THE EFFECT OF BLOOD POOLING IN THE LOWER LIMBS DURING PROLONGED SITTING ON CEREBRAL ARTERIAL STIFFENING: TWO RANDOMIZED CROSS-OVER TRIALS

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A thesis proposal submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Masters of Arts in the Department of Exercise and Sports Science (Exercise Physiology).

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ABSTRACT

Alexander Pomeroy: The Effect of Blood Pooling in the Lower Limbs During Prolonged Sitting on Cerebral Arterial Stiffening: Two Randomized Cross-Over Trials
(Under the direction of Lee Stoner)

Prolonged sitting (PS) can induce arterial stiffening, which is a precursor of atherosclerosis of the cerebral arteries. Heart-Middle Cerebral Artery pulse wave velocity (hmPWV) was assessed in eleven participants were examined over two randomized cross-over trials (SIT: n = 5, 23.6 [5.3] y, 40% F, 23.1 [3.2] kg/m2; LAY: n = 6, 26.5 [7.6] y, 66.7% F, 22.5 [2.3]). Participants underwent two conditions on two separate visits in each trial: Cuff, where bilateral occlusive cuffs were applied to the legs and Non-Cuff, a control condition. In the Cuff and Non-Cuff conditions in each trial, hmPWV decreased over time. In the SIT trial, the random effects model generated a large time effect (β = -97.17, 95% CI: -125.1 to -69.28, ES = 1.55). Regardless of posture, venous pooling was not associated with hmPWV.

KEY WORDS
Pulse wave velocity, executive function, sedentary behavior, transcranial doppler
ACKNOWLEDGEMENTS

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>CAR</td>
<td>Cerebral Autoregulation</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral Blood Flow</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>fNIRS</td>
<td>Functional Near-Infrared Spectroscopy</td>
</tr>
<tr>
<td>HHb</td>
<td>Deoxygenated hemoglobin</td>
</tr>
<tr>
<td>hmPWV</td>
<td>Heart-MCA Pulse Wave Velocity</td>
</tr>
<tr>
<td>MCA</td>
<td>Middle Cerebral Artery</td>
</tr>
<tr>
<td>NIRS</td>
<td>Near-Infrared Spectroscopy</td>
</tr>
<tr>
<td>NIBP</td>
<td>Non-Invasive Blood Pressure</td>
</tr>
<tr>
<td>NVC</td>
<td>Neurovascular Coupling</td>
</tr>
<tr>
<td>O2Hb</td>
<td>Oxygenated hemoglobin</td>
</tr>
<tr>
<td>PWV</td>
<td>Pulse Wave Velocity</td>
</tr>
<tr>
<td>tHb</td>
<td>Total hemoglobin</td>
</tr>
<tr>
<td>TMT</td>
<td>Trail-Making Test</td>
</tr>
<tr>
<td>TSI</td>
<td>Tissue Saturation Index</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular Dementia</td>
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<tr>
<td>VFT</td>
<td>Verbal Fluency Test</td>
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### LIST OF TERMS

