 20 cm/sec; p = 0.0001) (*Figure B.9*). There were no observed differences in PWV between old mice (417 ± 15 cm/sec) and old mice supplemented with NR (402 ± 18 cm/sec; $p = 0.754$) (Figure B.9).

Blood Pressure Measures

There were no differences in systolic blood pressure between young (99 ± 6 mmHg) and old (100 ± 9 mmHg; $p = 0.99$) and between old controls and old mice supplemented with NR (111 ± 5 mmHg; $p = 0.5$) (Table A.2). There were no differences in diastolic blood pressure between young (69 ± 7 mmHg) and old mice (73 ± 7 mmHg; $p = 0.972$), and between old controls and old mice supplemented with NR (79 ± 6 mmHg; $p = 0.877$). There were no differences in mean arterial pressure between young (79 ± 7 mmHg) and old mice (81 ± 8 mmHg, $p = 0.95$), and between old controls and old mice supplemented with NR (90 ± 6 mmHg; $p = 0.675$).

CHAPTER IV

DISCUSSION

The objective of this thesis project was to determine if 1.) age-related loss of SIRT3 contributes to aortic stiffness, due to impaired NAD+ bioavailability and 2.) NR supplementation is effective in boosting age-related NAD^+ and normalizing aortic stiffness in old mice. As expected we demonstrated that old male C57BL/6 mice have increased aortic stiffness, which is accompanied with aortic collagen abundance and reduced SIRT3 expression. Several novel findings emerge from this work. First, decreased aortic SIRT3 protein is correlated with aortic stiffness, which is further supported by animals deficient in SIRT3 having higher aortic stiffness. These data suggest that functioning SIRT3 may be necessary for regulating vascular stiffness. Second, renal SIRT3 protein is reduced in old mice and we are the first to show here an age-related loss of renal NAD^+ bioavailability. Chronic NR supplementation is effective in increasing renal NAD^+ levels to that of young control mice, however, NR had no effect on normalizing aortic stiffness.

Age-related changes in physiological function

Aging is associated with declines in vascular and renal function. Limited NAD^+ levels with age may be mediated by several mechanisms, including increased activity of NAD^+ consuming pathways (PARPs, CD38, SIRTs) or age-related decreases in NAD^+ biosynthetic pathways (NAMPT, NRK1). Consistent with this, we show for the first time that NAD^+ is reduced in the kidneys of old mice (Figure B.6), suggesting it is a therapeutic target for improving age-related cardiovascular and renal diseases. Coupled with decreased renal SIRT3 protein (Figure B.5) this decrease in NAD^+ is likely mediated by increased activity of the other NAD^+ consumers PARPs and CD38.

Age-related vascular dysfunction

Consistent with the work of others we found that aging is associated with increased large elastic artery stiffness (Figure B.1) due in part to increased collagen deposition in the arterial walls (Figure B.2). Increased stiffness could be due to both functional and structural modifications due to excessive arterial oxidative stress, specifically from dysfunctional mitochondria. Aortic and renal SIRT3 is reduced in old mice (Figure B.3)(Figure B.5), however we were not able to determine oxidative stress or mitochondrial function in the current study. Since SIRT3 regulates the activity of MnSOD (Tao, Vassilopoulos, Parisadou, Yan, & Gius, 2014), FOXO3a (Tseng, Shieh, & Wang, 2013), and the electron transport chain (van de Ven, Santos, & Haigis, 2017) the age-related increase in stiffness could be mediated by reduced SIRT3 protein due to increased mitochondrial stress, leading to aortic remodeling resulting in collagen increases with aging. Furthermore, even though young animals have lower PWV than old, individual SIRT3 protein expression is related to their PWVs (Figure B.4). Our finding that mice deficient in SIRT3 exhibit stiffer vessels (Figure B.7) further supports the necessity of SIRT3 in maintaining healthy arterial stiffness. However, genotyping of these animals is not complete, and our preliminary data suggests the animals may not be complete KO's (Supplemental Figure 1) but rather a mixed group of heterozygous and homozygous knockouts.

