EFFECTS OF ALT-711 TREATMENT ON AGE-RELATED CEREBROVASCULAR DYSFUNCTION AND COGNITIVE IMPAIRMENT IN AGED MICE

by

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A THESIS

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As the life expectancy for humans continues to increase, the prevalence of age-related diseases is rising. Specifically, humans are becoming more at risk for cardiovascular and cerebrovascular diseases. As a result, it is important to understand the physiology behind age-related cognitive impairment so that we can propose solutions and treatments for these diseases. There are many factors that can lead to aging of the brain. Specifically, large artery stiffness is a strong predictor of cerebrovascular dysfunction. Large artery stiffness occurs because of the accumulation of advanced glycation end-products, or AGEs, in blood vessels. This study used wild type C57BL/6 mice to determine the impact that Alagebrium Chloride, or ALT-711 - a drug that breaks AGEs - has with regards to improving cerebrovascular function and cognition in aged mice. C57BL/6 mice treated with Alt-711, via oral gavage, were compared to C57BL/6 mice without treatment. We found that ALT-711 did not impact large artery stiffness, nitric oxide mediated dilation in cerebral endothelial cells, cognition, or motor coordination ($p>0.05$ for all). However, the data revealed that posterior cerebral artery (PCA) elastic modulus is correlated with motor coordination and instinctual behavior. In addition, cerebral artery endothelial function is correlated with motor coordination. The results of this study give insight about the efficacy of ALT-711 treatment on preserving cerebrovascular function and cognition, and it also helps expand our knowledge about which treatments work for large artery stiffness prevention in mice.

Acknowledgements

I would like to thank Dr. Ashley Walker for the opportunity to conduct research in the Aging and Vascular Physiology Laboratory. This lab was an integral part of my college experience, and I feel lucky and honored to have been a part of it. I would also like to thank Emily Reeve for serving as the third reader on my thesis committee and being the absolute best mentor that I could ask for. Her continuous guidance and advice were invaluable to my pursuit of becoming a better scientist and a better person. Lastly, thank you to Brian McWhorter for serving as the CHC advisor on my thesis committee. His flexibility, communication and availability made the thesis process smooth and manageable.

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Introduction

With increasing standards of living and a growing body of knowledge on health and physiology, humans are living longer than ever. In developed countries, life expectancy has nearly doubled in the past century (Shetty et al., 2018). In fact, the number of people over 65 years old in the U.S. is projected to become ~25% of the population within the next 40 years (Shetty et al., 2018). This has significantly raised the prevalence of age-related diseases and cardiovascular ailments (Yazdanyar, A. & Newman A. B., 2009). To promote the maintenance of overall health as well as push for a higher quality of life, there is an urgent need to understand these age-related pathologies so that medical interventions can be employed.

As humans age, evidence suggests that their large arteries, namely the carotid artery and the aorta, become stiffer. This is due to the breakdown or increased fragmentation of elastin, an extracellular matrix protein important for arterial elasticity and dilation, as well as an increase in crosslinking of collagen, a main structural protein that provides support and strength (Walker et al., 2021). In healthy individuals, collagen and elastin are present in proper amounts, which allows for sufficient elasticity within the large arteries. This large artery elasticity dampens the pulsatile flow of blood before it enters the brain. In turn, it protects the brain's sensitive microvasculature from damage and degradation (Chirinos, J. A. et al., 2019). However, stiffer large arteries cannot dampen this pulsatile flow as effectively, leading to increased pulse pressure to smaller, systemic arteries (Chirinos, J. A. et al., 2019). As a result, the increased pulse pressure to the brain causes damage to the cerebrovasculature and results in impaired neuronal communication, cerebral hypoperfusion, and ultimately cognitive impairment (Cooper & Mitchell 2016).

Notably, there is evidence that suggests that arterial stiffness may cause dysfunction of the endothelial cells of cerebral arteries (Donato et al., 2018). Endothelial cells play a key role in the dilation and constriction of arteries (Cyr et al., 2020). In doing so, they act as effective blood flow regulators to the brain - an important and essential function.

An understanding of the mechanism by which they promote dilation and constriction is crucial to this study. At large, endothelial cells regulate cerebral blood flow by releasing chemical signals that will communicate with the smooth muscle surrounding the arteries to either dilate or constrict the vessel (Luscher & Barton, 1997). Specifically, one signal that causes endothelial cells to release vasodilators is acetylcholine (ACh) (Luscher & Barton, 1997). When ACh enters the endothelium, it activates endothelial nitric oxide synthase (eNOS) (Elhusseiny et al., 1999). Once activated, eNOS produces nitric oxide (NO) - a vasodilator. After NO is produced, it passes through to smooth muscle cells where it causes relaxation and thus dilation of the arteries. This mechanism may be impaired in individuals whose arteries are stiffer. The entrance of unregulated, high pulsatile flow into the brain promotes dysfunction of cerebral endothelial cells (Thorin-Trescases et al., 2018). As a result, their function is impaired and their ability to cause dilation, or respond to doses of ACh, could be impaired or diminished. For this reason, ACh is a useful tool to evaluate the function of endothelial cells in *ex vivo* studies. These results can be helpful for elucidating the relationship between large artery stiffness and cerebrovascular dysfunction.

In addition to large artery stiffness and cerebrovascular dysfunction, another common characteristic of vascular aging is the accumulation of oxidative stress (Ionescu-Tucker & Cotman 2021). The various complex physiological processes that occur in the human body have necessary metabolic byproducts. Among the most common and impactful metabolic byproducts

are reactive oxygen species (ROS). ROS primarily results from mitochondrial respiration as they are byproducts of the electron transport chain (Ionescu-Tucker & Cotman 2021). At basal levels, they can function as useful cellular messengers and mediators of the cell cycle (Dumas & Knaus, 2021). However, accumulation of ROS throughout a lifespan can have harmful effects such as inducing cell apoptosis, promoting chronic inflammation, and causing mitochondrial dysfunction (Jahan et al., 2022). However, perhaps one of the most relevant consequences of ROS is its role in reducing NO bioavailability. ROS are unstable free radicals, so they are very reactive. As a result, excess circulating ROS will react with NO ultimately impairing the ability of endothelial cells to induce dilation (Csiszar et al., 2002). For this reason, decreasing the amount of ROS and preserving NO bioavailability is a focal point for potential treatment options targeted towards age-related cerebrovascular dysfunction and cognitive impairment.

Evidence from animal studies support the idea that age-related arterial stiffening is highly related to the accumulation of AGEs (Sajithlal et al., 1998). AGEs are a group of compounds that are formed via the combination of glucose with proteins or lipids in the blood (Rowan et al., 2018). There are two primary ways that they accumulate; endogenous and exogenous pathways. Endogenous AGEs are formed as byproducts from glycolysis and oxidative stress conditions within the body. Exogenous AGEs come from outside sources -- that is, they are diet derived. Specifically, a higher sugar diet and the consumption of highly processed food will accelerate the accumulation of AGEs in the body (Sharifi-Zahabi et al., 2021).

As these AGEs circulate within the vasculature, they can have various harmful physiological effects. Namely, they can promote crosslinking of collagen, which leads to vessel stiffness. They also alter crucial programmed cell death pathways (Waghela et al., 2020). In addition, the interaction of AGE with its transmembrane receptor, RAGE, is a mechanism that

can lead to a buildup of oxidative stress within the body. Once RAGE and AGE are bound together, this can lead to a buildup of reactive oxygen species (ROS) as well as the induction of inflammatory signaling cascades (Sharifi-Zahabi et al., 2021). As discussed previously, ROS can decrease the bioavailability of NO and thus result in dysfunction of endothelial cells. Without healthy endothelium, blood flow to the brain cannot be effectively regulated. Overtime, this can lead to chronic hypoperfusion and potentially result in cognitive impairment (Wang et al., 2018).

With increasing evidence indicating that there is an association between large artery stiffness and cognitive impairment, breaking these AGEs are proposed to be a new method for alleviating cognitive decline by maintaining arterial elasticity and ultimately preserving cerebrovascular function (Kass et al., 2001). Alagebrium Chloride, or ALT-711, is a molecule that serves to break the crosslinks caused by AGEs. As a result, this drug diminishes collagen cross linking and therefore decrease arterial stiffness (Zieman et al., 2007). Further, employment of ALT-711 could inhibit the pathological AGE-RAGE interactions; this would decrease oxidative stress, restore NO bioavailability, and thus preserve endothelial function. At large, cognition and overall executive function could be improved after the administration of Alt-711.

The mechanisms of AGE formation and oxidative stress are well-understood phenomena. In addition, there is evidence supporting the association between age-related increases in large artery stiffness and cerebrovascular dysfunction. However, it is unknown whether preventing age-related large artery stiffness, via an AGE breaker such as Alt-711, will also preserve cerebrovascular function. This study aims to reveal this relationship and shed light on the mechanisms of cerebrovascular dysfunction and cognitive impairment associated with aging.

Methods

Animals and Tissues

Mice were obtained from the National Institute on Aging colony at Charles River. An equal number of male (n = 10) and female (n =10) C57BL/6 mice were randomly assigned to a treatment group by cage. 4 animals were euthanized during the intervention making the total n=16. All mice were on a normal chow diet with ad libitum food and water and were housed in an animal care facility on a 12/12-hour light-dark cycle at 24°C. Alt-711 (1 mg/kg/day) or vehicle control (distilled H_2O) were administered, via oral gavage, to mice once daily for three weeks, followed by 1 week without treatment. This continued for a total of 4 months of study. Mice were treated from 20 to 24 months of age. After the mice were euthanized, tissues from posterior cerebral arteries and carotid arteries were immediately used to conduct ex-vivo vascular function analysis. All animal procedures conformed 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.