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Arterial stiffness</td>
<td>The lack of elasticity of an artery. The stiffness of an artery is dependent on its structural composition.</td>
</tr>
<tr>
<td>Cerebral Autoregulation</td>
<td>The system by which the cerebral vasculature maintains cerebral perfusion pressure despite a varying central blood pressure. The mechanisms for cerebral autoregulation function at the level of large arteries.</td>
</tr>
<tr>
<td>Neurovascular Coupling</td>
<td>The system by which the cerebral vasculature directs blood flow to metabolically active regions of the brain. The mechanisms for neurovascular coupling function at the level of the small cerebral arteries and arterioles.</td>
</tr>
<tr>
<td>Cerebral Blood Flow</td>
<td>The total volume of blood that flows through the cerebral vasculature.</td>
</tr>
<tr>
<td>Pulse Wave Velocity</td>
<td>The arterial path length travelled by the systolic pressure wave, divided by the time it takes to travel. This is a measure of arterial stiffness.</td>
</tr>
<tr>
<td>Prolonged Sitting</td>
<td>A single bout of sitting lasting at least 30 minutes.</td>
</tr>
<tr>
<td>Venous Blood Pooling</td>
<td>Blood pooling in the veins of the calves, such as the peroneal and tibial veins.</td>
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Vascular Dementia (VaD) affects an estimated 4% of U.S. seniors (65+ years old),¹ and increases to 15% for those 80+ years old.² One of the major risk factors for development of VaD is atherosclerosis, specifically in the cerebral arteries.³–⁵ While several factors can contribute to atherosclerotic development, hemodynamic causes, including acute arterial stiffening, have been suggested to have the greatest contribution.⁶ Cerebral arterial stiffening, including that due to atherosclerosis, also increases with age.⁷,⁸ During a bout of prolonged sitting (uninterrupted sitting greater than 1 hour in duration), central and peripheral arterial stiffening is well-documented.⁹,¹⁰ It has been hypothesized that venous pooling in the lower limbs (VP), a phenomenon known to occur during prolonged sitting, contributes to the observed disruptions to central hemodynamics.¹¹ However, the physiological relationships potentially linking cerebral hemodynamic changes and venous blood pooling has not been thoroughly investigated. If these relationships are better understood through research, treatments could be developed that target this relationship, potentially reducing an individual’s rate of cerebral atherosclerotic development.

This study aimed to elucidate the relationship between VP during an acute bout of prolonged sitting and changes in cerebral hemodynamics, and determine how much VP
contributes to cerebral hemodynamic changes during sitting. In the long-term, establishment of a potential mechanism would allow researchers to study the mechanism in vulnerable populations, such as those at risk for cerebrovascular dysfunction, and come up with sitting interruption strategies. This study is novel, as there is limited evidence into the role of venous pooling in affecting cerebral arterial function. While this study did not seek to establish sitting interruption strategies for cerebral hemodynamic disruptions, a mechanism could inform future studies of what physiological variables need to be addressed in the development of interruption strategies.

The primary hypothesis for this study was that VP causes arterial stiffening in the cerebral arteries. To test the hypothesis, the study assessed arterial stiffness during a bout of prolonged sitting, and during a bout of prolonged sitting with a sub-diastolic tourniquet to augment VP. Changes in arterial stiffness and VP were compared between conditions and over time within-subjects.

**Aim 1: Determine the effect of venous pooling manipulation on the cerebral arterial stiffening response.**

Six healthy, young adults were recruited for study in the first randomized crossover trial. The participants completed the first trial (LAY), with a control session of two hours uninterrupted supination (2 hours, CONTROL), and an experimental condition with increased venous pooling via dual, bilateral tourniquets inflated that fully occluded the calves (CUFF).

The primary outcome was cerebral arterial stiffness (AS) and the independent variable was venous pooling (VP). Mechanistic outcomes can provide context to the relationship.
Research hypotheses:

1. In the condition with bilateral leg tourniquets, VP would increase beyond that measured in the control condition, and cerebral AS would increase beyond what would be measured in the control condition.

Aim 2: Determine the effect of venous pooling manipulation during a bout of prolonged sitting on the cerebral arterial stiffening response.

Six healthy, young adults were recruited for study in the second randomized crossover trial. The participants would complete the second trial (SIT), consisting of a control session of prolonged sitting (2 hours, CONTROL), and prolonged sitting with dual, bilateral tourniquets inflated that fully occlude the calves (CUFF).

The primary outcome was cerebral arterial stiffness (AS) and the independent variable was venous pooling (VP). Mechanistic outcomes provided context to the relationship.

Research hypotheses:

1. During a bout of prolonged sitting, VP would increase, and cerebral AS would increase.

2. In the condition with bilateral leg tourniquets, VP would increase beyond that measured in the respective control condition, and cerebral AS would increase beyond what would be measured in the control condition. Both VP and cerebral AS in the prolonged sitting trial CUFF condition would increase beyond the VP and cerebral AS in the supinated trial CUFF condition.
### Assumptions

- Participants accurately follow pre-assessment guidelines and give truthful information to questionnaires.
- Placement of devices, such as Transcranial Doppler (TCD) and fNIRS, was similar between the two visits.