NR supplementation normalized age-associated loss of NAD^+

Importantly, we show here for the first time that supplementation with NR, a clinically and commercially available supplement, can ameliorate age-related reductions of NAD^+ levels in mouse kidney tissue (Figure B.8). This finding may have important implications for the prevention and treatment of age-related declines in renal function, AKI, CKD, and glomerular mesangial cell hypertrophy, one of the abnormalities observed in diabetic nephropathy (Morigi,

et al., 2015) (Zhuo, et al., 2011). Increased NAD^+ in the kidney by NR supplementation could result in increased substrate for SIRT3 allowing for increased SIRT3 activity, which has been shown to protect from cisplatin-induced AKI (Morigi, et al., 2015). Increased NAD^+ has also been shown to inhibit high-glucose induced hypertrophy in rat glomerular mesangial cells (Zhuo, et al., 2011). Currently, the strongest evidence exists for a role of SIRT3 mediating the beneficial effects of NAD^+ therapy in the kidney (Hershberger, Martin, & Hirschey, 2017). Because CKD risk increases with advancing age and patients with CKD die of CVD, this has broad therapeutic potential in the treatment of both vascular and renal dysfunction.

Even though we did not measure NAD^+ levels in the aorta, it is reasonable to expect that NAD^+ levels in aged mice were increased with NR supplementation. If so, this could perhaps indicate that arterial stiffness is not due to decreased NAD+ substrate, but rather the decrease of SIRT3 protein present in the aorta (Figure B.3) as we saw no change in arterial stiffness with NR supplementation (Figure B.9). It is also possible that the increased NAD^+ was being utilized by the PARPs, resulting in better DNA repair, but not leaving enough substrate for SIRT3. PARPs demonstrate a higher catalytic turnover than the Sirtuins and their inhibition increases NAD^+ pools (Bai, et al., 2011). Similarly, the NADase CD38 has been shown to increase with age, causing a reduction in NAD+ levels in other tissues (Camacho-Periera, et al., 2016). If PARPs or CD38 were upregulated in the aorta due to the aged phenotype of our mice, the disproportionate consumption of NAD^+ would leave less substrate for SIRT3 to deacetylate downstream targets, limiting the effects of NR supplementation on arterial stiffness.

Previously, de Picciotto (2016) showed that supplementation with NMN, another NAD^+ precursor, for eight weeks ameliorated age-associated arterial stiffening and reduces collagen in thoracic aorta segments. Here we used long-term NR supplementation because NR is a clinically

available nutraceutical. The overall goal of our work is to translate into a clinical population therefore we sought to test the efficacy of NR on improving aortic stiffness. It has been recently discovered that nicotinamide riboside kinase (NRK1) is the rate-limiting enzyme for NR and NMN driven NAD^+ synthesis (Ratajczak, et al., 2016). It is possible that due to the long-term supplementation in this study or the age of the mice an inhibitory mechanism was induced decreasing the activity of NRK1, resulting in the observed lack of change in arterial stiffness.

Limitations

Measures of NAD^+ levels in the aorta were not assessed, therefore we cannot determine whether NR supplementation had any effect on NAD^+ levels in large elastic arteries. NAD^+ levels are very unstable and require the tissue to be harvested and frozen immediately to provide interpretable data. The combination of the anatomical location of the aorta and the need to remove perivascular fat and other visceral tissue limit the ability to make these measures. Although the tissue measurements of NAD^+ presented here are not direct measurements of aortic NAD^+, the kidney demonstrated decreased NAD^+ with age which was normalized with NR supplementation, serving as a viable proof of concept that our intervention did in fact increase NAD^+ levels.

Conclusion and Future Study

The above findings provide novel evidence that SIRT3 may be required for the maintenance of reduced aortic stiffness in aging. In this case, attempting to provide SIRT3 more substrate via long-term NR supplementation had no effect on age-associated arterial stiffness even though NAD^+ was increased. Although arterial stiffness was not reduced after six months of NR supplementation mitochondrial quantity & quality, overall oxidative stress, SIRT3 activity, NRK1 and the expression of downstream targets such as MnSOD may still have changed in the