Cerebrovascular function

Cerebrovascular function was measured via pressure myography by assessing endothelial-dependent dilation (EDD). Posterior cerebral arteries (PCAs) were excised and placed in myograph chambers (DMT Inc., Denmark) with physiological salt solution 145 mM NaCl, 4.7 mM KCl_2, 2.0 mM $CaCl_2$, 1.17 mM $MGSO_4$, 1.2 mM NaH_2PO_4, , 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. (Walker, 2015) Once cannulated, arteries were warmed to 37°C, pressurized to 50 mmHg, and allowed to

equilibrate for approximately 1 hour. All arteries were sub maximally pre-constricted with phenylephrine (1-6 μM to obtain ~20-40% pre-constriction). Lumen diameter was measured in response to increasing concentrations of the endothelium-dependent dilator, ACh (1x10-9 to 1x10-4 M) or endothelium-independent dilator, sodium nitroprusside (SNP: 1x10-10 to 1x10-4 M).

Passive stiffness

Passive arterial stiffness was measured ex-vivo in carotid arteries and PCAs. Arteries were isolated and cannulated between glass pipette tips on a myograph as previously described (Michiels, 2003). Prior to assessment, the vessels were incubated for 60-minutes in calcium-free solution to eliminate effects of myogenic tone. Lumen diameter and medial wall thickness were measured to increases in intraluminal pressure (Donato, Eskurza, Silver, et al. 2007). Measurements for each artery were recorded from 5 to 100 cmH_2O (3.7– 73.5 mmHg) in 5 cmH_2O increments. Stress was calculated as:

$$\sigma = PD/2\mathrm{WT}$$

where P is pressure in dyne cm^{-2}, D is lumen diameter and WT is wall thickness.

Strain was calculated as:

$$\varepsilon = (D - D_i)/D_i$$

where D_i is the initial starting diameter.

Data for each artery were fit to the curve:

$$\sigma = \sigma_i e^{\beta\varepsilon}$$

where σ_i is the initial starting stress (5 cmH_2O) and β is the slope of tangential elastic modulus versus stress. A higher β represents a stiffer artery (Lesniewski et al., 2011 & Walker et al., 2014 & Singh et al., 2001).

Artery Histology

Sections of carotid and middle cerebral arteries (MCAs) were frozen in optimal cutting temperature (OCT) and then– sliced into 8 μm sections using a cryostat (Nikon, Minato, Tokyo, Japan) and adhered to a charged slide (Michiels, 2003). Carotid and MCA slides were stained for collagen 1 (ab270993, 1:250 μL) using immunofluorescence. Elastin content was acquired using autofluorescence. Arteries were fluorescently tagged using Alexa Fluor 647 (1:1000 μL) goat anti-rabbit IgG (H+L). Imaging of artery slides was captured using Leica Microsystems microscope (Wetzlar, Germany). These methods were quantified using FIJI by ImageJ software.

Accelerating Rotarod Test

To assess motor coordination, mice were tested using a Rotarod apparatus (Ugo Basile., Italy) over a two-day period adapted from Xhako et al (2020). Before each testing day mice were habituated to the testing room for one hour prior to the beginning of testing. On the first day, mice were placed on an elevated rod rotating at 4 rpm to acclimatize them to testing conditions. Mice needed to stay on the rod for 90 seconds to acclimatize to the rotarod rest. If the mice fell off before 90 seconds elapsed, they were placed on the rod again. On the second day, the mice were placed on the rod that accelerated 4 rpm to 40 rpm over the course of 5 minutes. Mice ran on the rod until they fell off or spun around two consecutive times and the time of fall was

recorded by the apparatus. Mice received three trials spaced 10 minutes apart on the second day. Data was analyzed using GraphPad Prism.

Nest Building

Nest building protocol was utilized to assess cognition. Mice were left with a cotton nestlet in an individual cage overnight with food and water and assessed on their ability to construct a nest. They were assessed the next morning according to a five-point nestling scale with various parameters **(Table 1)**.

Score	Parameters
1	Neslet is >90% intact
2	Neslet is partially torn up
3	Neslet is <50% intact but <90% is within a quarter of cage floor area
4	Flat nest with >90% of neslet torn up, nest is flat with walls higher than mouse's body weight
5	>90% of nestlet torn up, nest is a crater with walls higher than mouse's body height

Table 1: Nest Building Score Parameters (Deacon 2012).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 9. Group differences were determined via a student's t test. A Bonferroni correction was used to compare maximum endothelium dependent dilation across groups and dose conditions. Significance was determined by a p value of less than 0.05. All data was assessed for normal distribution using Shapiro-Wilks tests. All data is presented as mean ± standard deviation. To determine correlations, a Pearson Correlation test was performed on all the data together, including both treatment and control groups.

Results

Ex vivo cerebral artery endothelial-dependent dilation and endothelial-independent dilation were not different between ALT-711 and control groups

In PCA's, maximal vasodilation to ACh was not different across groups (p =0.3896, **Figure 1**). The administration of L-NAME, an eNOS inhibitor, resulted in minimal dilation. This result was found in both the ALT-711 group (p = <0.0001, **Figure 1**) and control group (p = <0.0001, **Figure 1**). The maximal dilation to doses of ACh while L-NAME was present was similar between groups (p = 0.3346), **Figure 1**). To examine the effects of ALT-711 treatment on endothelial independent dilation, we examined the PCA's response to increasing doses of SNP, a chemical that supplies the smooth muscle with NO directly. There were no differences between groups (p = 0.1309 **Figure 2**).

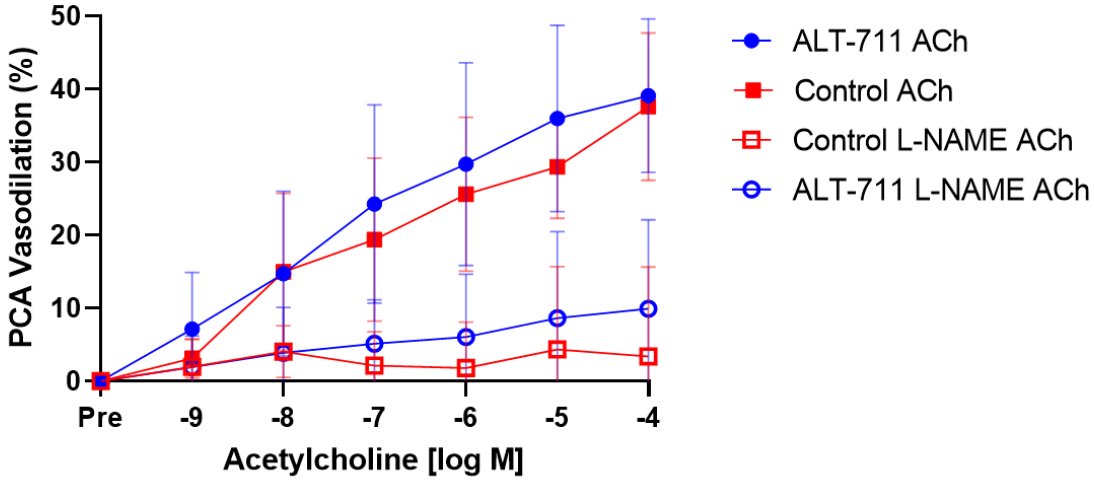

Figure 1: Vasodilation response to doses of ACh in the presence and absence of L-NAME, measured in PCAs in ALT-711 (n=8) and control (n=7) groups.

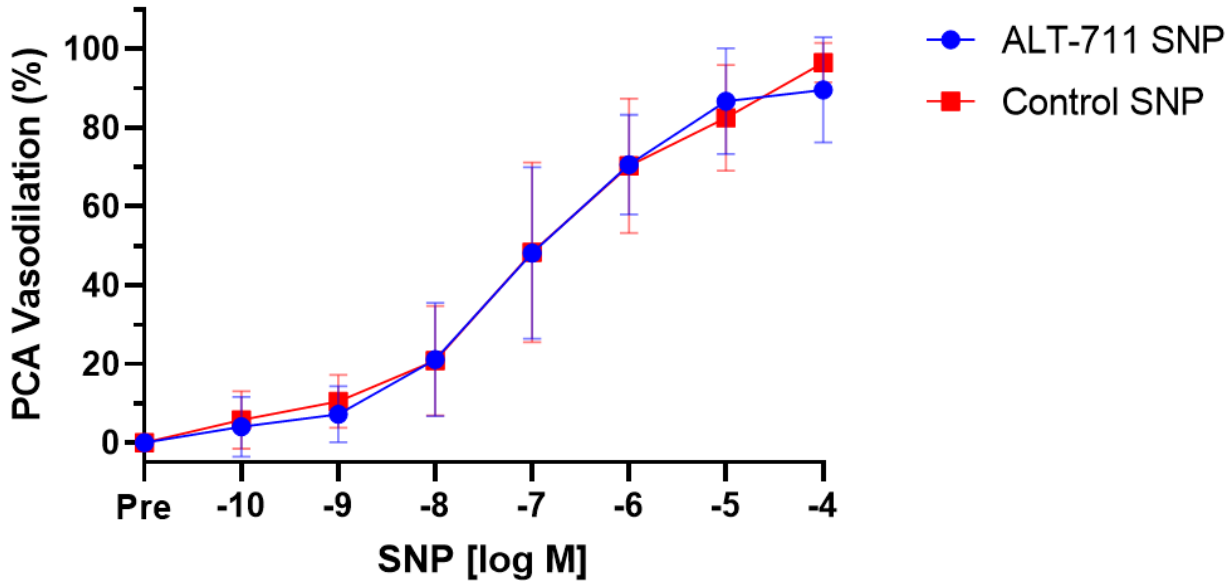

Figure 2: Endothelial independent dilation to doses of SNP, measured in PCA's in ALT-11 (n=8) and control (n=7) groups.