### Delimitations

- Experimental visits occurred at the same time of day to mitigate diurnal effects.
- Participants were fasted for 8 hours to reduce the influence of glycemic hormones.

### Limitations

- TCD probe placement may have varied slightly between visits.
- To reduce participant burden, the length of the sitting bout was set at 2 hours of sitting. It is possible that this duration may not elicit as large of a change in the function of the cardiovascular system as would occur with a longer sitting bout.
CHAPTER II: REVIEW OF LITERATURE

INTRODUCTION TO TOPIC

The purpose of this literature review is to summarize the current knowledge related to prolonged sitting and acute deficits in cerebrovascular function, and explain why this research points to the value of establishing a mechanism for cerebral hemodynamic disturbances.

Atherosclerosis of the cerebral arteries can develop in arteries exposed to high pulse pressure, which occurs when arteries undergo acute stiffening. Arterial stiffening can be observed by elevated pulse wave velocity (PWV) through an arterial segment. Studies have shown aortic PWV elevations of 0.3-0.4 m/s after 3 hours of prolonged sitting. While this does not directly reflect changes in the cerebral arteries, changes in aortic PWV have been previously correlated to changes in cerebral arterial stiffening as measured by pulsatility index. However, the mechanism for how these changes occur during sitting is unclear. With limited knowledge of a mechanism, physiologists are unable to establish guidelines for to reduce sitting exposure for people, especially those in at-risk populations. The literature review explains the basis for investigation into the role of prolonged sitting and its effect on cerebral arterial stiffening, as well as exploratory analysis into the impact of potential cerebral hemodynamic changes on executive function. The potential physiological mechanism from the literature is depicted in Figure 1.
**SEDENTARY BEHAVIOR**

Sedentary behavior is behaviors that are 1.5 metabolic equivalents (METs) or less while awake, and includes standing still, laying, and most forms of sitting. Sedentary behavior is different than being physically inactive, and is considered a separate risk factor for cardiovascular and cerebrovascular diseases. Time spent in sedentary behavior is a risk even in those who are highly physically active, showing that there is a need to address its risks in all sedentary populations. In western countries, time spent sedentary for adults is increasing. Between 1970 and 2000, the prevalence of sedentary jobs doubled, from 20% to 40%. From

*Figure 1. Proposed Mechanism for Blood Pooling’s Effects.*
1992 to 2006, non-occupational sedentary time has stayed stable, but 90% of non-occupational
time was spent in sedentary behavior.\textsuperscript{21} In combination, these stats show in a steady rise in time
spent sedentary, which means people are exposed to an increasing amount of risk for
cerebrovascular disease. Fortunately, sedentarism is a modifiable lifestyle factor. While some
risk factors for cardiometabolic disorders are based on genetics or uncontrollable variables,\textsuperscript{22}
modifiable lifestyle factors can be altered by changing present behaviors to healthier ones. For
example, instead of participating in uninterrupted sedentary behavior, a person could break up a
single bout by getting up and walking or doing light exercise.\textsuperscript{23} Strategies to mitigate the effects
of sedentary behavior are most limited by a lack of knowledge about its effects and mechanisms,
as it wasn’t clearly defined until 2017.\textsuperscript{16}

\section*{GAPS IN KNOWLEDGE}

While sedentary behavior has been linked to a variety of vascular pathologies, including
cerebral atherosclerosis, there is still a lot of physiological information to be discovered, which
could indicate the aspects of sedentary behavior that have the greatest impact on disease
developments. This study assessed one aspect of prolonged sitting, a prevalent form of sedentary
behavior, on cerebral arterial function.

\section*{EXECUTIVE FUNCTION}

Executive function describes a wide variety of cognitive behaviors, ranging from control
of attention to reasoning and working memory.\textsuperscript{24} Most executive functions are impulses that
originate in the prefrontal cortex,\textsuperscript{24} and are considered higher-order thought processes.\textsuperscript{25} These
processes are critical for all life activities, including activities of daily living and occupational
attentiveness, to name a few. Executive function can be altered by behaviors such as exercise, and events such as ischemic brain injury.