aorta. Endothelial function in the large elastic arteries and resistance vessels may have changed as well. These changes may be relevant to the development of other vascular pathologies associated with age and deserve further study. With the increased NAD^+ levels from NR supplementation, renal function may improve which would have implications in the treatment and prevention of AKI, CKD, and diabetic nephropathy. It is possible that the arterial stiffness may be related to reduced SIRT3 rather than reduced NAD^+. Further study of the effects of a more direct inducer of SIRT3 such as caloric restriction, cold exposure, exercise, or honokiol (Pillai, et al., 2015) on arterial stiffness is warranted. The inability to measure aortic NAD^+ reduces our understanding of changes in arteries, and there may be differences between conduit and resistance arteries. Further investigation into the potential inhibitory mechanisms of NAD^+ biosynthetic enzymes such as NRK1 or the DNA repair status as the result of increased PARPs activity are worth investigating as well. NAD^+ metabolites will provide mechanistic insight as to the pathways involved as well and is to be the focus of future work and experiments. Unfortunately, due to the time constraints of a Master's thesis in we were unable to investigate these proposed mechanisms here. However, we do show for the first time that long term NR supplementation rescues age-related reductions in NAD^+ levels in the kidney, demonstrating potential for the therapeutic use of NR in preventing acute kidney injury, chronic kidney disease, and diabetic nephropathy.

APPENDIX A: TABLES

Table A.1 Animal Characteristics

Characteristic	**Young**	**Old**	**Old+NR**
Bodyweight (g)	40.0 ± 1.3	31.1 ± 2.0**	34.8 ± 2.1
	n = 6	n = 6	n = 5
Kidney Weight (mg)	204 ± 10	223± 18	242 ± 15
	n = 5	n = 4	n = 5
Soleus Weight (mg)	9.0 ± 0.5	6.0 ± 0.2**	6.0 ± 0.4*
	n = 8	n = 6	n = 7
Gastrocnemius Weight (mg)	175 ± 9.2	134 ± 6.1*	141.5 ± 8.8
	n = 8	n = 6	n =7
Tibia/Fibula Length (cm)	2.0 ± 0.0	2.1 ± 0.0	2.1 ± 0.0
	n = 8	n = 6	n = 7
Food Intake (g/day)	3.0 ± 0.2	3.8 ± 0.4	3.3 ± 0.5
	n = 4	n = 3	n = 4

Values are Mean ± SEM

*$p < 0.05$ vs Young

**$p = 0.005$ vs Young

Table A.2 Blood Pressure Measures for Young, Old, and Old +NR Mice

Group	**N**	**Systolic** (mmHg)	**Diastolic** (mmHg)	**MAP** (mmHg)
Young	9	99 ± 6	69 ± 7	79 ± 7
Old	8	100 ± 9	73 ± 7	81 ± 8
Old + NR	9	111 ± 5	79 ± 6	90 ± 6

Values are Mean ± SEM

APPENDIX B: FIGURES

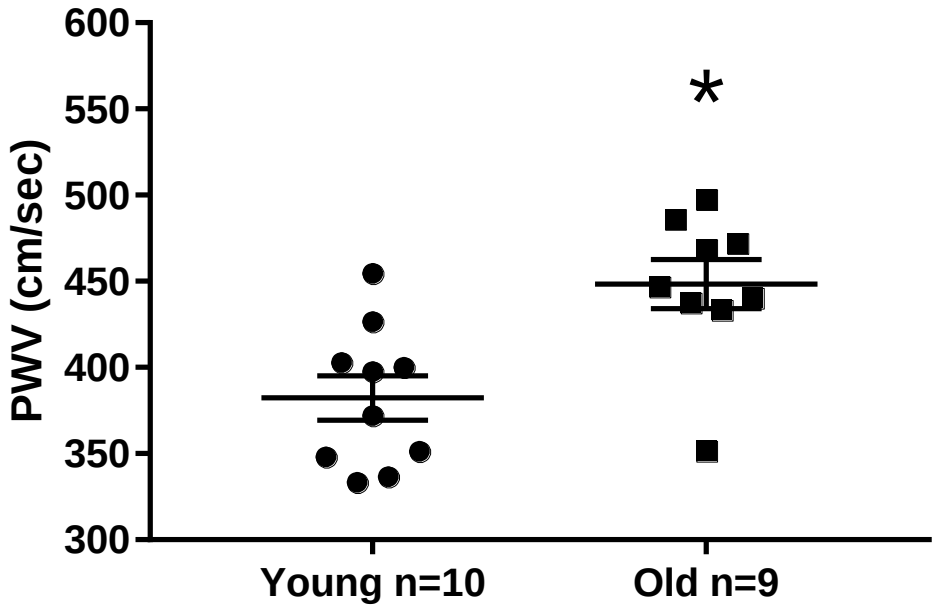


Figure B.1: Age-related increase in PWV

Aortic pulse wave velocity of Young (~6-month-old) and Old (~26-month-old) C57BL/6 mice
*$p < 0.005$ vs Young