Passive stiffness showed that wall thickness in the PCA and carotid artery was not different between ALT-11 and control groups. Collagen-1 immunofluorescence revealed that the thickness of the media layer of the carotid artery and MCA was not different across groups, and that the sum of the media layer and the adventitia layer of the carotid artery was thicker in the control group compared to the ALT-711 group, but this was not different for the MCA's.

Wall thickness was assessed via *ex vivo* passive stiffness measures as well as a collagen-1 immunofluorescence. Passive stiffness measure revealed that wall thickness in carotid arteries was not different across groups ($p = 0.1961$, **Figure 3**) and wall thickness of PCA's was not different across groups ($p = 0.3558$, **Figure 3**). Additionally, collagen-1 immunofluorescence revealed that the thickness of the media layer of the carotid artery and MCA was not different across groups ($p = 0.1658$, $p = 0.0824$ **Figure 4**). However, collagen-1 immunofluorescence showed that the sum of the media layer and the adventitia layer of the carotid artery was thicker in the control group compared to the ALT-711 group, but the this was not different for in MCA's ($p = .0389$, $p = 0.3835$ **Figure 5**)

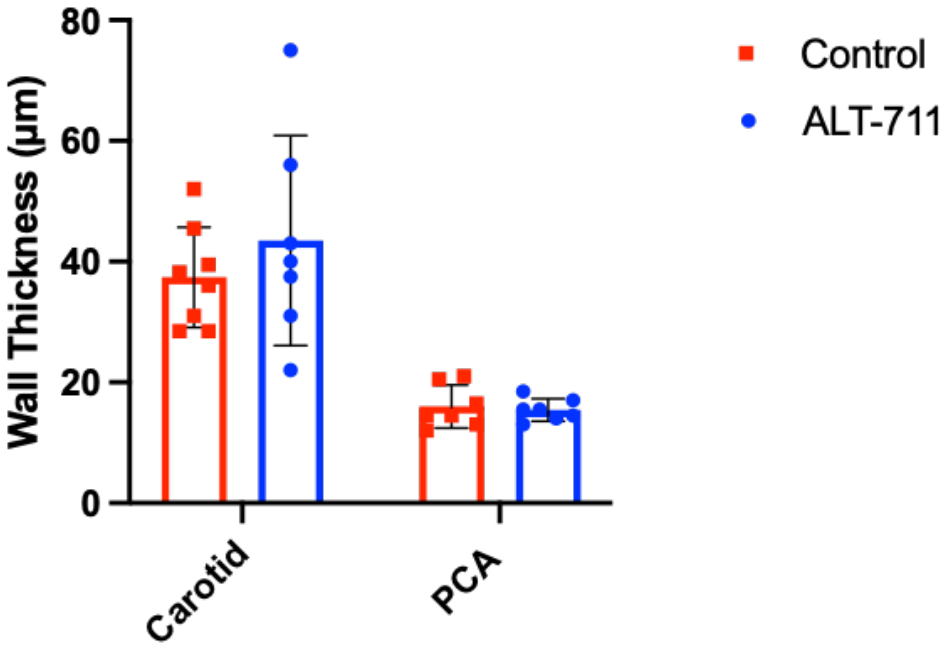

Figure 3: *Ex vivo* arterial wall thickness in carotid and PCA's in ALT-711 (n=8) and control (n=7) groups.

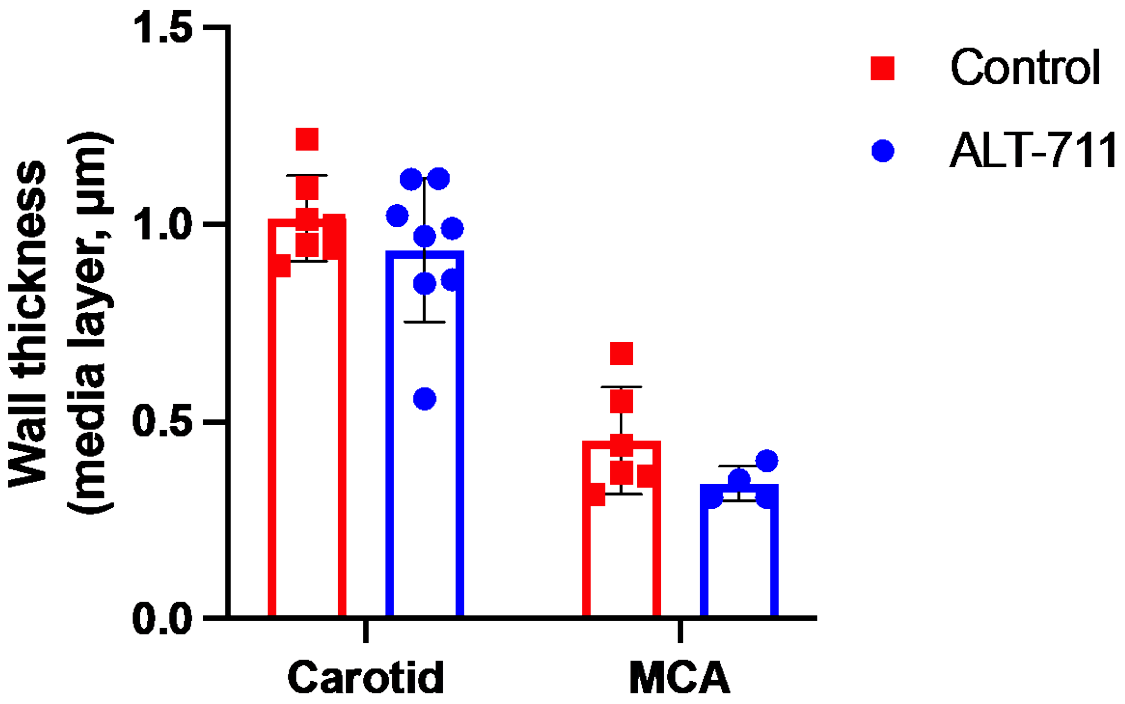

Figure 4: Thickness of the media layer of the carotid artery and MCA, via collagen-1 immunofluorescence, in ALT-711 (n=8) and control (n=7) groups.

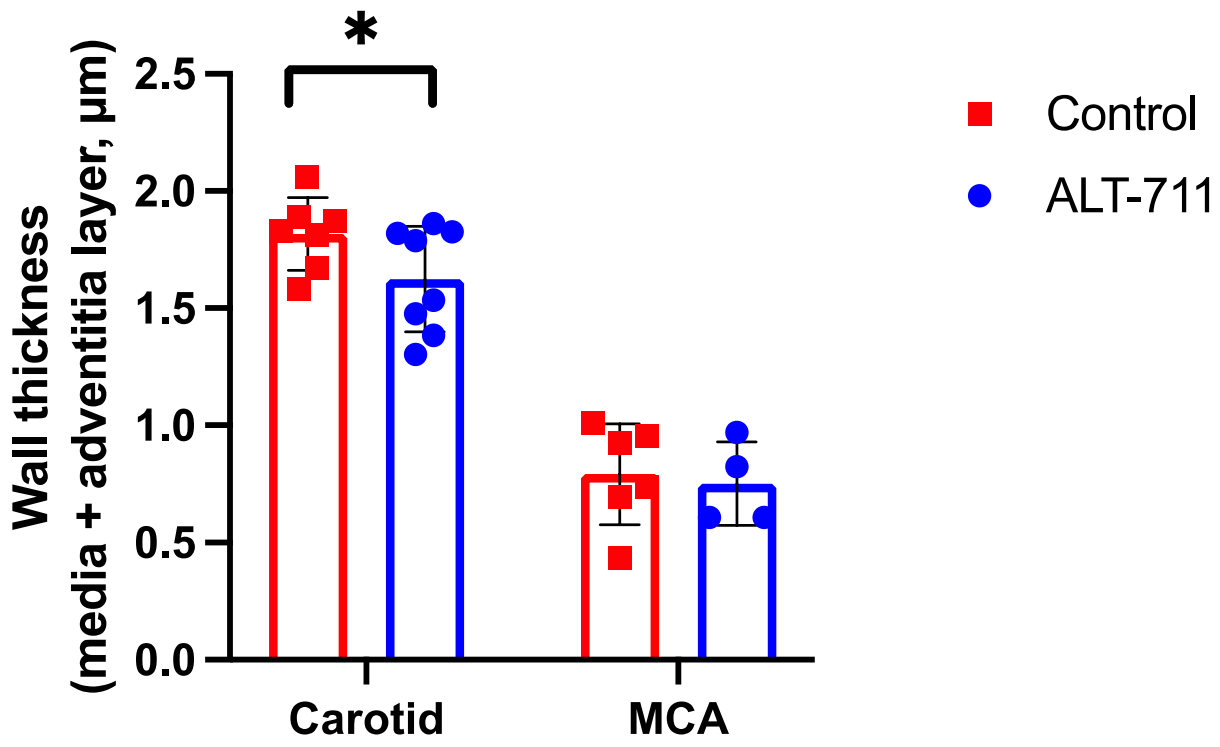

Figure 5: Thickness of the media and adventitia layer of the carotid artery and MCA, via collagen-1 immunofluorescence, in ALT-711 (n=8) and control (n=7) groups.

