ASSOCIATION BETWEEN A NON-INVASIVE

ASSESSMENT OF FRAILTY AND VASCULAR

DYSFUNCTION IN OLD MICE

By

JAZMIN COLE

A THESIS

Presented to the Department of Human Physiology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

September 2020

An Abstract of the Thesis of

Jazmin Cole for the degree of Bachelor of Science
in the Department of Human Physiology to be taken September 2020

Title: Association between a Non-Invasive Assessment of Frailty and Vascular
Dysfunction in Old Mice

Approved: _____*Dr. Ashley Walker*_____
Primary Thesis Advisor

Advancing age is characterized by not only an increased risk for cardiovascular diseases (CVDs), but also a decline in functional reserve and impaired adaptive capacity across multiple physiologic systems, also known as frailty. Impaired vascular function is a known contributor to CVDs and potentially has a role in increased frailty. In patients with overt disease, measures of frailty are related to vascular endothelial cell dysfunction. However, the relation between vascular endothelial function and frailty in a non-disease population is unknown. It is also unknown if dysfunction of a particular vascular bed is more closely related to frailty. This study aimed to correlate vascular dysfunction with age and frailty and examine possible mechanisms in genetically and environmentally identical mice. The major finding of this study is that frailty is correlated with age and mesentery artery endothelial cell dysfunction. The driver of this dysfunction appears to be oxidative stress and lower antioxidant enzyme expression. In contrast to mesentery arteries, middle cerebral artery endothelial dysfunction was not correlated with frailty index or age. These results suggest that frailty index could be a non-invasive marker of vascular impairment or improving mesentery artery health may be a possible way to reduce frailty in older adults.

Acknowledgements

I would like to first thank the three members of my thesis committee, who have been there to see my struggle and stress about completing this thesis and encouraged me to continue. Dr. Ashley Walker has been so important in helping me stay on track and become a confident scientist over the last three years. Professor Melissa Graboyes inspired me to challenge myself with doing a multi-disciplinary thesis that approached physiology from a public health lens. Nick Winder has personally witnessed me stressing over writing and working long hours in my research lab and was always a source of relief (even though we argued over everything).

My aunts, uncles, and cousins have also been so supportive of my college journey and I wouldn't have been able to finish college without them. Kate Stoysich and Suzie Stadelman have helped me find what career paths I want to take and steered me in the direction that best fit me while also reminding me that I have the ability to do anything I want.

Finally, I want to mention my mom, Julie, who would have been so proud of all that I accomplished had she lived to see my college graduation. She was the true inspiration for me to join my research lab because she passed away in July 2017 of Alzheimer's Disease, which I have now spent 3 years researching. As a first-generation college student/graduate, I know that my mom would have been there every step of the way to make sure that I graduated.

Table of Contents

List of Figures

List of Tables

Foreword

COVID-19 was first confirmed in Wuhan, China in December 2019. A travel-related case was reported in the US in January 2020 and the first case of nontravel-related COVID-19 was confirmed in late February 2020. Spreading rapidly across the world, the pandemic reached Hillsboro, Oregon on February 28. On March 11, the University of Oregon emailed all students, notifying them that the first four weeks of spring term would be completely online. I remember joking with a friend and saying, "What if graduation was all online? Wouldn't that suck?" Little did I know, that was exactly what happened. Over the next three months, the entirety of my last quarter of college, restaurants and bars closed, campus became a ghost town, and toilet paper was completely out of stock.

I spent most of my days in the bedroom of my house, where all four of my roommates were either told to work from home or lost their job completely. All of the plans I had made for spring term were canceled. A suicide prevention walk I had been planning for a year and received funding for was canceled. I celebrated my 21st birthday in May with a bottle of champagne in my backyard. I hadn't seen any members of my family since December and did not know when I would see them again. My research lab was close indefinitely, with all my samples stored in the freezer, waiting to be tested. Two abstracts I was a part of had been accepted to a large biology conference in San Diego that was cancelled and then moved online. The dream post-grad job working for the CDC I had applied for in January was delayed and then they stopped responding to me emails. The term when I had expected all of the hardships I had experienced in college would finally resolve was suddenly the hardest three months of my life. To say I

became extremely depressed is an understatement. I stopped attending the last two classes I needed to graduate, I missed meetings I was supposed to be running, and my unread emails grew and grew.

I write this foreword to explain why this thesis is not what I imagined it would be. In spring 2019 I had decided to take on the challenge of writing a multi-disciplinary, two-part thesis that combined my interests in global health and physiology. In the summer of 2019, I had the opportunity to travel to the West African country of Ghana. I stayed in the capital, Accra, for 7 weeks with a host family while taking classes and completing a volunteer internship at the West African AIDs Foundation (WAAF). The focus of this study abroad program was global health, so my classes, internships, and group field trips were designed to enhance my understanding of local and global factors that influence the health status of people. Before I left for my study abroad program, I spent several weeks working with my advisors to create a multi-disciplinary thesis project, submitting a prospectus, and applying for grants to fund my study abroad. I learned how to conduct, record, and interpret ethnographic observations and returned from Ghana with a notebook full of my daily experiences and specific observations that related aging, the focus of my laboratory and the other half of my proposed thesis. Over the course of my final fall and winter quarter, I transcribed my hand-written notes to electronic documents, added thoughts, identified themes, and began to construct chapters that would make up the second part of my thesis. In the background of my thesis work, the pandemic was getting worse and worse.

When spring quarter began, I was unmotivated to graduate college, despite being 8 credits and a thesis away from graduating with two majors and a minor.

Eventually I decided to withdraw from the term and finish my classes and thesis in the summer. My research was stalled because I was not able to complete my gene expression experiments. I had not opened any documents related to my thesis since March and was not sure how I was going to complete it. After some very supportive calls from my advisors, I came up with a plan to finish my last few credits and complete my thesis project. I managed to conduct close to 80 hours of research in my lab to finish my data collection and analysis.

The biggest challenge became the global health section of my thesis. While I had my ethnographic observations, writing chapters based on them was well out of my field of expertise. As the deadlines for my drafts approached, the anxiety grew. Between the time I finished my first draft and my final draft, my cat and I moved two times: Eugene to Portland for two weeks, and then Portland to Boston permanently. After going back and forth for months if I should stress about trying to write up at least three chapters about my experiences in Ghana, I made the decision to not continue with the additional sections. Coming to that conclusion was not easy for me because I had spent so much time trying to combine my two passions and I thought a multi-disciplinary project would reflect myself and my college experiences the best. Had things been different in the spring of 2020, I could have completed both parts, but I have learned to let go of things that are out of my control and be grateful for what I do have: a completed honors thesis and a bachelor's degree. Thus, this completed thesis focuses entirely and solely on the lab-based portion of my research on aging, and doesn't seek to integrate my ethnographic observations on again in Ghana, despite that being my original intent.

Introduction

Gerontology, the study of aging, has gained in importance and popularity as a medical/research specialty in the last few decades as older people (aged 65+) begin to represent a substantial proportion of the global population. This multi-disciplinary field is complicated by the fact that aging affects every individual in different ways. Geroscience is focused on identifying the biological mechanisms and strategies of aging to compress morbidity and increase healthspan, rather than lifespan extension. The key difference between these two paradigms is that compression of morbidity promotes the concept of health span, or the period healthy aging free from chronic clinical diseases and disabilities (Seals, Jablonski, & Donato, 2011). On the other hand, lifespan extension focuses on increasing the number of years a population can live. To slow the vicious cycle of declining physiological function leading to reduced functional status, medical research focuses on how physiological processes change with aging. While aging is not a disease state itself, there are many diseases associated with aging: arthritis, cardiovascular diseases, cataracts, type 2 diabetes, and Alzheimer's Disease. Historically, the increase in these chronic diseases has been a sign of a population experiencing an epidemiological transition from low to high life expectancy and rapid-growth. With improved standards of living, hygiene, and nutrition, a population experiences less incidences of infectious diseases. The result is that mortality due to infectious diseases decreases, life expectancy increases, and the prevalence of chronic diseases of the aged increases. Understanding the changes and mechanisms behind aging is important to create therapies and treatments for associated diseases.

Literature Review

Endothelial Function and Dysfunction

The ability for arteries to change the diameter of their lumen is important for maintaining blood flow and regulating blood pressure. Vasoconstriction, or when an artery contracts, decreases the diameter of the lumen and subsequently increases the blood pressure and decreases the blood flow through the artery. Vasodilation, or when an artery relaxes, increases the diameter of the lumen and subsequently decreases the blood pressure and increases the blood flow through the arterial wall. The endothelium is a single layer of cells that lines the innermost part of an artery and is primarily responsible for vasodilatory processes (Cines et al., 1998), making it the focus of vascular research.

