ASSOCIATION BETWEEN A NON-INVASIVE ASSESSMENT OF FRAILTY AND VASCULAR DYSFUNCTION IN OLD MICE

By

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A THESIS

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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.
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Table 1. Clinical Assessment of Deficits in Aging Mice to Create a Frailty Index. 28
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 shear 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
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,
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, TEMPO (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

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 1
Correlation of age with frailty index score for male and female mice across young and old ages.
When considering only old mice (23-32 months of age, \( n = 49 \)), a positive correlation is still observed \( (r = 0.3105, p = 0.0168, \text{Figure 2A}) \) and is further observed in only old male mice \( (r = 0.3498, p = 0.0314, \text{Figure 2B}) \).

**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 \( (p > 0.1) \).
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

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).

![Young and Old Male and Female](image1)

![Young and Old Male and Female](image2)

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).
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

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).
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
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, Rodríguez-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
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)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clinical Assessment of deficit</th>
<th>Scoring</th>
</tr>
</thead>
</table>
| 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                           |
<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
<th>0 = absent</th>
<th>0.5 = focal lesions (ears, neck, under chin)</th>
<th>1 = widespread or multifocal lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatitis</td>
<td>Document skin lesions, open sores anywhere on the body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of whiskers</td>
<td>Inspect the animal for signs of a reduction in the number of whiskers</td>
<td>0 = no loss, long whiskers</td>
<td>0.5 = reduced number or only short whiskers</td>
<td>1 = absence of whiskers</td>
</tr>
<tr>
<td>Coat condition</td>
<td>Inspect the animal for signs of poor grooming</td>
<td>0 = smooth, sleek, shiny coat</td>
<td>0.5 = coat is slightly ruffled</td>
<td>1 = unkempt, ungroomed, matted appearance</td>
</tr>
<tr>
<td>Tumors</td>
<td>Observe the mice to look for symmetry. Hold the base of the tail and manually examine mice for visible or palpable tumors.</td>
<td>0 = absent</td>
<td>0.5 = &lt; 1 cm, single tumor</td>
<td>1 = &gt;1 cm, multiple tumors</td>
</tr>
<tr>
<td>Distended abdomen</td>
<td>Hold the mouse vertically by the base of their tail and tip backwards over your hand</td>
<td>0 = absent</td>
<td>0.5 = slight bulge</td>
<td>1 = abdomen clearly distended</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>Inspect the mouse for curvature of the spine or hunched posture. Run your fingers down both sides of the spine to detect abnormalities</td>
<td>0 = absent</td>
<td>0.5 = mild curvature felt upon touch</td>
<td>1 = clear visual evidence of hunched posture</td>
</tr>
<tr>
<td>Tail stiffening</td>
<td>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</td>
<td>0 = whole tail curls</td>
<td>0.5 = tail responsive but does not curl, or only curls at the very end</td>
<td>1 = tail completely unresponsive</td>
</tr>
<tr>
<td>Gait disorders</td>
<td>Observe the freely moving mouse to detect abnormalities such as hopping, wobbling, circling, wide stance, or weakness</td>
<td>0 = no abnormality</td>
<td>0.5 = abnormal gait, animal can walk fast</td>
<td>1 = marked abnormality, impaired ability to walk fast</td>
</tr>
<tr>
<td>Tremor</td>
<td>Observe the freely moving animal to detect tremor, both at rest and when the animal is trying to climb up an incline</td>
<td>0 = no tremor</td>
<td>0.5 = slight tremor</td>
<td>1 = marked tremor, animal cannot climb</td>
</tr>
<tr>
<td>Forelimb grip</td>
<td>Hold the mouse. Allow it to</td>
<td>0 = sustained grip</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
<td>Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------------------------------------------------</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
| **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 |
<table>
<thead>
<tr>
<th>Condition</th>
<th>Examination Method</th>
<th>Scoring System</th>
</tr>
</thead>
</table>
| 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 |
Additional Figure 1: A Clinical Frailty Index in Mice

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


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