Elastin and collagen content in the arterial wall of the carotid artery and MCA was not different between groups

Elastin content was in the arterial wall was not different between groups in the carotid artery and MCA, respectively ($p = 0.0539$, $p = 0.1224$, **Figure 6**). Additionally, collagen content in the arterial wall was not different between groups in the carotid artery and MCA, respectively ($p = 0.3470$, $p = 0.3147$), **Figure 7**.

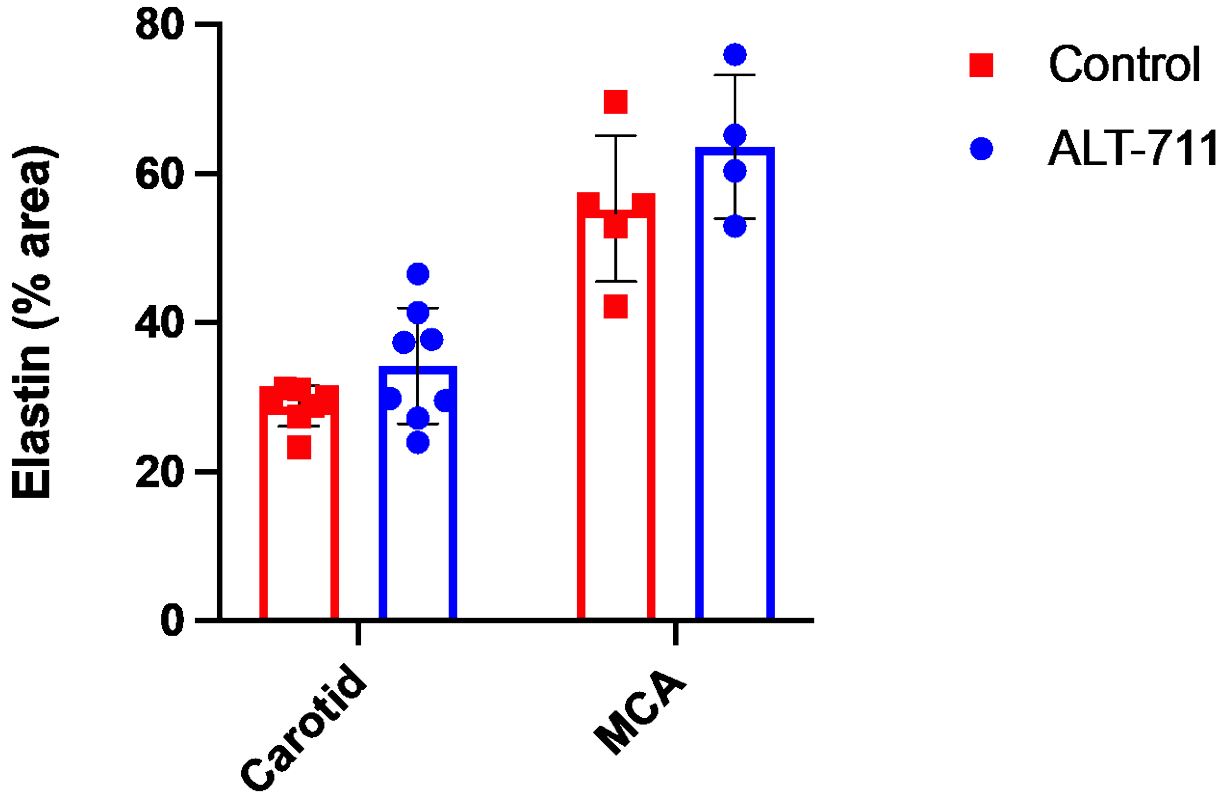

Figure 6: Percent area of the arterial wall made up of elastin in the carotid artery and MCA, via autofluorescence, in ALT-711 and control groups.

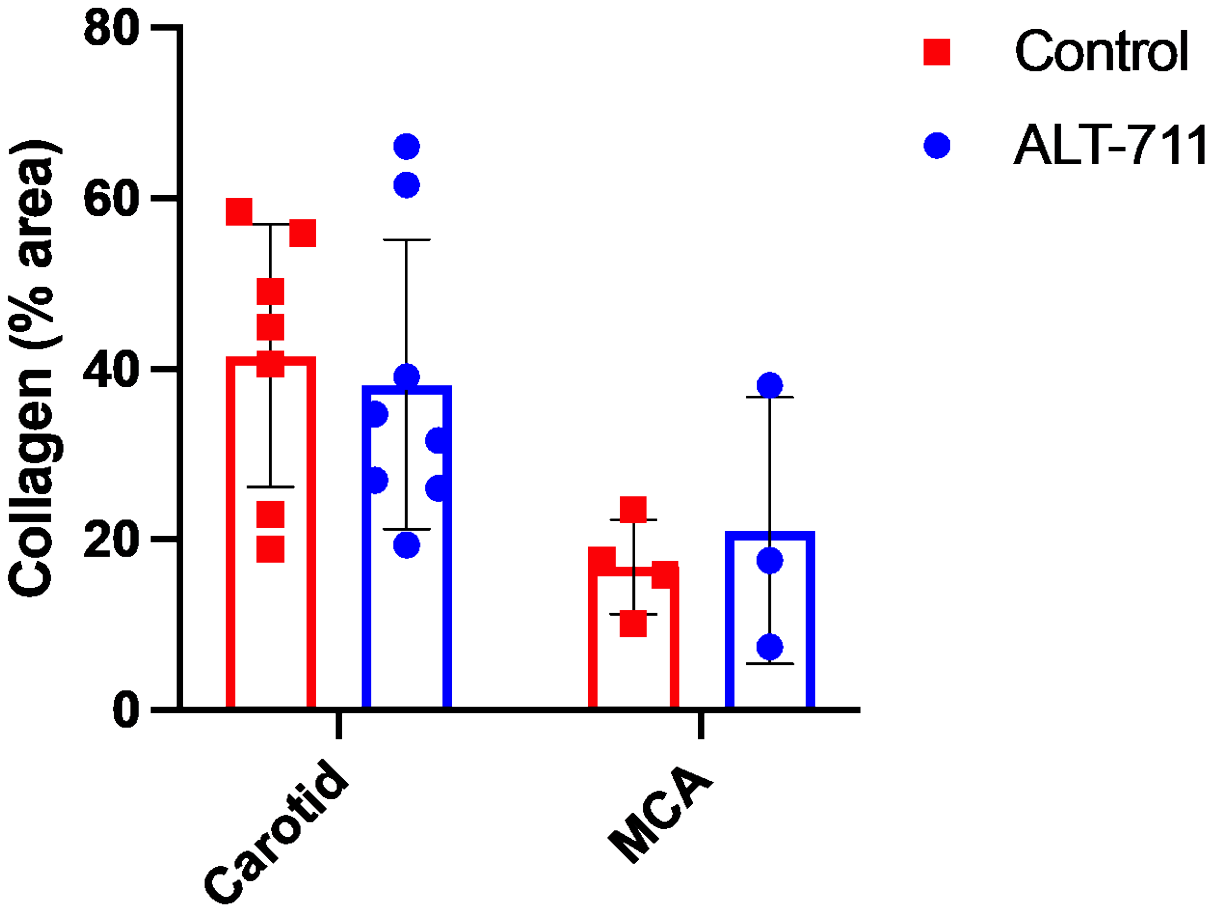

Figure 7: Percent area of the arterial wall made up of collagen in the carotid artery and MCA, via collagen-1 immunofluorescence, in ALT-711 and control groups.