Endothelial-independent dilation is caused by relaxation of smooth muscle cells in the tunica media while endothelial-dependent dilation is caused by signaling in the endothelial cells that triggers smooth muscle cell relaxation (Taddei et al., 2001). There are two mechanisms by which endothelial cells facilitate dilation of arteries: mechanical and chemical. Endothelial cells contain muscarinic receptors on the luminal side that allow for acetylcholine (ACh) binding. ACh is a small molecule called a neurotransmitter that is released from neurons to control different bodily functions (Wessler & Kirkpatrick, 2009). When ACh binds to receptors on endothelial cells, it activates the enzyme endothelial nitric oxide synthase, eNOS (Elhusseiny, Cohen, Olivier, Stanimirović, & Hamel, 1999) which in turn produces nitric oxide, NO, (Palmer, Ashton, & Moncada, 1988) a very reactive molecule called a free radical. Production of NO results in cyclic guanosine monophosphate-induced vascular smooth

muscle relaxation (Xu et al., 2015). This endothelium signaling causes the smooth muscle cells that form the tunica media to relax and the diameter of the lumen to expand, giving this process the term endothelial-dependent dilation. A similar process can also be initiated by sheer stress or increased blood flow through an artery without the release of ACh.

Vascular impairment is reflected by reduced endothelium dependent dilation (Durrant et al., 2009), decreased NO availability (Harrison, 1997), and increased large artery stiffness (Fleenor et al., 2014), all of which can predict future cardiac events (Gokce et al., 2002). There are two interactive mechanisms by which aging is thought to cause dysfunction in the vasculature. The first is oxidative stress, which is mediated by the increased production of reactive oxygen species (ROS) superoxide (O2-) outcompeting decreased antioxidant defenses seen with aging (Katusic & Vanhoutte, 1989). Antioxidants are ROS scavenging proteins that have been shown to counteract the vascular dysfunction seen with aging (Niwa, Carlson, & Iadecola, 2000). O2- is mainly produced in the body by the protein NADPH oxidase (Donato et al., 2007) or the mitochondria (Ungvari et al., 2007) and it reduces NO availability by interacting with it to create another ROS, peroxynitrite (ONOO-) (Dröge, 2002). With less NO available to endothelial cells, the signaling pathway that allows for relaxation of smooth muscle cells is decreased and reduced endothelial-dependent dilation is observed.

The second aging mechanism that has been shown to cause vascular dysfunction is chronic low-grade inflammation caused by activation of the master pro-inflammatory transcription factor, nuclear factor-kB (NF-kB). Aged mice and humans have shown increased expression of NF-kB compared with young controls and resulting increased

6

release of secondary pro-inflammatory cytokines (Lesniewski et al., 2011) and

increased NADPH oxidase activation leading to increased O2- production (Walker,

Kaplon, Pierce, Nowlan, & Seals, 2014). Impaired endothelium dependent dilation was

also reflected in these studies and was rescued with treatment of an inhibitor of the

upstream activator of NF-kB.

Frailty Assessments

Frailty indexes are typically used in medical settings to measure the health status

of older patients to assess potential risk for poor outcomes following a procedure. The

first frailty index was developed by Dr. Kenneth Rockwood and since then other

clinical scales have been published with varying results, which can potentially lead to

uncertainty about the term frailty and the different components that should be used to

measure (Rockwood et al., 2005). Outside of medical and clinical trials, multiple studies

have focused on using frailty indexes to predict development of cardiovascular diseases

(Sergi et al., 2015), vascular dementia (Avila-Funes et al., 2012), and Alzheimer's

Disease (Buchman, Boyle, Wilson, Tang, & Bennett, 2007).

Only two studies have investigated the correlation between endothelial cell

dysfunction and frailty (Alonso-Bouzón et al., 2014; Mansur et al., 2015). While both

studies found an association between endothelial dysfunction and frailty, they

determined endothelial dysfunction via an inferior technique of measuring levels of

asymmetric dimethylarginine, an inhibitor of eNOS, or in a diseased population. Both

studies also presented frailty as a discrete variable (healthy, pre-frail, frail). Discrete

variable models, while can be easier to use, don't allow researchers to look at individual

deviations or outliers and are generally more specific. While human clinical trials for

frailty have been used, few experimental animal models have used frailty to investigate the mechanisms by which frailty is contributing to the effects seen. Therefore, integrating animal models into experiments allows researchers to introduce stressors and add to the understandings of how interventions affect fundamental aging processes (Kirkland & Peterson, 2009). Therefore, my research hoped to use a different methodology to evaluate endothelial dysfunction to correlate with frailty on a continuous scale. We aimed to further investigate the relative abundance of pro-oxidant, anti-oxidant, and pro-inflammatory genes in correlation to frailty score and endothelial dysfunction.

Methods

Animals and Tissues

Old male and female C57BL/6J mice with no genetic modifications were purchased from the National Institute on Aging colony at Charles River. Young (4-10 months, n=36) and old (23-32 months, n=49) mice were used for this study. All mice were on a normal chow diet with free access to food and water. Mice were housed in an animal care facility on a 12/12-hour light-dark cycle at 24°C. All mice were euthanized by exsanguination under inhaled isoflurane prior to vascular reactivity studies. Mesentery arteries were used for vascular reactivity studies because of their importance in systemic vascular resistance, tissue perfusion specifically to the intestines, and blood pressure regulation. Middle cerebral arteries (MCAs) were also used to evaluate endothelial function in arteries that are susceptible to large elastic artery stiffness seen with aging. Both of these vascular beds have been shown to have distinct functional changes with aging in mouse models (Walker et al., 2015). At the time of vascular function measurements, samples of mesentery arteries were collected and stored in a -80°C freezer until gene expression studies were conducted. All animal procedures conform to the *Guide to the Care and Use of Laboratory Animals* (8th edition, revised 2011) and were approved by the Institutional Animal Care and Use Committees at the University of Oregon.

Vascular Reactivity

EDD is a measurement of endothelial cell function and provides insight not only into the regulation of vascular tone, but also overall arterial health (Vita & Keaney,

2002). EDD was assessed *ex vivo* in isolated, pressurized mesentery arteries by the

dilation to increased doses of Acetylcholine (ACh, 10^{-9} to 10^{-4} M) after pre-constriction

(by phenylephrine, 2 uM). Endothelium-independent dilation (EID) was assessed by the

dose response to sodium nitroprusside (SNP, 10^{-10} to 10^{-4} M) after preconstriction

(Walker et al., 2015; Walker, Henson, et al., 2014). To do so, mesentery and middle

cerebral arteries were excised and placed in myograph chambers (DMT Inc.) with

physiological salt solution containing 145 mM NaCl, 4.7 mM CaCl2, 1.17 mM MgSO4,

1.2 mM NaH2PO4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM

MOPS buffer, and 1 g/100 mL BSA, pH 7.4 at 37°C, cannulated onto glass

micropipettes and secured with nylon (11-0) sutures. Once cannulated, arteries were

warmed to 37°C, pressurized to 50 mmHG, and allowed to equilibrate for ~1 hour.

Changes in luminal diameter with ACh and SNP doses were recorded in notebooks and

later inputted into an Excel sheet. To determine the effect of superoxide (oxidative

stress) on EDD, responses to ACh were measured following a 60-min incubation in the

presence of the superoxide scavenger, TEMPOL (1mM) (Walker et al., 2015).

Mouse Frailty Assessments

Mouse frailty was assessed using a previously studied 31-item frailty index

based on established clinical signs of deterioration in C57BL/6J mice developed in

consultation with a veterinarian (Whitehead et al., 2014). One item was removed from

this index (vision loss). Body mass and surface temperature were measured but did not

receive a score. Two investigators were trained by a veterinarian on baselines clinical

assessments and performing assessments with young mice of the same breed (not

included in this study). Clinical assessments included evaluation of the integumentary,

musculoskeletal, vestibulocochlear/auditory, ocular, nasal, digestive, urogenital, and respiratory systems as well as signs of discomfort, body weight, and body surface temperature. Each mouse was examined at approximately the same time in the day, following the same order of assessments within 14 days of their planned vascular reactivity study. Table 1 details the methods of clinical assessment and scoring. The severity of each deficit was assessed and assigned either a 0, 0.5, or 1, with a higher score indicating more severe frailty.