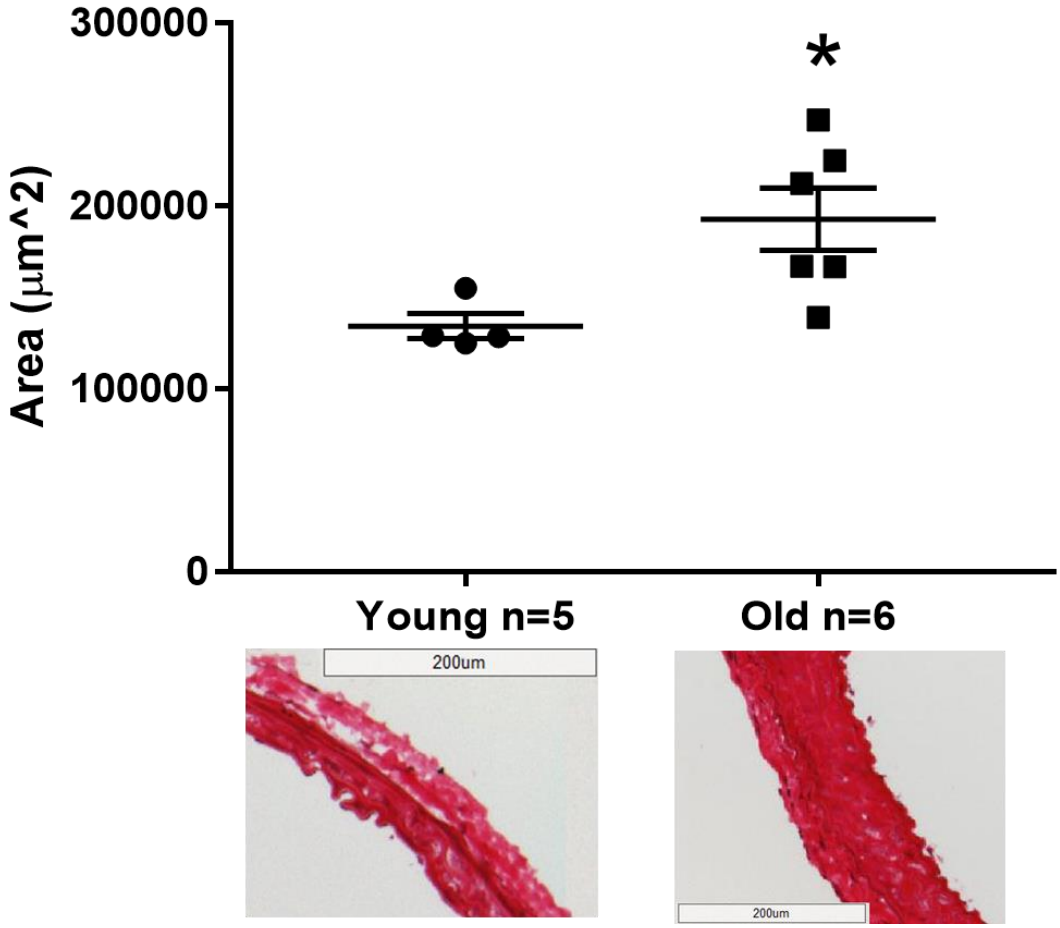


Figure B.2: Age-related increase in collagen staining

Picrosirius Red stained aorta collagen area measures in young (Y, ~6 months old), old (O, ~26 months old) mice with representative images.
*$p < 0.05$ vs young