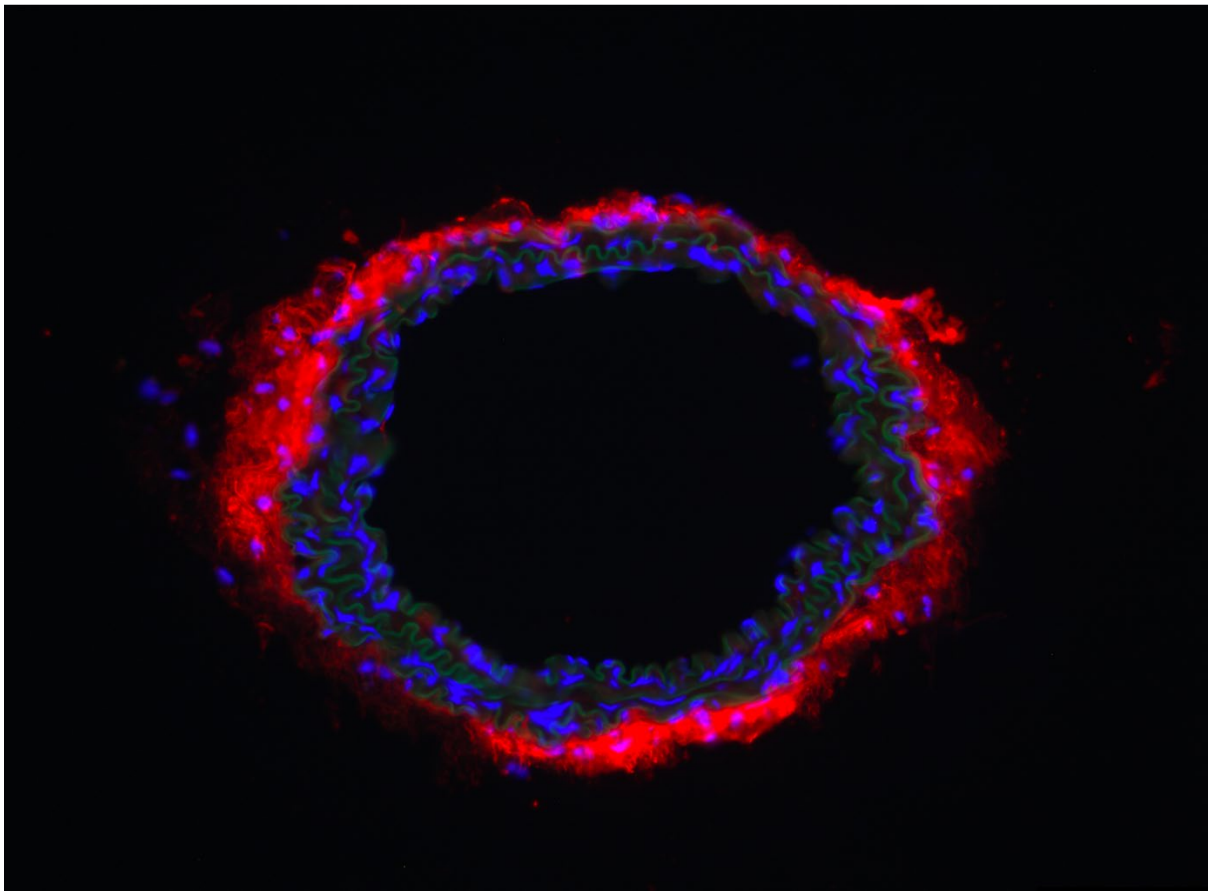

Figure 8: Cross section of carotid artery after collage-1 immunofluorescence in control group (n=7).

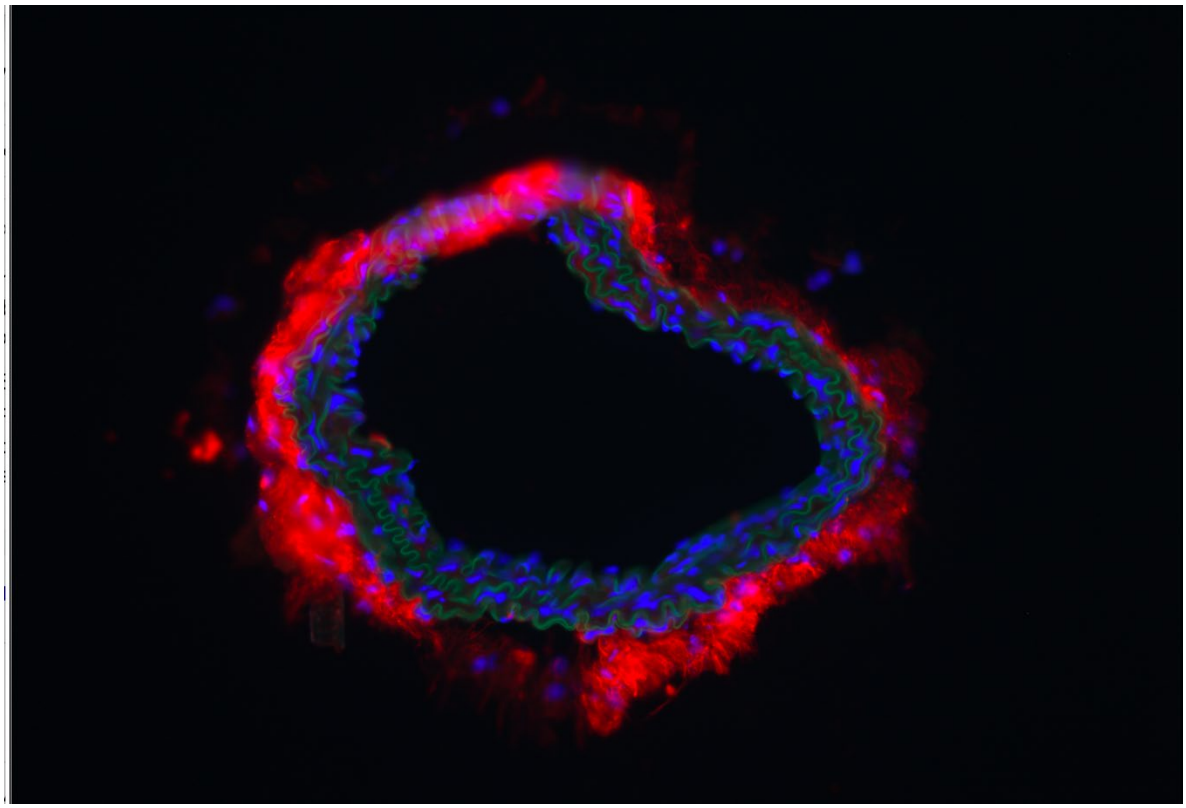

Figure 9: Cross section of carotid artery after collagen-1 immunofluorescence in ALT-711 group (n=8).

Arterial stiffness in PCA and carotid arteries was not different between ALT-711 and control groups

To determine if treatment with ALT-711 reduced arterial stiffness, β stiffness, elastic modulus, tangential stress and strain, and changes in internal diameter with increasing pressure were measured in carotid arteries and PCA's. Carotid artery β stiffness was not different between groups (p = .0642, **Figure 10**). Also, PCA β stiffness was not different between groups (p = 0.3828, **Figure 10)**. Additionally, the elastic modulus at lower pressures of carotid arteries was not different across groups (p = 0.1906, **Figure 11**). The elastic modulus of PCA's at lower pressures was not different across groups (p = 0.2293, **Figure 11**). The elastic modulus of carotid arteries at high pressures was not different across groups (p = 0.4168, **Figure 12**). The elastic modulus of PCA's at high pressures was not different across groups (p = 0.1949, **Figure 12**).

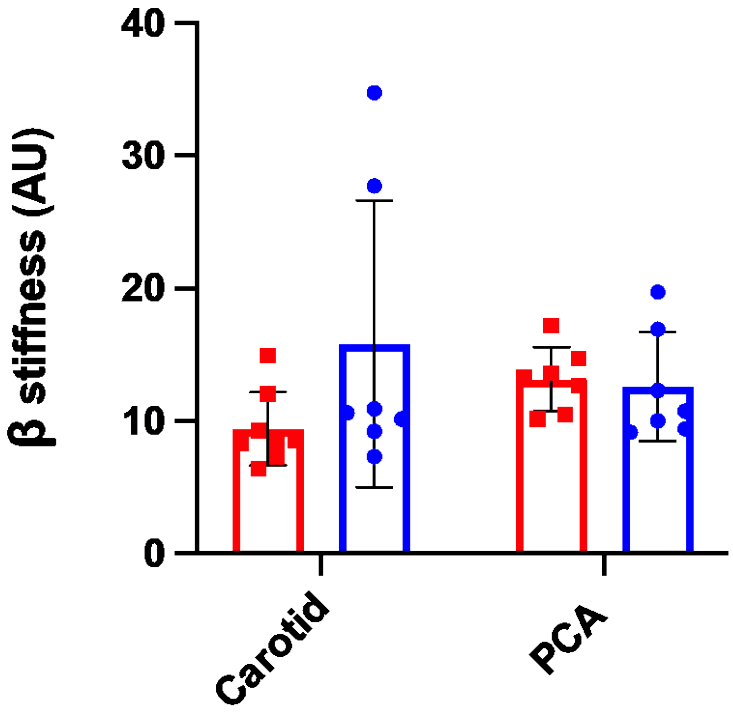

Figure 10: β stiffness in carotid and PCA's in ALT-711 (n=8) and control (n=7) groups.

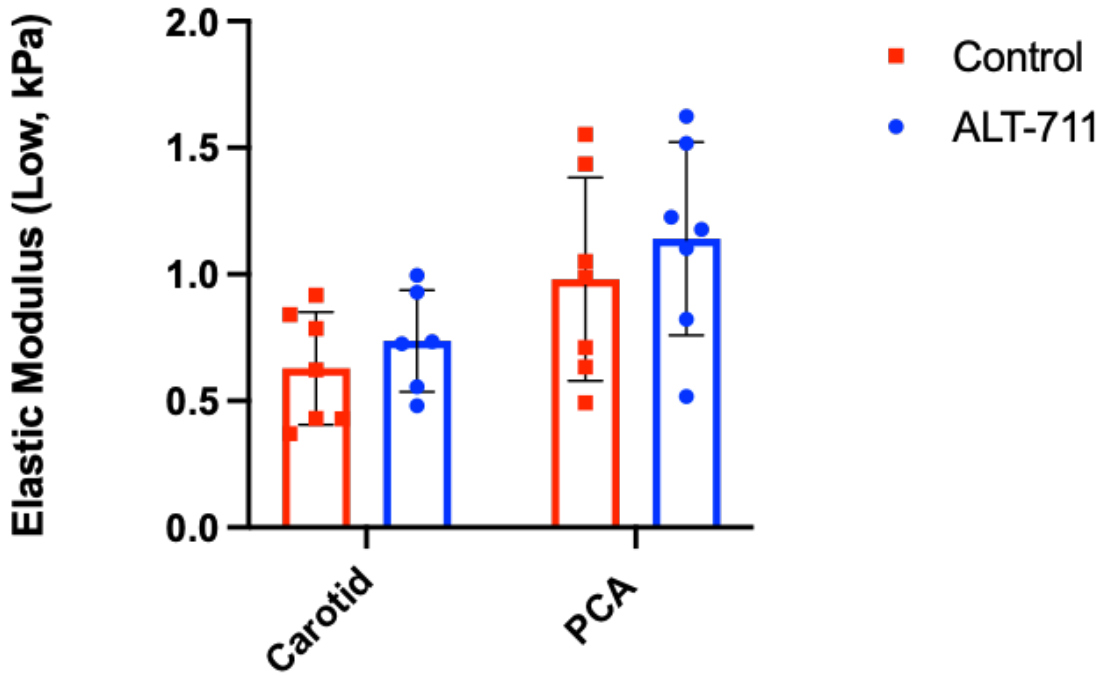

Figure 11: Elastic modulus at low pressures in carotid and PCA's in ALT-711 (n=8) and control (n=7) groups.