Gene Expression

Stored mesentery samples for 63 mice samples were used for gene expression of pro-inflammatory and pro-oxidant genes. mRNA expression was measured by real-time qPCR in mesentery arteries. RNA was isolated by a standard protocol utilizing Qiazol and RNAeasy Mini Kits (Qiagen). The RNA was then quantified using a NanoDrop 2000. Reverse transcription was performed to produce cDNA with a Qiagen Quantitect Reverse Transcription Kit. The cDNA samples underwent real-time qPCR using ThermoFisher PowerUp Sybr Green. 18S rRNA was used as a housekeeping gene transcript to control for tissue concentration. Gene expression was quantified for pro-inflammatory gene *IL-1beta*, pro-oxidant gene *Nox2*, and antioxidant genes *Sod1, Sod2,* and *Sod3*.

Statistical Analysis

Single linear regression analysis was used to assess associations between frailty index scores, age, maximal artery dilation, and change in artery dilation. For all dose responses, group differences were determined by repeated-measures ANOVA and

presented as mean ± SEM. Significance was set at $p<0.05$, and all reported probability

tests were one-sided. Person Correlations were conducted using GraphPad Prism

version 8.0.0 for Windows, GraphPad Software, San Diego, California USA,

www.graphpad.com.

Results

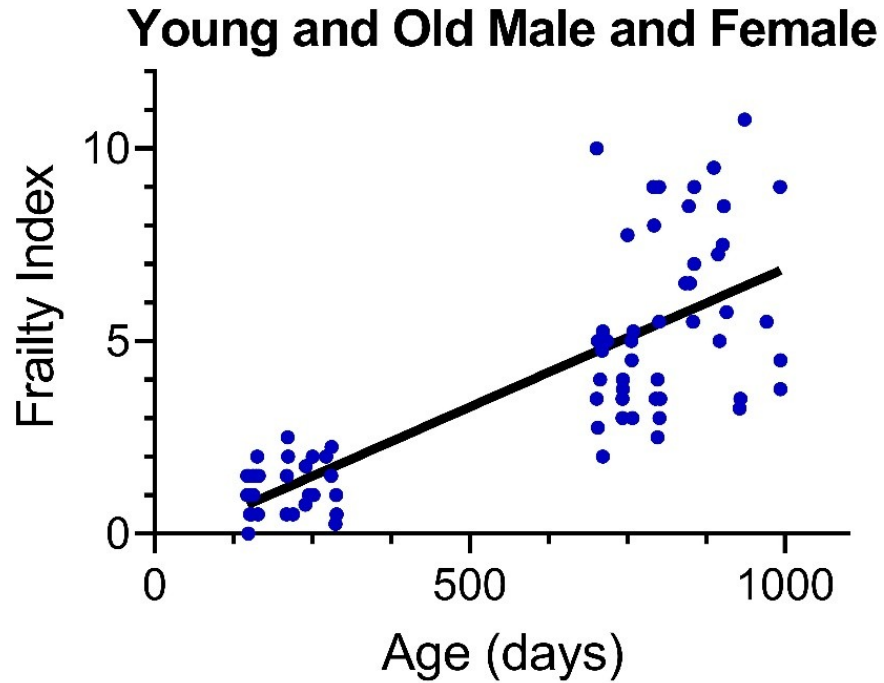## Age is Positively Correlated with Frailty Index

Figure 1

Correlation of age with frailty index score for male and female mice across young and old ages.

As previously reported (Whitehead 2014), age is positively correlated with an increased frailty index score. This study aimed to include both females (n=28) and males (n=57) from a wider range of ages, 4 to 32 months. Figure 1 demonstrates that age is still positively correlated across all ages, regardless of sex (r=0.796 p <0.0001).
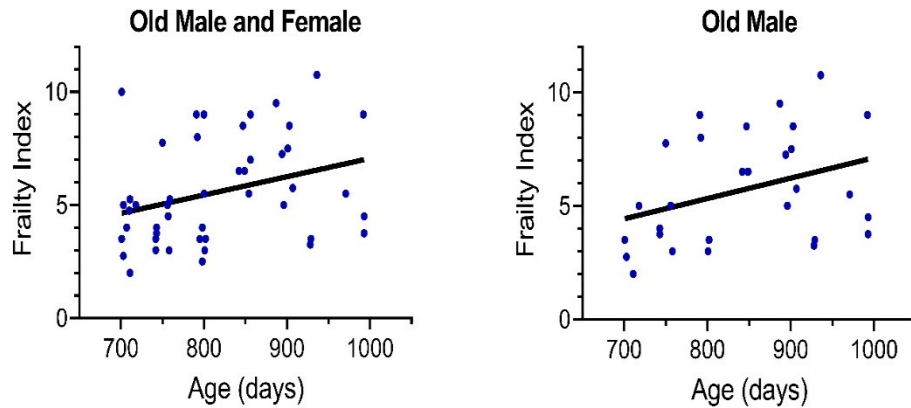
Figure 2

A: Correlation of frailty index with age in old male and female mice. B: Correlation of frailty index with age in old male mice

When considering only old mice (23-32 months of age, n =49), a positive correlation is still observed (r= 0.3105, p=0.0168, Figure 2A) and is further observed in only old male mice (r=0.3498, p=0.0314, Figure 2B).

Age is Associated with Decreased Anti-Oxidant Gene Expression

Old male and female mice (n=56) had significantly decreased gene expression for SOD1 (p=0.0361), SOD2 (p=0.0007), and SOD3 (p=0.0001), as shown in Figure 3. Age was also associated with increased expression of the pro-oxidant gene NOX2 (p=0.0131) but was not associated with the increased expression of the inflammatory marker IL-1beta (p>0.1).
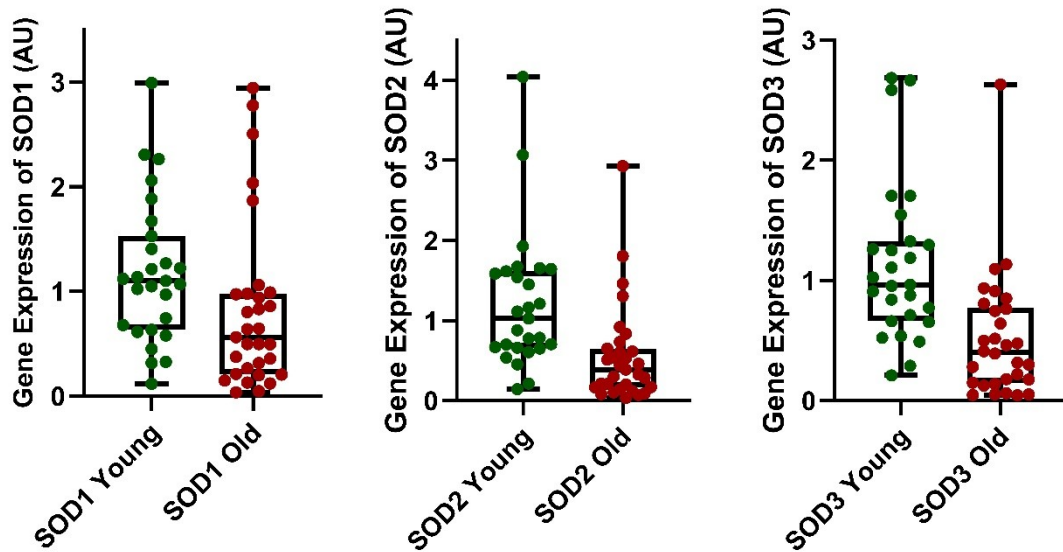
Figure 3

Expression of anti-oxidant genes SOD1, SOD2, SOD3 between young and old male and female mice.