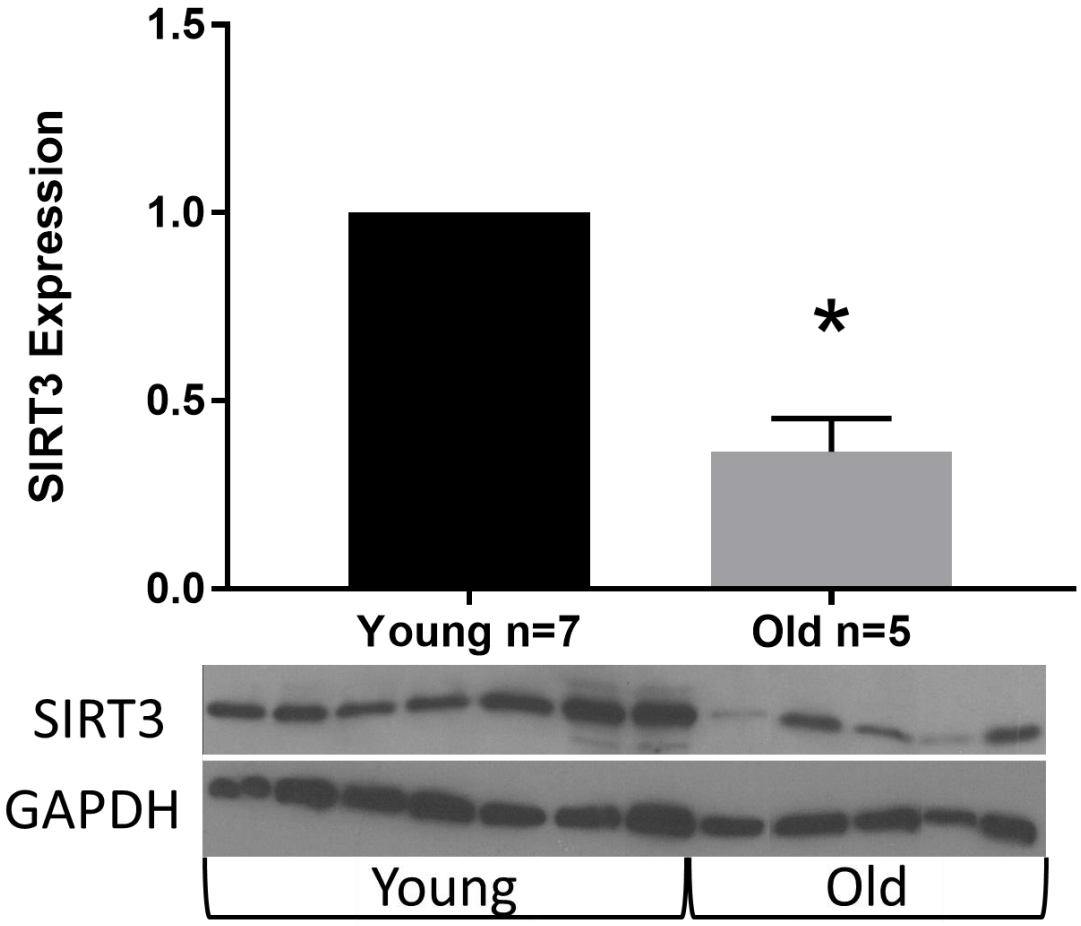


Figure B.3: Age-related decrease in aortic SIRT3

SIRT3 protein in the aorta of young (~6-month-old) and old (~26-month-old) male C57BL/6 mice. Graph is quantification of displayed blot. SIRT3 protein was normalized to GAPDH, average of young mice was standardized to equal 1, old measures relative to young
*$p < 0.05$ vs young

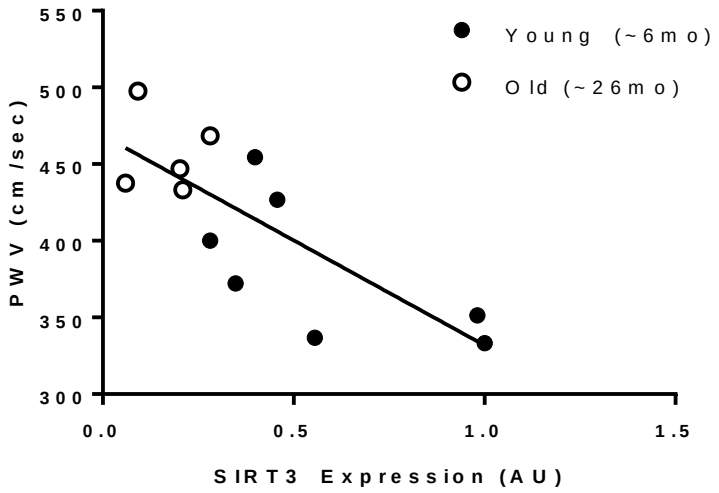


Figure B.4: Aortic stiffness is related to aortic SIRT3

Pearson product-moment correlation of SIRT3 protein in young and old male C57BL/6 mouse aorta with aortic pulse wave velocity
Total Cohort ($r = -0.7798$, $n = 12$, $p = 0.0028$)
Young Only ($n = 7$, $p = 0.12$)