Healthy executive function is heavily reliant on a process known as neurovascular coupling. Neurovascular coupling is a process by which the brain sends nutrients to active regions of the brain based on their metabolism. For example, while performing a pattern-recognition task, which would require processing from the frontal lobe, metabolism of oxygen and glucose by the frontal lobe would increase, and neurons would signal local arterioles to dilate and perfuse more blood to the tissue. Because of the relationship between brain function and neurovascular coupling, functional outcomes, such as performance tests, can provide evidence for healthy or dysregulated cerebral hemodynamic function.

An important covariate of executive function is sleep. After an acute bout of sleep deprivation, a group was found to perform worse on a Spatial Stroop task, which involved participants looking at a blank screen until a left or right-pointing arrow appeared, then required them to press the corresponding arrow key on a keyboard. The group saw an increase in both time to completion and number of errors. Some people naturally sleep more or less, but acute changes in sleep duration were used to covary with executive function changes. Furthermore, sleep quality can influence executive function measures. Both can be measured with accelerometry for objective measurement or questionnaire, and compared to changes in executive function.
KEY POINT

Executive function changes are one potential consequence of cerebral hemodynamic changes. In the proposed study, executive function was assessed to determine the functional impact of prolonged sitting on participants.

HEMODYNAMIC PRECURSORS OF CEREBRAL ATHEROSCLEROSIS

Atherosclerosis is a gradual process that can occur in the arteries, and the middle cerebral artery, one of the primary arteries delivering blood to the cerebrum, is particularly vulnerable. While genetic and metabolic factors can contribute to this process, hemodynamic factors may play a larger role in atherosclerotic development. Two hemodynamic contributors to atherosclerosis that can occur in the cerebral vasculature are exposure to acute arterial stiffening and decreased cerebral blood flow. Both work synergistically to contribute to atherosclerosis by exposing the cerebral arteries to potentially damaging pulse pressures and promoting dysfunction of the arterial endothelium in cerebral arteries via a reduction in shear stress respectively.

High arterial stiffness in the carotid artery has been shown to be associated with increased atherosclerosis of the intracranial cerebral arteries. Healthy arteries are not stiff, instead being highly elastic, and absorb some of the pulse pressure by way of the Windkessel effect. This effect is highly dependent on the function of the arterial endothelium. When exposed to high pulse pressures, the function of the endothelium can be impaired, and begins to constrict the blood vessels. These damages stimulate thickening in the arterial wall which can be pro-
atherosclerotic in the cerebral arteries, and with continued exposure can lead to remodeling of the arterial wall. This remodeling can include atherosclerotic development.

Decreases in cerebral blood flow (CBF) could mediate the changes in pro-atherogenic endothelial dysfunction that occurs with arterial stiffening. The function of the arterial endothelium is dependent on the shear stress on the innermost layer of the blood vessel. When arteries are subjected to decreased flow, the endothelium will constrict the artery. In healthy individuals, total CBF is controlled by a process known as cerebral autoregulation (CAR). Due to variance in blood pressure throughout the day, the cerebral arteries adapt to maintain a continuous supply of oxygen and glucose to the brain. However, the cerebral autoregulatory system responds effectively to changes in mean arterial pressure (MAP, a weighted average of systolic and diastolic blood pressures), but it does not respond as well to changes in systolic pulse pressure alone. When arteries are subjected to high pulse pressures, the autoregulatory system will stimulate the cerebral arteries to constrict, even though MAP may not be increasing at the same rate. If arteries are constricting, but MAP is not increasing proportionally, shear stress on the arterial wall would decrease, and therefore the arterial endothelial function can become dysregulated. Repeated exposure to endothelial dysfunction is associated with atherosclerosis in central arterial tissues, but it has not been thoroughly explored in cerebral arteries. However, in an elderly population, higher resistance to flow, which could be due to arterial stiffening, was associated with decreased blood flow through the middle cerebral artery. The combination of increased cerebral arterial stiffening and decreased CBF provide a physiological pathway by which local endothelial dysfunction creates a pro-atherosclerotic state in the cerebral vasculature.
GAPS IN KNOWLEDGE

The specific aspects of prolonged sitting that impact CBF are not well understood. Understanding how CBF changes could indicate a mechanistic connection between prolonged sitting changes and atherosclerosis development risk. This study aims to assess how CBF is impacted by prolonged sitting.