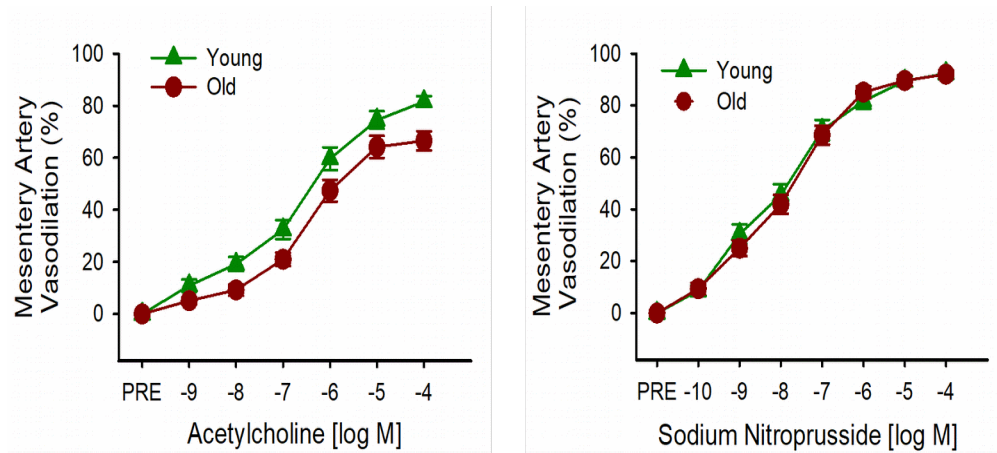## Frailty Index is Negatively correlated with Mesentery Artery Maximal Dilation

Figure 4

A: Mesentery artery dose response to endothelium-depended dilator acetylcholine in young and old male and female mice. B: Mesentery artery dose response to endothelium-independent dilator sodium nitroprusside in young and old male and female mice.

The response to endothelium-independent dilator SNP did not differ between young and old mesentery arteries (Figure 4B, p>0.05). In mesentery arteries, the maximal dilation to the endothelium-dependent dilator ACh in old mice was 15% lower than young (dose response: p=0.015 and max: p=0.004, Figure 4A).
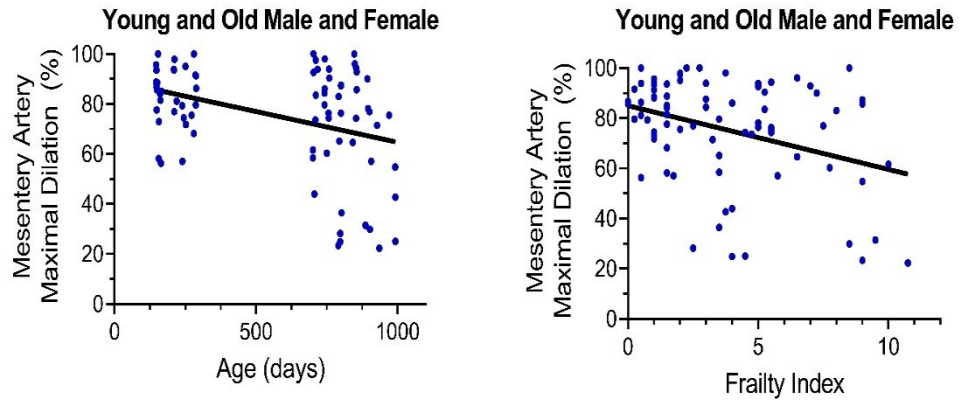
Figure 5

A: Correlation of age with mesentery artery maximal endothelium-dependent dilation (EDD). B: Correlation of frailty index with mesentery artery maximal endothelium-dependent dilation (EDD).

Age was negatively correlated with mesentery artery maximal EDD following ACh administration (r=-0.364, p=0.0005, Figure 5A). Frailty was similarly negatively correlated with maximal mesentery artery EDD for the same set of mice (r=-0.3471, p=0.0009, Figure 5B). Among old male and female mice, there was no significant correlation between frailty in mesentery artery EDD (p>0.05). Old male mice were observed to have a strong correlation of frailty with mesentery maximal EDD (r=-0.4394, p= 0.0097, Figure 6).
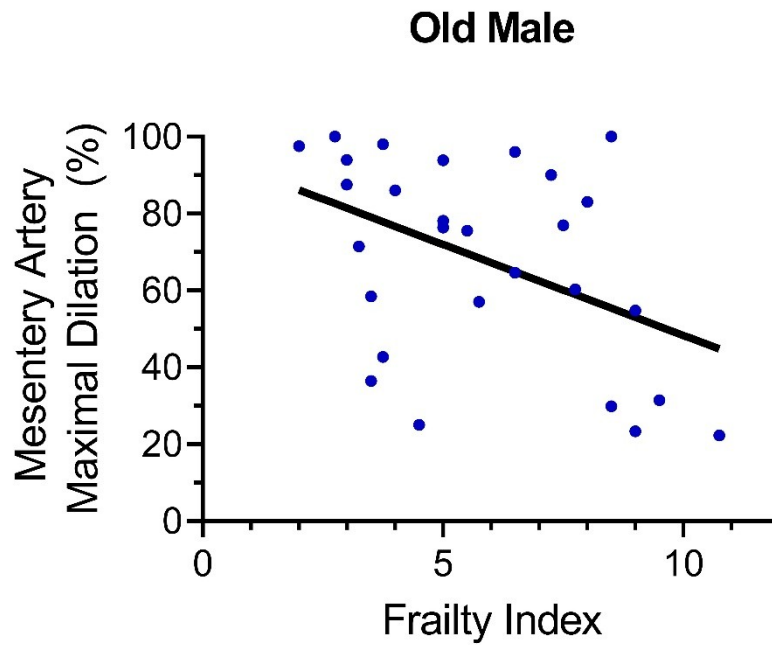
Old Male

Figure 6

Correlation of frailty index with mesentery artery maximal endothelium-dependent
dilation (EDD) in old male mice.

Additionally, mesentery artery maximal EDD was correlated with decreased expression
of anti-oxidant genes SOD2 (p=0.0436) and SOD3 (p=0.0142) in male and female mice
(n=52, data not shown).

Frailty Index Score is not Correlated Middle Cerebral Artery Maximal Dilation
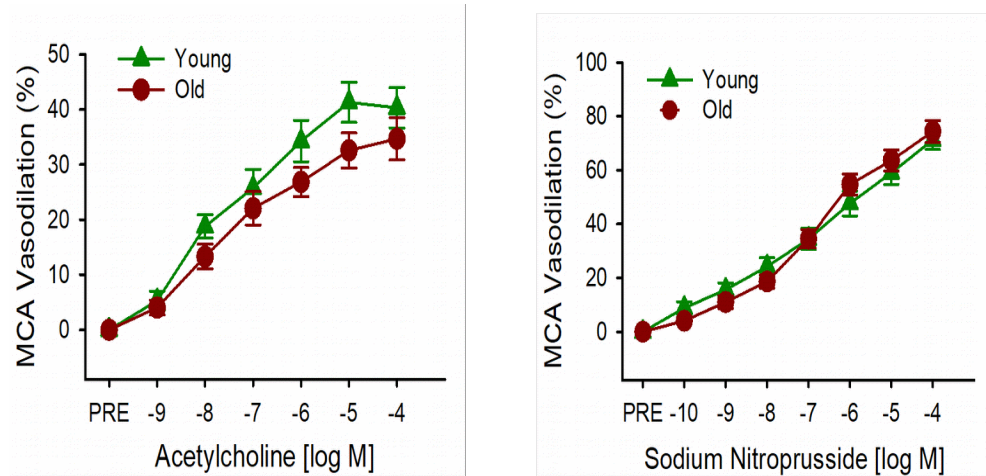
Figure 7

Middle cerebral artery dose response to endothelium-dependent dilator acetylcholine in young and old male and female mice. B: Middle cerebral artery dose response to endothelium-independent dilator sodium nitroprusside in young and old male and female mice.

The response to SNP did not differ between young and old for middle cerebral arteries (Figure 7B, p>0.05). In MCAs, the maximal dilation to ACh in old mice was 12% lower than young but the dose response and maximal dilation were not significantly different between young and old (Figure 7A, p>0.05).

For all ages, both age and frailty index were not significantly correlated with MCA maximal EDD (p>0.05) Among old male and female mice, both age and frailty were not significantly correlated with MCA maximal EDD (p>0.05), and not correlated among only old males (p>0.05).