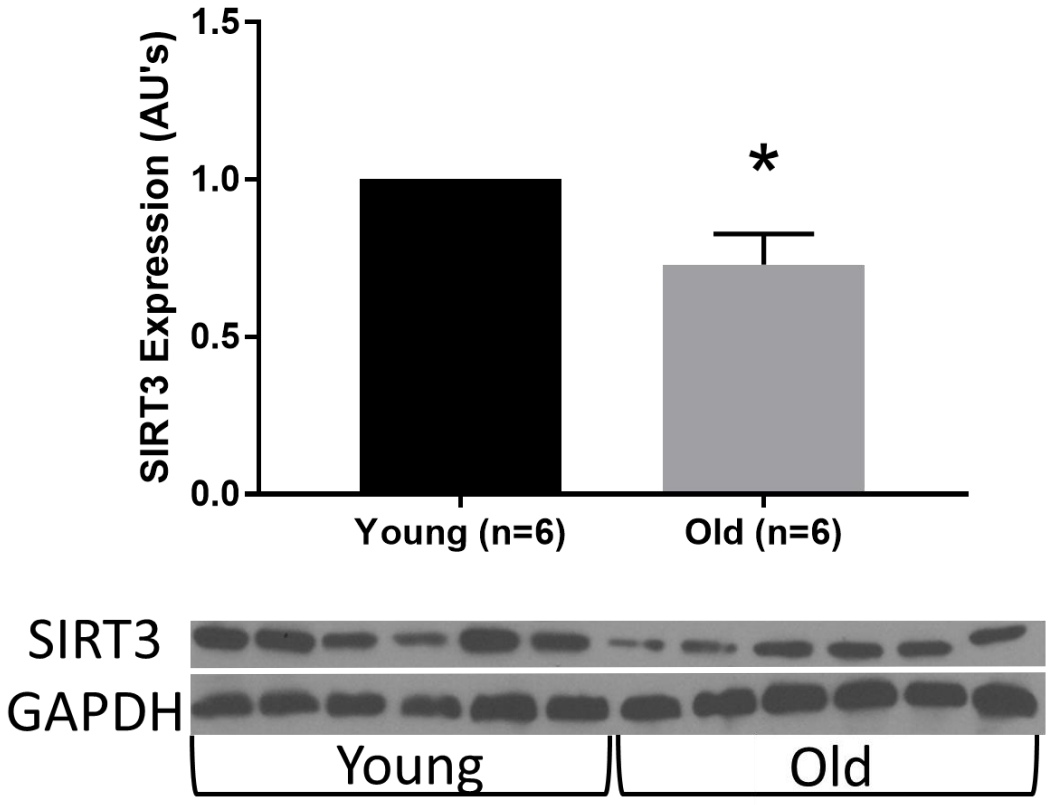


Figure B.5: Age-related decrease in renal SIRT3

SIRT3 protein in the kidneys of young (~6-month-old) and old (~26-month-old) male C57BL/6 mice. Graph is quantification of displayed blot. SIRT3 protein was normalized to GAPDH, average of young mice was standardized to equal 1, old measures relative to young
*$p < 0.05$ vs young