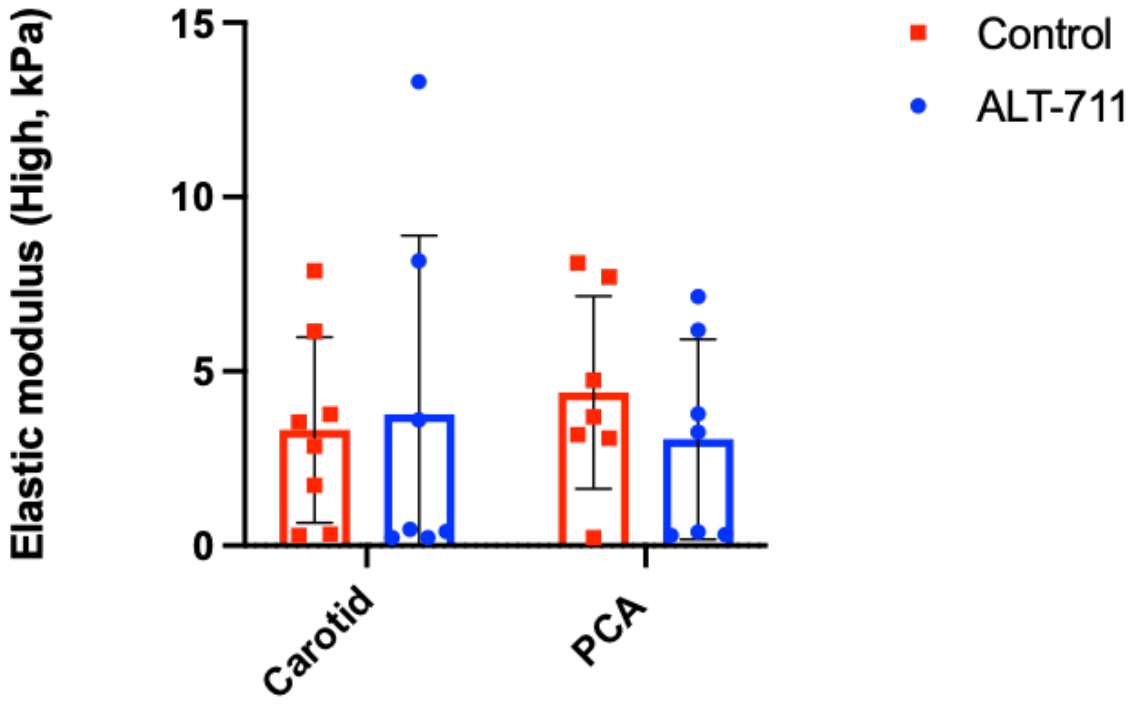

Figure 12: Elastic modulus at high pressures in carotid and PCA's in ALT-711 (n=8) and control (n=7) groups.

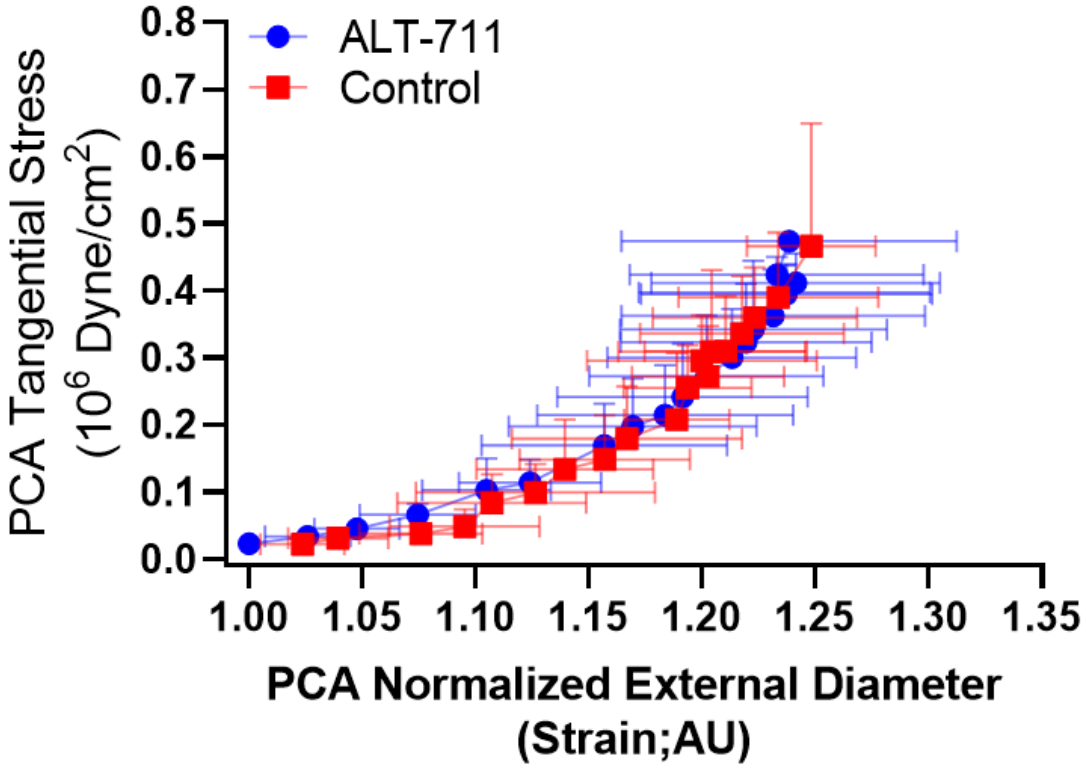

Figure 13: PCA *ex vivo* stress-strain curves in ALT-711 (n=8) and control (n=7) groups.

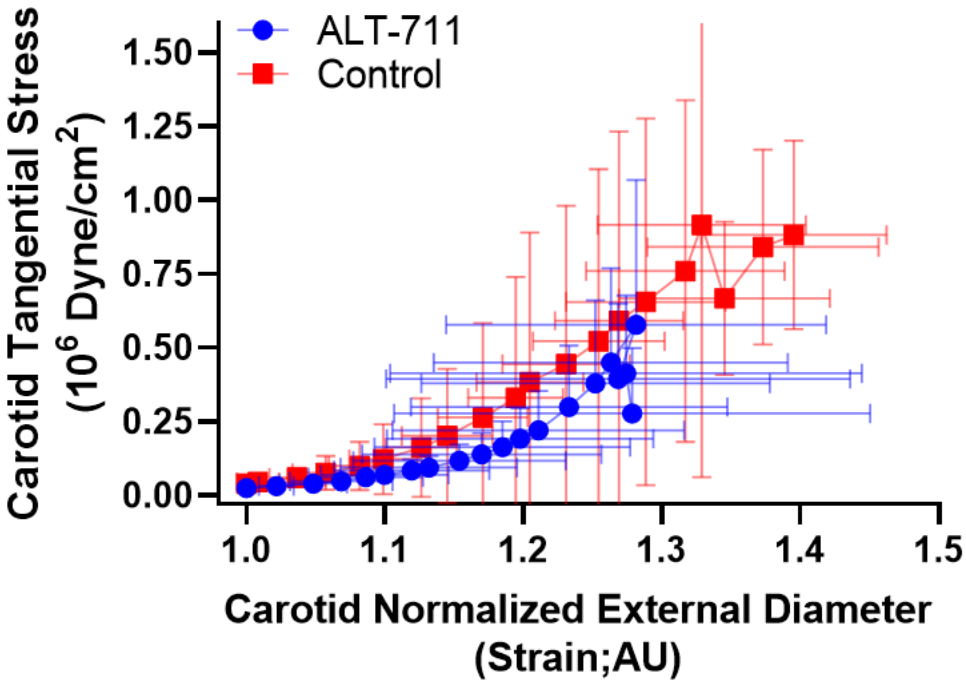

Figure 14: Carotid artery *ex vivo* stress-strain curves in ALT-711 (n=8) and control (n=7) groups.