Improvement of Maximal Dilation following TEMPOL Incubation
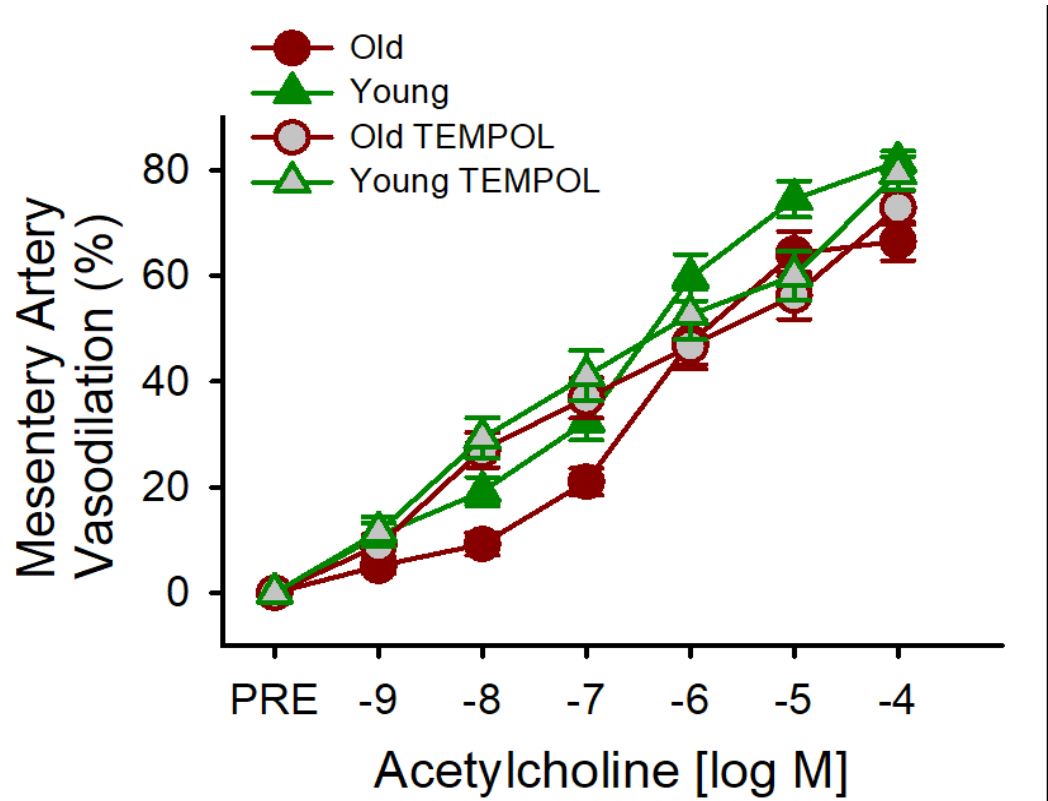
Figure 8

Endothelium-dependent dilation to acetylcholine in the absence or presence of the superoxide scavenger TEMPOL measured by pressure myography in mesentery arteries in young (n=27) and old (n=44) male and female mice.

For old mice, scavenging superoxide with TEMPOL increased the EDD response to ACh in the mesentery arteries (dose response: $p<0.001$ and max: $p=0.26$, Figure 8) but not in old MCAs ($p>0.05$, data not shown). TEMPOL did not affect the EDD response to ACh in young mesentery arteries or MCAs ($p>0.05$).

Changes to Maximal Dilation with TEMPOL in Mesentery Arteries (Age)

Changes to Maximal Dilation with TEMPOL in Mesentery Arteries (Frailty Index)
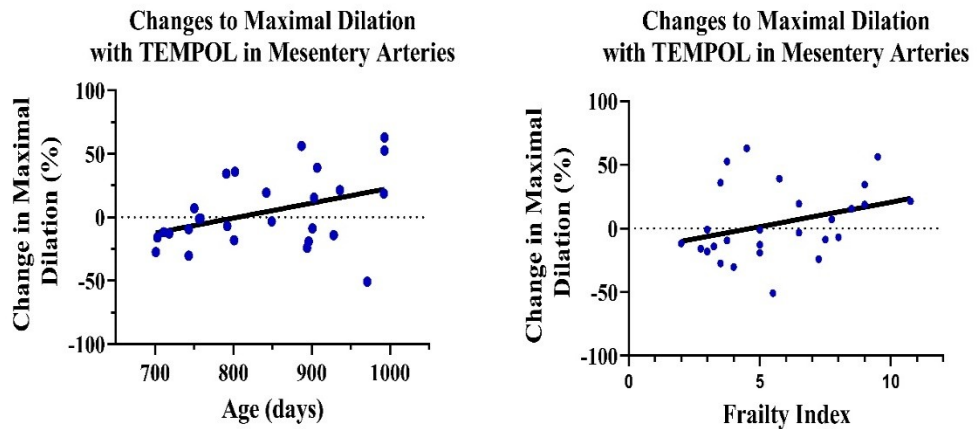
Figure 9

A: Correlation of age with change in maximal endothelium-dependent dilation following TEMPOL incubation in mesentery arteries of old male mice. B: Correlation of frailty index with change in maximal endothelium dependent-dilation following TEMPOL incubation in mesentery arteries of old male mice.

Among old male mice, both age (r=0.39622, p=0.0408, Figure 9A) and frailty index (r=0.3244, p=0.0494, Figure 9B) were positively correlated with an improvement in mesentery artery maximal EDD following incubation with superoxide scavenger TEMPOL.

Discussion

Chronological vs Physiological Health

Aging research is primarily focused on increasing healthspan, the amount of years spent in good health, rather than increasing lifespan, the total amount of years lived. Frailty indexes have become an essential assay in aging research and are now encouraged to be used to evaluate the overall health across several domains before aging interventions can be described as increasing healthspan (Richardson et al., 2016) Previous research has aimed at providing a clinical definition of frailty in both humans (Rockwood et al., 2005) and mice (Whitehead et al., 2014). A 2004 conference held by the National Institute of Aging called for more integrative, system biology approaches to understand the mechanisms of frailty, and in the larger scope, aging (Walston et al., 2006). In this study, we aimed to correlate vascular dysfunction with age and frailty in genetically and environmentally identical mice. Unsurprisingly, our study showed that frailty and age have a positive relationship in that older mice are generally frailer. However, frailty varied among highly genetically similar mice who were studied within days of each other. When controlling for age by only examining old mice in endothelial dysfunction studies, frailty was a significant predictor of impaired maximal dilation in mesentery arteries. Together, these results demonstrate the importance of frailty status in vascular dysfunction and diseases. The heterogeneity among old mice was also observed in our vascular reactivity studies, both when correlating for age and frailty, which has not been reported in the only other studies investigating endothelial dysfunction and frailty (Alonso-Bouzón et al., 2014; Mansur et al., 2015). The data

presented shows vascular aging is heterogeneous and can be independent of chronological age.

Impaired Vascular Abilities in Older/Frailer Mice

Vasodilation is facilitated in two ways: endothelial-independent and endothelium dependent. The former is controlled by the relaxation of smooth muscle cells in the outermost layer of an artery while the latter is controlled by signaling in the endothelial cells that line the inner layers (Taddei et al., 2001). Chemical signaling is mostly facilitated by binding of the neurotransmitter ACh, which causes a series of reactions that induce vascular smooth muscle relaxation (Wessler & Kirkpatrick, 2009; Xu et al., 2015). Vascular dysfunction is described as a reduced EDD in response to increasing doses of ACh (Durrant et al., 2009). Aging is thought to facilitate reduced EDD through two mechanisms: oxidative stress and chronic inflammation. Oxidative stress is primarily mediated by overproduction of O2- outcompeting antioxidant defenses in the body (Katusic & Vanhoutte, 1989). Chronic inflammation is primarily influenced by the master pro-inflammatory transcription factor NF-kB (Donato, Black, Jablonski, Gano, & Seals, 2008).

These mechanisms work together to decrease the availability of NO to endothelial cells, an intermediate product that is necessary to propagate the signaling pathway to increase vessel diameter (Dröge, 2002). This study used ACh to demonstrate EDD and SNP to demonstrate endothelial-independent dilation. In this study, we found mesentery arteries exhibited impaired EDD in older and more frail mice than young and less frail mice while endothelium-independent dilation was not changed. This demonstrates that arterial dysfunction was localized to the endothelial cells inability to

22

respond to ACh rather than the smooth muscle cells inability to dilate. Furthermore, endothelial cell dysfunction could be mediated by increased O2- production and decreased NO availability, as shown by improvements in mesentery EDD after incubation with TEMPOL, an O2- scavenger. Consistent with other studies, TEMPOL was only effective in old mice (de Picciotto et al., 2016; Fleenor, Seals, Zigler, & Sindler, 2012; Gano et al., 2014), indicating that O2- does not impair EDD in young mice.