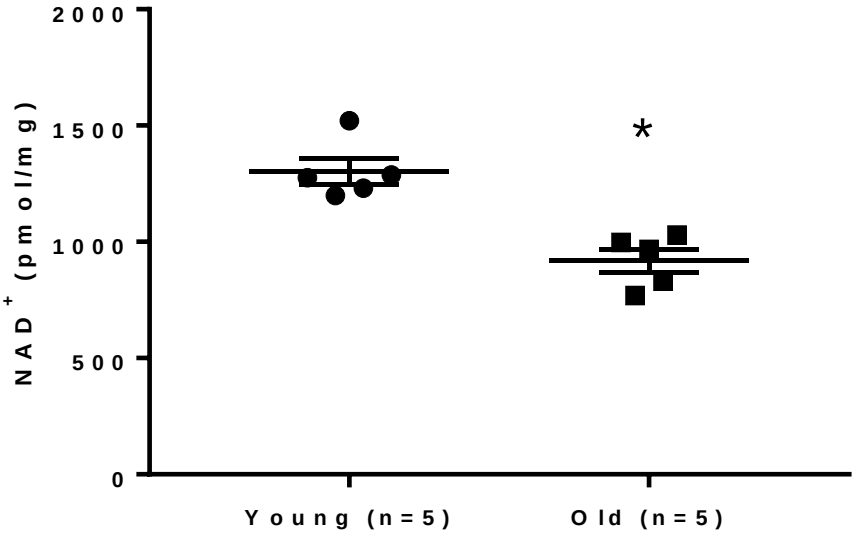


Figure B.6: Age-related decrease in renal NAD^+

NAD^+ in young (~6-months-old) and old (~26-months-old) mouse kidney tissue.
*$p < 0.005$

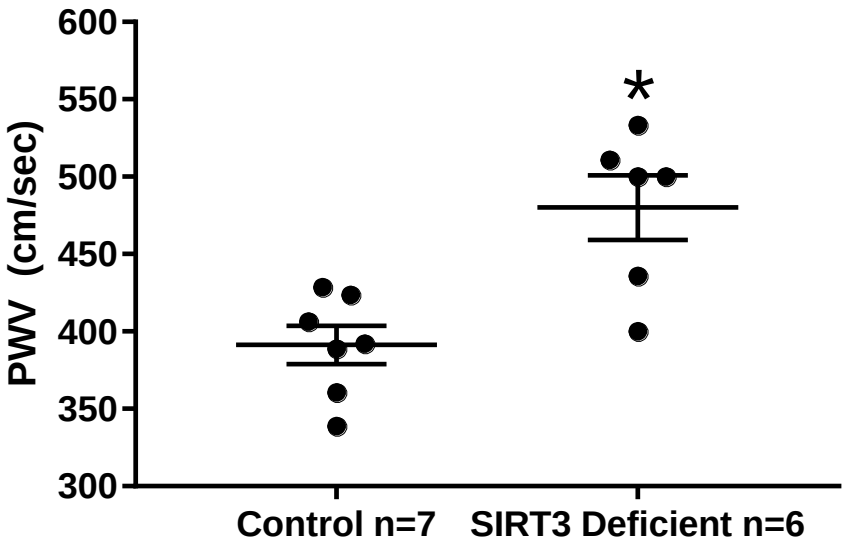


Figure B.7: SIRT3 deficient animals have increased PWV

Aortic pulse wave velocity of 10-month old male C57BL/6 control and SIRT3 deficient mice
* $p < 0.005$ vs Control

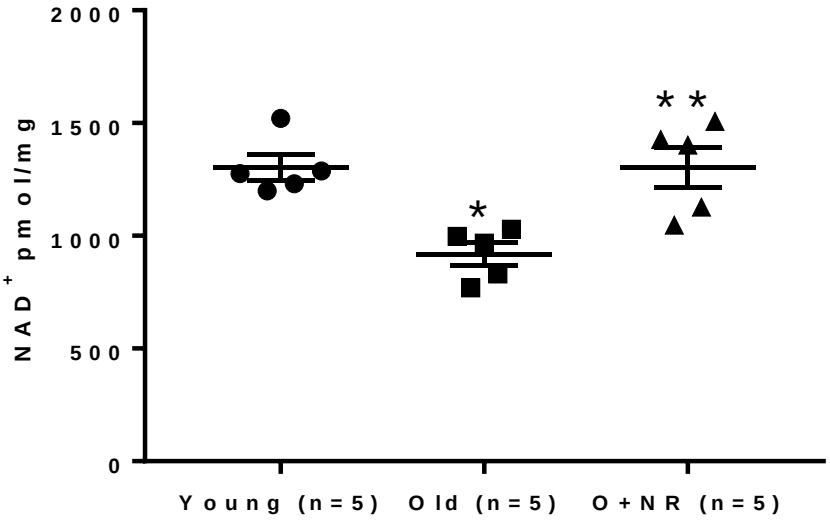


Figure B.8: NR supplementation increased NAD^+ in the kidneys

NAD^+ levels in the kidney of young (~6-months-old), old (~26-months-old), and old mice supplemented with NR for 6 months (O+NR).
*$p < 0.005$ vs. young
**$p < 0.005$ vs. old