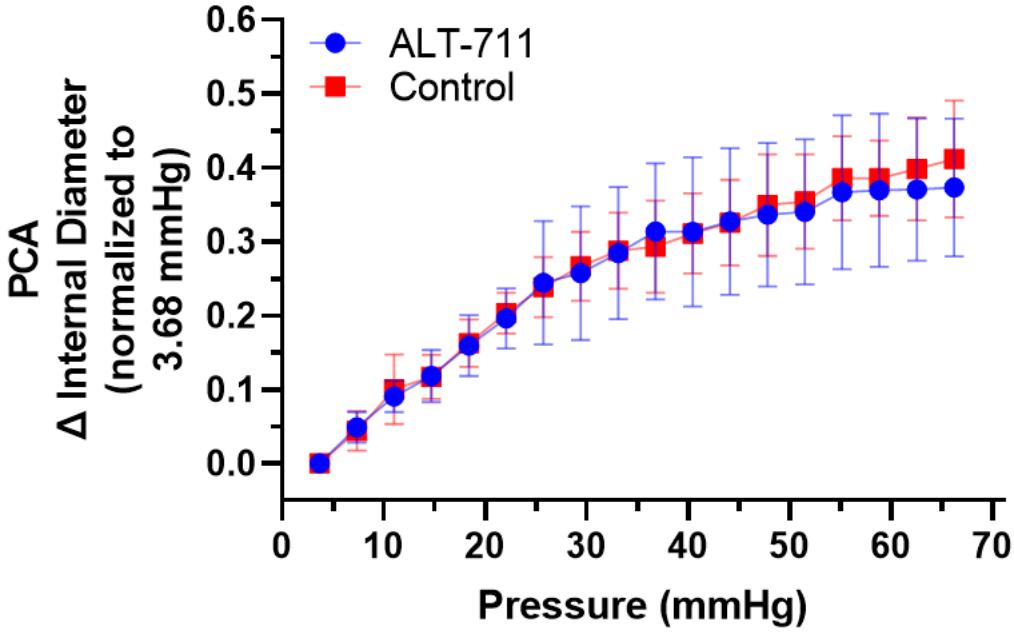

Figure 15: Change in internal diameter of PCA to increasing pressure in ALT-711 (n=8) and control (n=7) groups.

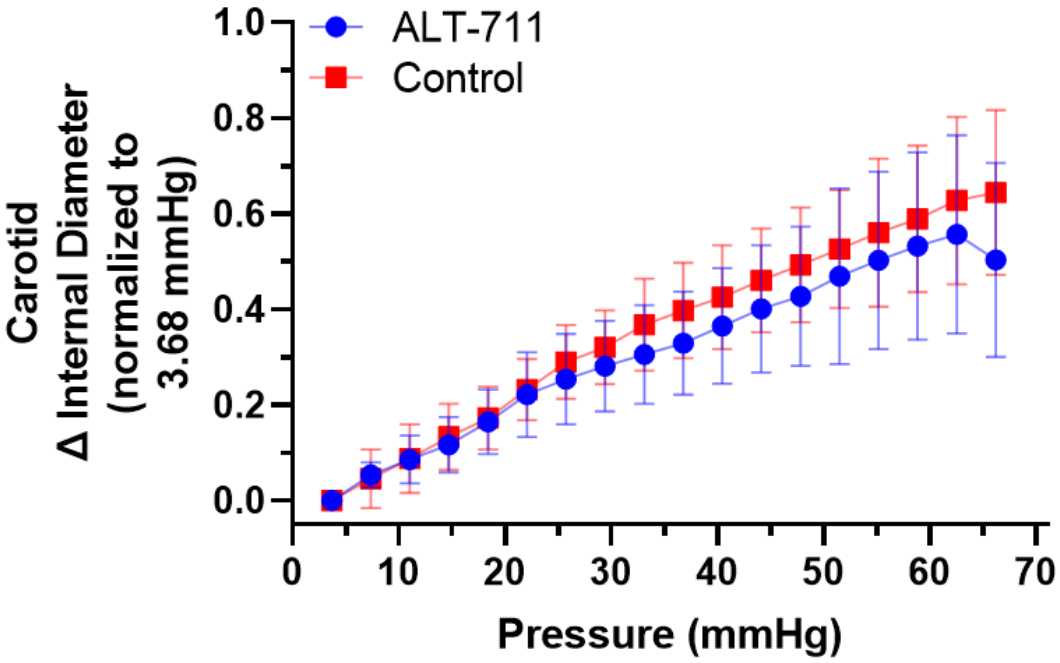

Figure 16: Changes in internal diameter of carotid artery to increasing pressure in ALT-711 (n=8) and control (n=7) groups.

Instinctual behavior was not different between ALT-711 and control groups

To assess cognitive behavior, nest building scores were evaluated. There were no significant differences between nest building scores across groups ($p = 0.5$, **Figure 17**)

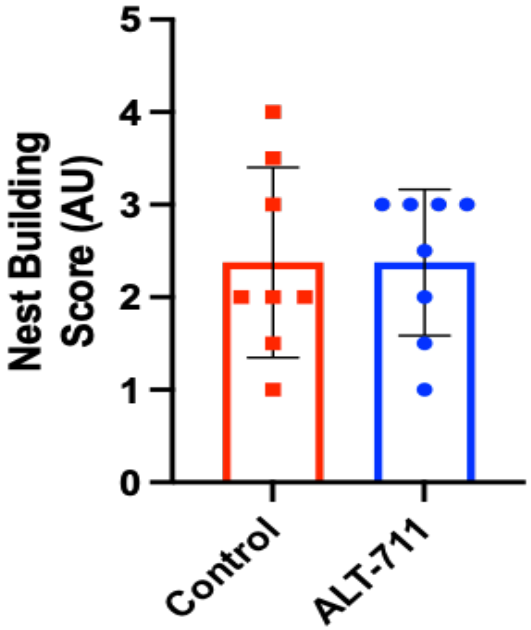

Figure 17: Nest building scores for ALT-711(n=8) and control groups (n=7).

Motor coordination

To assess motor coordination, time spent on the rod during the accelerator rotarod test was measured and analyzed. There were no significant differences between time spent on the rod across groups (p = 0.3151, **Figure 18**).

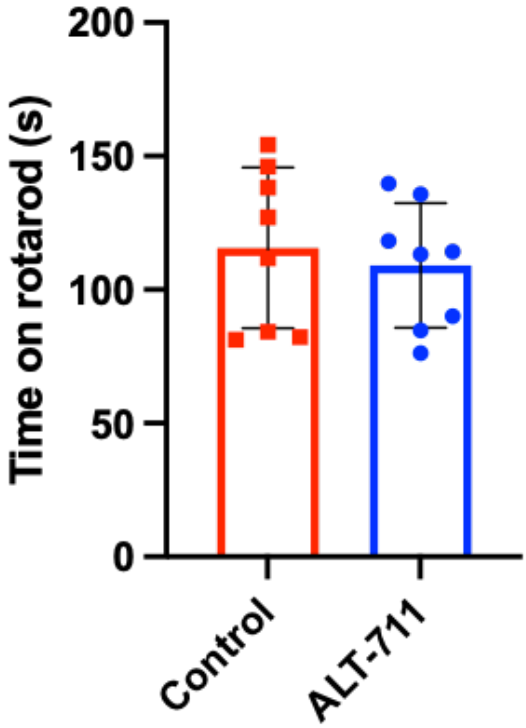

Figure 18: Average time spent on rotarod for ALT-711 (n=8) and control groups (n=7).

Correlations

To assess for correlations of interest, the treatment and control groups were pulled together, and a Pearson Correlation test was performed. This test revealed several notable correlations. The average maximum dilation to ACh is positively correlated with average rotarod time ($p = 0.049$, $r = 0.44$, **Figure 19A**). PCA low elastic modulus is positively correlated with PCA β stiffness ($p = 0.019$, $r = 0.56$), and negatively correlated with average rotarod time ($p = 0.042$, $r = -0.48$, **Figure 19B**), and nest building scores ($p = 0.035$, $r = -0.50$, **Figure 19C**).

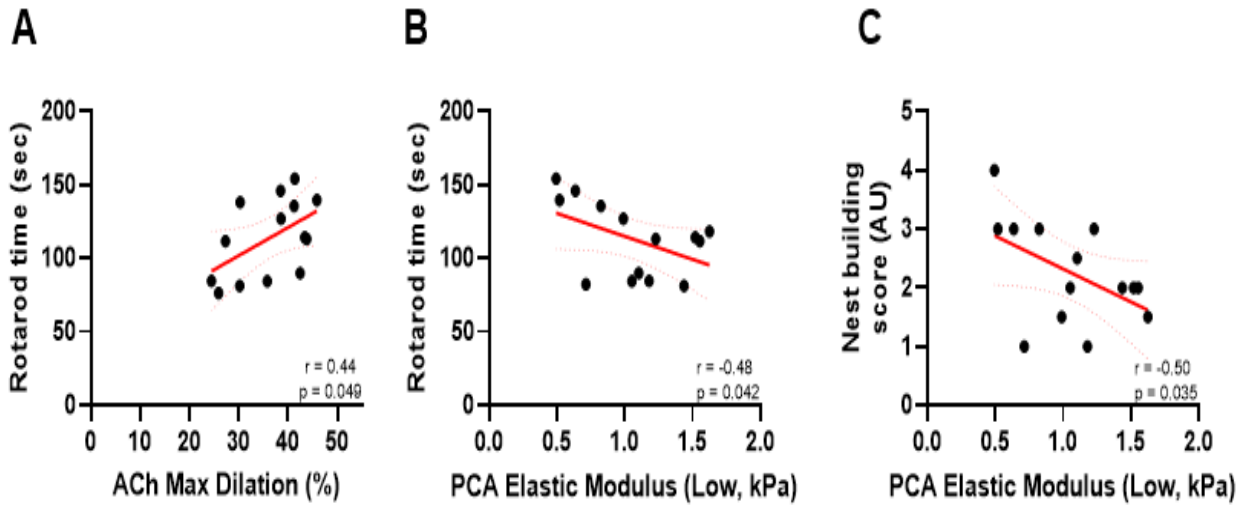

Figure 19: (A) The average maximum dilation to ACh is positively correlated with average rotarod time and (B) PCA low elastic modulus is negatively correlated with average rotarod time and nest building scores and (C) PCA low elastic modulus is negatively correlated with nest building scores *p<0.05

Discussion

One of the strongest predictors of cognitive impairment as humans age is the stiffening of their large arteries, specifically the aorta and carotid artery (Scuteri, 2007). Several animal studies have shown that increasing the stiffness of these arteries results in cerebrovascular dysfunction and impaired cognition (Muhire et al., 2019). However, the effect of *reducing* large artery stiffness, via ALT-711, on cerebrovascular function and cognitive behavior has not been studied. The stiffening of large arteries is largely associated with the accumulation of AGEs (Mcnulty et al., 2007). Therefore, the effect of large artery stiffness reduction can be studied through the employment of a drug that breaks AGEs, such as ALT-711. The investigation of this phenomenon could help elucidate a mechanism of age-related cognitive impairment and cerebrovascular dysfunction.