Possible Mechanisms to Explain Impaired Vascular Abilities

To further look at mechanisms related to age, frailty, and vascular reactivity we conducted expression of pro-oxidant (NOX), anti-oxidant (SOD), and inflammatory (IL-1beta) enzymes in mesentery arteries. Inflammatory cytokine release (such as IL-1beta) activates an intracellular signaling cascade which increases the activity of NADPH oxidases (NOXs). NOXs are one of the enzymes responsible for producing superoxide (O2-) from oxygen molecules. The NOX enzyme is made of multiple subunits, each encoded for by a different gene. In this study, we focused on NOX2, the catalytic subunit of NOX most highly expressed in endothelial and smooth muscle cells. Superoxide dismutase (SOD) is an enzyme that converts O2- into H2O2 (Valko et al., 2007). There are 3 different isoforms of SOD found in different parts of a normal cell: SOD1 is found in the cytosol, SOD2 in the mitochondria, and SOD3 is extracellular. This study found significant correlations in several steps of the oxidative stress pathway. Decreased expression of all the isoforms of SOD and increased expression of NOX2 in old compared with young mice indicates that with aging O2- is being produced at higher rates without equivalent clearance from all parts of the cell.

Furthermore, lower SOD2 and SOD3 capacity was associated with decreased EDD, indicating that endothelial dysfunction in these arteries may result from a reduced ability to mitigate O2- in mitochondria or a reduction in blocking the transfer/intercommunication of in O2- between mesentery artery cells through the extracellular space.

Observed Variations Worth Mentioning

This study looked at two vascular beds because previous studies have shown they have distinct functional changes with aging in other mouse models (Walker et al., 2015). MCAs were used because they are highly susceptible to large artery stiffness seen with aging and is the main blood supply to the lateral surface of the cerebrum and the temporal lobe of the brain. Mesentery arteries were chosen because of their importance in blood pressure regulation and are susceptible to changes in the gut microbiota. The results shown in this study clearly show a variation in endothelial cell abilities between the vascular beds. While mesentery artery EDD dysfunction was associated with age, frailty, and anti-oxidant gene expression, MCA function was not significantly correlated with any of the variables presented in this study. A possible explanation for these results could be that mesentery arteries are more susceptible to endothelial cell dysfunction than MCAs because of their important role in blood pressure. Mesentery arteries could also influence frailty by changing blood flow to the intestines leading to modulations in digestion and metabolism. Additionally, it is possible that cognitive ability which was not part of the frailty index measurement in this study, is related to MCA endothelial dysfunction.

A second general observation made from this study is the difference between male and female mice. In this study, young and old female mice data were included because few studies conducted in this lab have examined females in vascular reactivity studies. The results of this study show that for a few correlations, old female mice were skewing data (see Figure 5B and 6). Colloquially, researchers observed several problems in young and old female mice that were not seen very often with male mice of the same ages, specifically eye swelling. It is well known that estrogen is a protective factor in mice, humans, and many other species because of its anti-oxidant effects (Viña, Gambini, García-García, Rodriguez-Mañas, & Borrás, 2013). In contrast to humans, mice don't experience a "menopause" and instead have a slow, more linear decline in estrogen levels with aging. In aged ovariectomized mice, estrogen agonists were shown to have a positive effect on aging-associated deficiencies in brain health and frailty index compared to controls (Said et al., 2018). Additionally, estrogen-regulated genes have biological functions in the electron transport chain of mitochondria, a major source of reactive oxygen species generation (O'Lone et al., 2007). The extent to which estrogen was impacting the results presented in this study is unknown and would need to be further investigated.

Limitations

Several limitations were unavoidable in this study. The research lab that these experiments were conducted only had the capability to house C57BL/6 mice and the findings of this study should be repeated in other strains of mice. While this study did represent a wider range of ages than many other studies, our cohort had a gap in the middle ages. Only gene expression protocols were possible in this study because of the

25

small size of mesentery arteries and thus no conclusions can be made about protein

concentration or enzyme activity. Finally, the results presented in this study are

correlations and do not provide any evidence towards causation.

Conclusion

The major finding of this study is that frailty is correlated with age and mesentery artery endothelial cell dysfunction. The driver of this dysfunction appears to be oxidative stress and lower antioxidant enzyme expression. In contrast to mesentery arteries, MCA endothelial dysfunction was not correlated with frailty index or age. These results suggest that frailty index could be a non-invasive marker of vascular impairment. These results also indicate that improving mesentery artery health may be a possible way to reduce frailty in older adults.

Further directions for this study would be focused on additional experiments with MCAs to recreate previous results from this lab. Additionally, more studies with female mice will further help understand the role of estrogen in vascular abilities and frailty. Finally, further characterizing the vascular and enzyme activity effects that are associated with increased frailty in mice can lead to better understanding of frailty in older adults.

Additional Materials

Table 1. Clinical Assessment of Deficits in Aging Mice to Create a Frailty Index.

Clinical Assessment of Deficits in Aging Mice to Create a Frailty Index. Adapted from (Whitehead et al., 2014)

Parameter	Clinical Assessment of deficit	Scoring
Temperature	Measure surface body temperature with an infrared thermometer directed at the abdomen (average of 3 times). Compare with reference values from sex-matched adult animals	0 = differs by <1 SD 0.5 = differs by 1-3 SD 1 = differs by >3 SD
Body weight	Weigh the mouse. Compare with reference values from sex-matched adult animals	0 = differs by <1 SD 0.5 = differs by 1-3 SD 1 = differs by >3 SD
Breathing rate/ depth	Observe the animal. Note the rate and depth of breathing as well as any gasping behavior	0 = normal 1 = labored breathing, rapid breathing or gasping behavior
Mouse grimace scale	Note facial signs of discomfort: orbital tightening, nose bulge, cheek bulge, ear position (drawn back), or whisker change	0 = no signs present 0.5 = 1 or 2 signs present 1 = 3 or more signs present
Piloerection	Observe the animal and look for signs of piloerection, in particular on the back of the neck	0 = no piloerection 0.5 = involves fur only on back of neck 1= widespread piloerection
Alopecia	Gently restrain the animal and inspect it for signs of fur loss *exclude potential signs of barbering or injury	0 = normal fur density 0.5 = focal areas of fur loss 1= widespread or multifocal fur loss
Loss of fur color	Note any changes in fur color from black to brown or gray	0 = normal color 0.5 = focal gray/brown changes 1= gray/brown fur throughout body

Dermatitis	Document skin lesions, open sores anywhere on the body	0 = absent 0.5 = focal lesions (ears, neck, under chin) 1= widespread or multifocal lesions
Loss of whiskers	Inspect the animal for signs of a reduction in the number of whiskers	0 = no loss, long whiskers 0.5 = reduced number or only short whiskers 1= absence of whiskers
Coat condition	Inspect the animal for signs of poor grooming	0 = smooth, sleek, shiny coat 0.5 = coat is slightly ruffled 1= unkempt, ungroomed, matted appearance
Tumors	Observe the mice to look for symmetry. Hold the base of the tail and manually examine mice for visible or palpable tumors.	0 = absent 0.5 = < 1 cm, single tumor 1= >1 cm, multiple tumors
Distended abdomen	Hold the mouse vertically by the base of their tail and tip backwards over your hand	0 = absent 0.5 = slight bulge 1= abdomen clearly distended
Kyphosis	Inspect the mouse for curvature of the spine or hunched posture. Run your fingers down both sides of the spine to detect abnormalities	0 = absent 0.5 = mild curvature felt upon touch 1= clear visual evidence of hunched posture
Tail stiffening	Grasp the base of the tail with one hand and stroke the tail with a finger of the other hand. The tail should wrap freely around the finger when mouse is relaxed	0 = whole tail curls 0.5 = tail responsive but does not curl, or only curls at the very end 1= tail completely unresponsive
Gait disorders	Observe the freely moving mouse to detect abnormalities such as hopping, wobbling, circling, wide stance, or weakness	0 = no abnormality 0.5 = abnormal gait, animal can walk fast 1= marked abnormality, impaired ability to walk fast
Tremor	Observe the freely moving animal to detect tremor, both at rest and when the animal is trying to climb up an incline	0 = no tremor 0.5 = slight tremor 1 = marked tremor, animal cannot climb
Forelimb grip	Hold the mouse. Allow it to	0 = sustained grip