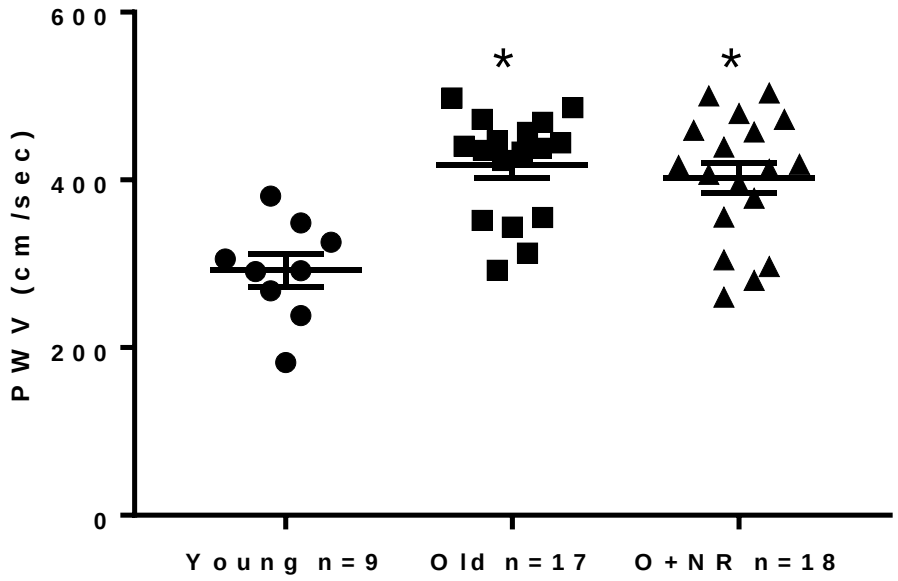
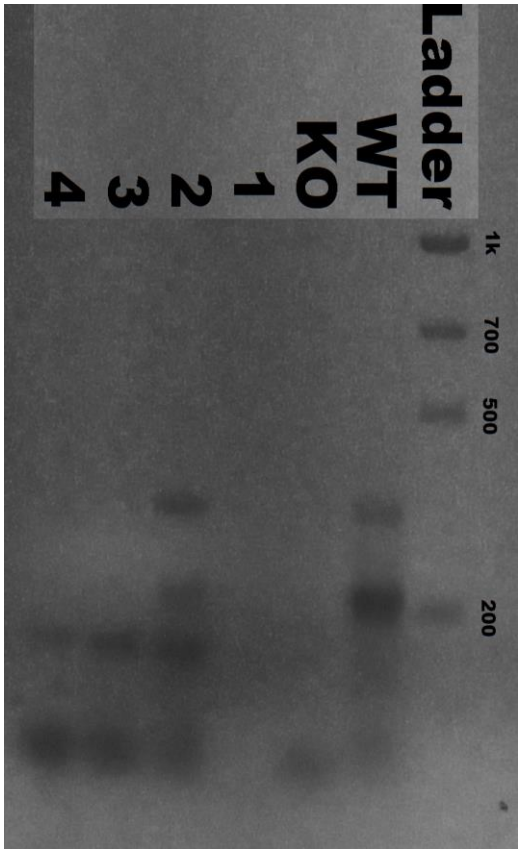


Figure B.9: NR supplementation had no effect on aortic pulse wave velocity

Aortic pulse wave velocity measures in young (~6-months-old), old (~26-months-old), and old mice supplemented with NR for 6 months (O+NR)
* $p < 0.001$ vs Young

APPENDIX C: SUPPLEMENTAL FIGURES



Supplemental Figure 1: Genotyping agarose gel of SIRT3 deficient animals

Inverted agarose gel image for the genotyping of SIRT3 deficient animals. Wild Type bands 336 base pairs, Knockout bands 160 base pairs. 1.5% agarose gel ran at 75 volts for 75 minutes on BIO-RAD Mini-Sub GT unit stained with ethidium bromide and imaged with UV light. Image inverted and contrast increased for clarity with ImageJ.

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