In this study, we examined the effect of large artery stiffness prevention, via ALT-711, on attenuating cerebrovascular dysfunction and cognitive impairment in aged mice. Contrary to our hypothesis, our data suggests that employing ALT-711 did not result in a reduction of large artery stiffness. Subsequently, ALT-711 did not improve cerebrovascular function, cognitive behavior, or motor coordination. However, we found that PCA low elastic modulus is correlated with average rotarod time and nest building scores. In addition, our data revealed that the maximum dilation to ACh in a PCA is correlated with average rotarod time.

ALT-711 did not improve cerebrovascular function

Contrary to what we hypothesized, ALT-711 did not improve cerebrovascular function. We expected the group that received ALT-711 to have a greater dilation response than the control group. We hypothesized this because previous studies have demonstrated that greater large artery stiffness reduces cerebrovascular function (Muhire et al., 2019). As a result, a

reduction in large artery stiffness, via breaking AGEs, could be a mechanism to preserve cerebrovascular function.

Our results may contradict our hypothesis because there was no evidence in our study that ALT-711 reduced large artery stiffness in the first place. One could reason that the arteries did not become less stiff, so they were not able to effectively dampen the pulsatile flow entering the brain. As a result, endothelial cell function was not preserved, and so the dilation response to ACh was the same in both groups.

A different phenomenon that our data supports is the concept of NO-mediated dilation. Administration of L-NAME, an eNOS-inhibitor, inhibits the ability of eNOS to produce NO after becoming activated by ACh. Accordingly, our data indicates that L-NAME inhibits the vessels ability to dilate. Treating the vessels with L-NAME allows us to confirm the primary mechanism by which cerebral vessels dilate; that is, NO travels to the smooth muscle and the smooth muscle facilitates expansion of the vessel walls. By confirming this mechanism, we know that making conclusions about a vessel's response to ACh is decidedly indicative of the vessel's ability to function and dilate properly.

ALT-711 did not influence large artery stiffness

Despite our expectations, treatment with ALT-711 did not have an impact on large artery stiffness. This was concluded by the interpretation of two values: β stiffness and elastic modulus. β stiffness quantifies the stiffness of an artery. Additionally, elastic modulus and elastic modulus at low and high pressures help specify the source of the stiffness. An increased elastic modulus at low pressures value indicates that elastin is the source of the stiffness - either from a decreased abundance or increased fragmentation. Conversely, an increased elastic modulus at high pressures indicates that collagen is the source of the stiffness, likely from an increased

crosslinking of collagen or increased collagen content. We predicted a lower β stiffness and a decreased elastic modulus in the ALT-711 group. This was expected because previous literature has demonstrated that AGEs promote collagen crosslinking and that ALT-711 reduces arterial stiffness (Bakris, 2004). However, collagen-1 immunofluorescence revealed that the media and adventitia layer of the carotid artery was thicker in the control group compared to ALT-711. This indicates that ALT-711 treatment impacted the thickness of the adventitia layer in some way, but this did not impact the overall arterial stiffness. Also, ALT-711 treatment did not impact the elastin content in the arterial wall. This was what we expected because AGEs promote crosslinking of collagen, not elastin. Nevertheless, ALT-711 did not impact the collagen content in the arterial wall either. This was contrary to our hypothesis. We expected that ALT-711 would decrease the collagen content because previous studies have shown that ALT-711 decreases collagen cross-linking (Candido et al., 2003).

ALT-711 did not alter instinctual behavior or motor coordination

The results of the nest building and accelerator rotarod test were inconsistent with our hypothesis; ALT-711 did not improve their ability to perform on these cognitive tests. Nest building measures the mice's instinctual behavior to build nests. The results of this test can be used to make conclusions about cognitive impairment and brain function. Additionally, the accelerator rotarod can reveal information about muscle coordination by timing how long the mice can stay on the rod. We hypothesized that treatment with ALT-11 would result in higher nest building scores and longer times on the rod. This was expected because previous studies have demonstrated that aged mice have impaired muscle strength and control (Hamieh et al., 2021). Similarly, prior animal studies have shown that ALT-711 treatment results in improved working memory (Zakaria et al., 2015). One possible explanation for why our data contradicts

previous literature could be that our sample size was not large enough. It is possible that the experiment would have yielded more significant differences between groups if there was a larger pool of data to account for individual variation.

Correlations

After the control and treatment data were combined, a Pearson Correlation test revealed four correlations between the variables; PCA elastic modulus low is correlated with PCA β stiffness, average rotarod time, and nest building scores. Additionally, average maximum dilation to ACh is correlated with average rotarod time. The correlations between these variables supports evidence found from previous studies.

A higher PCA elastic modulus at low pressures indicates that the source of the arterial stiffness is from elastin, either from increased fragmentation or decreased abundance. Because elastin is a protein that contributes to the elasticity of arteries, an increased low elastic modulus could allow the contributions of collagen, a structural protein, to effectively govern the properties of the vasculature. In turn, this could lead to an overall increase in arterial stiffness, or β stiffness. There is evidence from previous research that shows that increased elastin degradation leads to stiffer arteries (Wagenseil & Mecham, 2013). Additionally, a decrease in elasticity could impair motor coordination on a test such as the accelerator rotarod. This is a relationship that other studies have identified too. Experiments performed with elastin haploinsufficient Eln+/- mice indicated that old haploinsufficient Eln+/- mice performed significantly worse on rotarod tests in comparisons to young Eln+/+ mice as well as old Eln+/+. Notably, a correlation between PCA elastic modulus low and nest building scores is also a result that is supported by other studies. The link between increased arterial stiffness and decreased

cognitive function is a phenomenon that is well supported throughout research (Cooper & Mitchell, 2016).

These correlations support our original hypothesis on the mechanism of age-related cognitive impairment. That is, if there is increased fragmentation or decreased abundance of elastin within a vessel, it will lose its elasticity and its ability to effectively stretch; this will lead to increased arterial stiffness. As a result of this arterial stiffness, cerebrovascular function may be impaired, resulting in decreased performance in cognitive and motor coordination tests such as nest building and accelerator rotarod.

The correlation between average maximum dilation to ACh and average rotarod time can also be explained by this proposed mechanism. A vessels dilation response to ACh is indicative of the health and function of cerebral endothelial cells. In damaged endothelial cells, their ability to dilate in response to ACh will be impaired. This means that blood flow to the brain cannot be effectively regulated. The link between cerebrovascular function and cerebral blood flow with motor coordination is well documented throughout previous literature. Studies have shown that cerebral hypoperfusion underlies motor impairment in various human diseases (West, 2021).

Limitations and Future Research

It is important to consider some notable limitations of this study. First, there are many complex physiological phenomena that occur during the aging process. Because we studied old mice (~20-24 months), it is possible that these other mechanisms could confound the effects of the ALT-711 treatment and complicate the results of the experiment. Also, because we studied old mice, perhaps it was already too late to prevent large artery stiffness. Another important consideration is the fact that we did not test if the collagen crosslinks were actually broken. As a result, it is still possible that ALT-711 did effectively break the crosslinks but this did not impact the stiffness. Additionally, we only tested cerebral artery endothelial function in response to ACh. There are other mechanisms that elicit vasodilation that were not included in this study. Further, it is helpful to acknowledge that the standard chow diet that the mice were fed was extremely healthy. Therefore, perhaps this diet was so healthy that the drug didn't have much of an effect. Also, the C57BL/6 mice model that we used has its own limitations. Because these mice are immune to many age-related cerebrovascular pathologies such as Alzheimer's disease, it is important that the impact of ALT-711 is studied in animal models that are more representative of the complicated aging process in humans. Finally, the ALT-711 used in this study was from a different supplier from the one used in clinical trials and previous experiments. This may be a reason why we did not see the results that we expected.

Regarding future research, conducting studies on lowering the number of circulating AGEs could be another avenue for reducing large artery stiffness and examining its effects on brain function. Also, investigating the impact of preventing the formation of AGEs in the first place, rather than breaking the cross links after they are formed, is another possible study that could help elucidate the mechanism of age-related cerebrovascular dysfunction and cognitive

impairment. Further, looking at the impact of ALT-711 on neuroinflammation and circulating levels of ROS could help shed light on the brain aging process.

Conclusion

The results of this study revealed that large artery stiffness prevention, via ALT-711, did not attenuate age-related cerebrovascular dysfunction and cognitive impairment in aged mice. However, the data showed that there were correlations between arterial stiffness, motor coordination and cognitive behavior. More studies targeted towards breaking AGEs are needed to better understand the relationship between large artery stiffness and age-related decreases in brain function.

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