strength	grip the cage lid. Life animal by the base of the tail to assess grip strength	0.5 = reduction in grip strength 1 = no grip strength, no resistance
Body condition score	Place mouse on flat surface, hold tail base and manually assess the flesh/fat that covers the back and pubic bones	0 = bones palpable, not prominent 0.5 = bones are prominent or barely felt 1 = bones are visible or not felt due to obesity
Vestibular disturbance	Hold the base of the tail and lower mouse towards a flat surface, let animal walk while holding tail. Inspect for head tilt, spinning, circling, head tuck or trunk curling	0 = absent 0.5 = mild head tilt and/or spin when lowered 1 = severe disequilibrium
Hearing loss	Test startle reflex (mouse will flinch and blink). Hold a clicker near mouse, sound it 3 times and record responses.	0 = always reacts (3/3 times) 0.5 = reacts 1/3 or 2/3 times 1 = unresponsive (0/3 times)
Cataracts	Visual inspection of the mouse to detect opacity in the center of the eye	0 = no cataract 0.5 = small opaque spot 1 = whole lens is opaque
Corneal opacity	Visual inspection of the mouse to superficial white spots and/or clouding of the cornea	0 = no clouding/spots 0.5 = small, singular spot 1 = large or multiple spots/clouding
Eye discharge/swelling	Visual inspection of the mouse to detect ocular discharge and swelling of the eyes	0 = normal 0.5 = slight swelling and/or secretions 1 = obvious bulging and/or secretions
Microphthalmia	Inspect eyes.	0 = normal size 0.5 = one or both eyes slightly small or sunken 1 = one or both eyes very small or sunken
Nasal discharge	Visual inspection of the mouse to detect nasal discharge	0 = no discharge 0.5 = small amount of discharge or redness 1 = obvious discharge, both nares
Vision loss/menace reflex	Move an object towards the mouse's face 3 times. Record whether the mouse blinks in response	0 = always responds 0.5 = no response to 1 or 2 approaches 1 = no response to 3

		approaches
Malocclusions	Grasp the mouse by the neck scruff, invert and expose teeth. Look for uneven, overgrown teeth	0 = mandibular longer than maxillary 0.5 = teeth slightly uneven 1 = teeth very uneven and overgrown
Rectal prolapse	Grasp the mouse by the base of the tail to detect signs of rectal prolapse	0 = no prolapse 0.5 = small portion of rectum seen below tail or discharge 1 = entire rectum clearly visible below tail
Penile prolapse	Grasp the mouse by the base of the tail to detect signs of penile prolapse	0 = no prolapse 0.5 = small amount of prolapsed tissue visible 1 = prolapsed tissue clearly visible
Diarrhea	Grasp the mouse and invert it to check for signs of diarrhea. Also look for fecal smearing in home cage	0 = none 0.5 = some feces or bedding near rectum 1 = bloody feces and bedding near rectum, home cage smearing

Table 2. Mouse Frailty Assessment Form©

						Date: _____	

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

Rating: 0 = absent   0.5 = mild   1 = severe

				NOTES:
➤ **Respiratory system:**				
❖ Breathing rate/depth	0	0.5	1	_____
➤ **Discomfort:**				
❖ Mouse Grimace Scale	0	0.5	1	_____
❖ Piloerection	0	0.5	1	_____
➤ **Integument:**				
❖ Alopecia	0	0.5	1	_____
❖ Loss of fur colour	0	0.5	1	_____
❖ Dermatitis	0	0.5	1	_____
❖ Loss of whiskers	0	0.5	1	_____
❖ Coat condition	0	0.5	1	_____
➤ **Physical/Musculoskeletal:**				
❖ Tumours	0	0.5	1	_____
❖ Distended abdomen	0	0.5	1	_____
❖ Kyphosis	0	0.5	1	_____
❖ Tail stiffening	0	0.5	1	_____
❖ Gait disorders	0	0.5	1	_____
❖ Tremor	0	0.5	1	_____
❖ Forelimb grip strength	0	0.5	1	_____
❖ Body condition score	0	0.5	1	_____
➤ **Vestibulocochlear/Auditory:**				
❖ Vestibular disturbance	0	0.5	1	_____
❖ Hearing loss	0	0.5	1	_____
➤ **Ocular/Nasal:**				
❖ Cataracts	0	0.5	1	_____
❖ Corneal opacity	0	0.5	1	_____
❖ Eye discharge/swelling	0	0.5	1	_____
❖ Microphthalmia	0	0.5	1	_____
❖ Menace reflex	0	0.5	1	_____
❖ Nasal discharge	0	0.5	1	_____
➤ **Digestive/Urogenital:**				
❖ Malocclusions	0	0.5	1	_____
❖ Rectal prolapse	0	0.5	1	_____
❖ Penile prolapse	0	0.5	1	_____
❖ Diarrhoea	0	0.5	1	_____

❖ **Temperature score:** _____
❖ **Body weight score:** _____

Total Score/ Max Score:

© Susan E. Howlett, 2013

Additional Figure 1: A Clinical Frailty Index in Mice

Mouse Frailty Assessment form, adapted from (Whitehead et al., 2014)

Bibliography

Alonso-Bouzón, C., Carcaillon, L., García-García, F. J., Amor-Andrés, M. S., Assar, M. El, & Rodríguez-Mañas, L. (2014). Association between endothelial dysfunction and frailty: the Toledo Study for Healthy Aging. *Age*, *36*(1), 495. https://doi.org/10.1007/S11357-013-9576-1

Avila-Funes, J. A., Carcaillon, L., Helmer, C., Carrière, I., Ritchie, K., Rouaud, O., … Amieva, H. (2012). Is frailty a prodromal stage of vascular dementia? Results from the three-city study. *Journal of the American Geriatrics Society*, *60*(9), 1708–1712. https://doi.org/10.1111/j.1532-5415.2012.04142.x

Buchman, A. S., Boyle, P. A., Wilson, R. S., Tang, Y., & Bennett, D. A. (2007). Frailty is associated with incident Alzheimer's disease and cognitive decline in the elderly. *Psychosomatic Medicine*. https://doi.org/10.1097/psy.0b013e318068de1d

Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., … Stern, D. M. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood*, *91*(10), 3527–3561. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9572988

de Picciotto, N. E., Gano, L. B., Johnson, L. C., Martens, C. R., Sindler, A. L., Mills, K. F., … Seals, D. R. (2016). Nicotinamide mononucleotide supplementation reverses vascular dysfunction and oxidative stress with aging in mice. *Aging Cell*, *15*(3), 522–530. https://doi.org/10.1111/acel.12461

Donato, A. J., Black, A. D., Jablonski, K. L., Gano, L. B., & Seals, D. R. (2008). Aging is associated with greater nuclear NFκB, reduced IκBα, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. *Aging Cell*, *7*(6), 805–812. https://doi.org/10.1111/j.1474-9726.2008.00438.x

Donato, A. J., Eskurza, I., Silver, A. E., Levy, A. S., Pierce, G. L., Gates, P. E., & Seals, D. R. (2007). Direct Evidence of Endothelial Oxidative Stress With Aging in Humans. *Circulation Research*, *100*(11), 1659–1666. https://doi.org/10.1161/01.RES.0000269183.13937.e8

Dröge, W. (2002). Free Radicals in the Physiological Control of Cell Function. *Physiological Reviews*, *82*(1), 47–95. https://doi.org/10.1152/physrev.00018.2001

Durrant, J. R., Seals, D. R., Connell, M. L., Russell, M. J., Lawson, B. R., Folian, B. J., … Lesniewski, L. A. (2009). Voluntary wheel running restores endothelial function in conduit arteries of old mice: direct evidence for reduced oxidative stress, increased superoxide dismutase activity and down-regulation of NADPH oxidase. *The Journal of Physiology*, *587*(13), 3271–3285. https://doi.org/10.1113/jphysiol.2009.169771

Elhusseiny, A., Cohen, Z., Olivier, A., Stanimirović, D. B., & Hamel, E. (1999). Functional Acetylcholine Muscarinic Receptor Subtypes in Human Brain Microcirculation: Identification and Cellular Localization. *Journal of Cerebral Blood Flow & Metabolism*, *19*(7), 794–802. https://doi.org/10.1097/00004647-199907000-00010

Fleenor, B. S., Eng, J. S., Sindler, A. L., Pham, B. T., Kloor, J. D., & Seals, D. R. (2014). Superoxide signaling in perivascular adipose tissue promotes age-related artery stiffness. *Aging Cell*, *13*(3), 576–578. https://doi.org/10.1111/acel.12196

Fleenor, B. S., Seals, D. R., Zigler, M. L., & Sindler, A. L. (2012). Superoxide-lowering therapy with TEMPOL reverses arterial dysfunction with aging in mice. *Aging Cell*, *11*(2), 269–276. https://doi.org/10.1111/j.1474-9726.2011.00783.x

Gano, L. B., Donato, A. J., Pasha, H. M., Hearon, C. M., Sindler, A. L., & Seals, D. R. (2014). The SIRT1 activator SRT1720 reverses vascular endothelial dysfunction, excessive superoxide production, and inflammation with aging in mice. *American Journal of Physiology - Heart and Circulatory Physiology*, *307*(12), H1754–H1763. https://doi.org/10.1152/ajpheart.00377.2014

Gokce, N., Keaney, J. F., Hunter, L. M., Watkins, M. T., Menzoian, J. O., & Vita, J. A. (2002). Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study. *Circulation*, *105*(13), 1567–1572. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11927524

Harrison, D. G. (1997). Endothelial function and oxidant stress. *Clinical Cardiology*, *20*(11 Suppl 2), II-11–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9422847

Katusic, Z. S., & Vanhoutte, P. M. (1989). Superoxide anion is an endothelium-derived contracting factor. *American Journal of Physiology-Heart and Circulatory Physiology*, *257*(1), H33–H37. https://doi.org/10.1152/ajpheart.1989.257.1.H33

Kirkland, J. L., & Peterson, C. (2009). Healthspan, translation, and new outcomes for animal studies of aging. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *64*(2), 209–212. https://doi.org/10.1093/gerona/gln063

Lesniewski, L. A., Durrant, J. R., Connell, M. L., Folian, B. J., Donate, A. J., & Seals, D. R. (2011). Salicylate treatment improves age-associated vascular endothelial dysfunction: Potential role of nuclear factor κb and forkhead box o phosphorylation. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences, 66 A*(4), 409–418. https://doi.org/10.1093/gerona/glq233

Mansur, H. N., Lovisi, J. C. M., Colugnati, F. A. B., Raposo, N. R. B., Fernandes, N. M. da S., & Bastos, M. G. (2015). Association of frailty with endothelial dysfunction and its possible impact on negative outcomes in Brazilian predialysis patients with chronic kidney disease. *BMC Nephrology*, *16*(1), 157. https://doi.org/10.1186/s12882-015-0150-1

Niwa, K., Carlson, G. A., & Iadecola, C. (2000). Exogenous Aβ1–40 Reproduces Cerebrovascular Alterations Resulting from Amyloid Precursor Protein Overexpression in Mice. *Journal of Cerebral Blood Flow & Metabolism*, *20*(12), 1659–1668. https://doi.org/10.1097/00004647-200012000-00005

O'Lone, R., Knorr, K., Jaffe, I. Z., Schaffer, M. E., Martini, P. G. V., Karas, R. H., … Hansen, U. (2007). Estrogen Receptors α and β Mediate Distinct Pathways of Vascular Gene Expression, Including Genes Involved in Mitochondrial Electron Transport and Generation of Reactive Oxygen Species. *Molecular Endocrinology*, *21*(6), 1281–1296. https://doi.org/10.1210/me.2006-0497

Palmer, R. M. J., Ashton, D. S., & Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, *333*(6174), 664–666. https://doi.org/10.1038/333664a0

Richardson, A., Fischer, K. E., Speakman, J. R., de Cabo, R., Mitchell, S. J., Peterson, C. A., … Austad, S. N. (2016). Measures of Healthspan as Indices of Aging in Mice—A Recommendation. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *71*(4), 427–430. https://doi.org/10.1093/gerona/glv080

Rockwood, K., Song, X., MacKnight, C., Bergman, H., Hogan, D. B., McDowell, I., & Mitnitski, A. (2005). A global clinical measure of fitness and frailty in elderly people. *CMAJ : Canadian Medical Association Journal = Journal de l'Association Medicale Canadienne*, *173*(5), 489–495. https://doi.org/10.1503/cmaj.050051

Said, S. A., Isedowo, R., Guerin, C., Nar, N. N., Lillie, L., Bukovac, S., … Stuart, J. A. (2018). Effects of long-term dietary administration of estrogen receptor-beta agonist diarylpropionitrile on ovariectomized female ICR (CD-1) mice. *GeroScience*, *40*(4), 393–403. https://doi.org/10.1007/s11357-018-0038-7

Seals, D. R., Jablonski, K. L., & Donato, A. J. (2011). Aging and vascular endothelial function in humans. *Clinical Science*, *120*(9), 357–375. https://doi.org/10.1042/CS20100476

Sergi, G., Veronese, N., Fontana, L., De Rui, M., Bolzetta, F., Zambon, S., … Manzato, E. (2015). Pre-Frailty and Risk of Cardiovascular Disease in Elderly Men and Women: The Pro.V.A. Study. *Journal of the American College of Cardiology*, *65*(10), 976–983. https://doi.org/10.1016/J.JACC.2014.12.040

Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A., & Salvetti, A. (2001). Age-Related Reduction of NO Availability and Oxidative Stress in Humans. *Hypertension*, *38*(2), 274–279. https://doi.org/10.1161/01.HYP.38.2.274

Ungvari, Z., Orosz, Z., Labinskyy, N., Rivera, A., Xiangmin, Z., Smith, K., & Csiszar, A. (2007). Increased mitochondrial H 2 O 2 production promotes endothelial NF-κB activation in aged rat arteries. *American Journal of Physiology-Heart and Circulatory Physiology*, *293*(1), H37–H47. https://doi.org/10.1152/ajpheart.01346.2006

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007, January 1). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*. Pergamon. https://doi.org/10.1016/j.biocel.2006.07.001

Viña, J., Gambini, J., García-García, F. J., Rodriguez-Mañas, L., & Borrás, C. (2013). Role of oestrogens on oxidative stress and inflammation in ageing. *Hormone Molecular Biology and Clinical Investigation*, *16*(2), 65–72. https://doi.org/10.1515/hmbci-2013-0039

Vita, J. A., & Keaney, J. F. (2002). Endothelial Function. *Circulation*, *106*(6), 640–642. https://doi.org/10.1161/01.CIR.0000028581.07992.56

Walker, A. E., Henson, G. D., Reihl, K. D., Morgan, R. G., Dobson, P. S., Nielson, E. I., … Donato, A. J. (2015). Greater impairments in cerebral artery compared with skeletal muscle feed artery endothelial function in a mouse model of increased large artery stiffness. *The Journal of Physiology*, *593*(8), 1931–1943. https://doi.org/10.1113/jphysiol.2014.285338

Walker, A. E., Henson, G. D., Reihl, K. D., Nielson, E. I., Morgan, R. G., Lesniewski, L. A., & Donato, A. J. (2014). Beneficial effects of lifelong caloric restriction on endothelial function are greater in conduit arteries compared to cerebral resistance arteries. *Age (Dordrecht, Netherlands)*, *36*(2), 559–569. https://doi.org/10.1007/s11357-013-9585-0

Walker, A. E., Kaplon, R. E., Pierce, G. L., Nowlan, M. J., & Seals, D. R. (2014). Prevention of age-related endothelial dysfunction by habitual aerobic exercise in healthy humans: Possible role of nuclear factor κB. *Clinical Science*, *127*(11), 645–654. https://doi.org/10.1042/CS20140030

Walston, J., Hadley, E. C., Ferrucci, L., Guralnik, J. M., Newman, A. B., Studenski, S. A., … Fried, L. P. (2006). Research Agenda for Frailty in Older Adults: Toward a Better Understanding of Physiology and Etiology: Summary from the American Geriatrics Society/National Institute on Aging Research Conference on Frailty in Older Adults. *Journal of the American Geriatrics Society*, *54*(6), 991–1001. https://doi.org/10.1111/j.1532-5415.2006.00745.x

Wessler, I., & Kirkpatrick, C. J. (2009). Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *British Journal of Pharmacology*, *154*(8), 1558–1571. https://doi.org/10.1038/bjp.2008.185

Whitehead, J. C., Hildebrand, B. A., Sun, M., Rockwood, M. R., Rose, R. A., Rockwood, K., & Howlett, S. E. (2014). A Clinical Frailty Index in Aging Mice: Comparisons With Frailty Index Data in Humans. *The Journals of Gerontology: Series A*, *69*(6), 621–632. https://doi.org/10.1093/gerona/glt136

Xu, T., Lan, X., Guan, Y., Zhang, S., Wang, X., & Miao, C. (2015). Chronic nicotine treatment enhances vascular smooth muscle relaxation in rats. *Acta Pharmacologica Sinica*, *36*(4), 429–439. https://doi.org/10.1038/aps.2015.5