PROLONGED SITTING ACUTELY IMPAIRS CEREBROVASCULAR FUNCTION

During an acute bout of prolonged sitting, acute hemodynamic changes have been observed related to both arterial stiffening\(^{48,49}\) and cerebral blood flow.\(^{48,50}\) The effects were observed after 2-4 hours of sitting in the cited studies. However, the aspects of sitting that contribute to these changes have yet to be identified, and the rate at which they contribute is unknown.

Currently, evidence has shown a relationship between indicators of systemic arterial stiffening in the cerebral vasculature and sitting time.\(^{48,49}\) However, investigation into stiffening of specific cerebral arteries is limited. Furthermore, the evidence for central arterial stiffening is during prolonged sitting is well-established.\(^{9,10}\) While this does not directly point to a relationship between prolonged sitting and cerebral arterial stiffening, there is evidence that increased central arterial stiffness is correlated with increased stiffness in the common carotid artery,\(^{51}\) which are the primary arteries that supply blood to the head and neck as a whole.\(^{52}\) There is also evidence that higher stiffness in the aorta is correlated with higher systemic stiffness of the cerebral arteries.\(^{15}\) While there is not much evidence of a direct relationship between prolonged sitting and stiffening in the cerebral arteries, there are connections between
prolonged sitting and central arterial stiffening, and central arterial stiffening and cerebral arterial stiffening that point to an indirect relationship at least.

Cerebral blood flow has also been shown to decline during a bout of prolonged sitting. In two studies with 2- and 4-hour sitting bouts, mean cerebral blood velocity was shown to decrease by 2.2 and 1.8 cm/s, respectively. Mean cerebral blood velocity can be used to assess changes in cerebral blood flow. In general, periods of increased blood flow upregulate function of the vascular endothelium, and periods of decreased blood flow are associated with endothelial dysfunction. While the rate of endothelial dysfunction caused by changes in cerebral blood flow is not clear, a repeated cross-sectional study showed a negative association between cerebral blood flow and blood markers of endothelial dysfunction over a 33 year period. Since endothelial dysfunction is a precursor for atherogenesis in the cerebral arteries, cerebral blood flow decreases could also contribute to the rate of atherogenesis.

Lastly, the acute cerebrovascular dysfunction from prolonged sitting may result in changes in cognitive performance, demonstrating the connection between vascular and end-organ function. A cross-sectional study showed lower scores of executive function tests have also been linked to higher stiffness of the carotid arteries, which supply blood to the head and neck. Furthermore, changes in executive function decreases during sitting have been thoroughly researched, though largely in older adults. A connection between executive function and cerebrovascular disturbance has also been previously hypothesized.
While there is evidence for decreased cerebral blood flow during sitting, less has been done to establish the contributions of arterial stiffening that occurs during a single bout of sitting. Arteries become stiffened due to multiple physiological stimuli, and upon repeated exposure, can then become atherosclerotic. Evidence for a mechanism for cerebral arterial stiffening, mediated by changes in cerebral blood flow, would help determine how much and when sitting becomes pathological, and help experts to design strategies to mitigate these pathologies.

The cardiovascular system is complex, and relies on three mechanisms to generate pressure that drives blood through the body: the heart, the respiratory pump, and the skeletal muscle pump. Healthy function helps the body to deliver blood to all tissue as needed despite various forces that may act on the body throughout the day, including gravity. However, during an acute bout of sitting the role of the skeletal muscle pump is diminished due to decreased skeletal muscle activity. The heart and respiratory pump increase effort to compensate for the lack of muscle pump activity, but are unable to fully compensate for the reduction of blood pressure generation. Therefore, gravity causes blood to pool in the veins of the lower limbs, which are the lowest point on the body while sitting, and the farthest anatomical distance away from the heart. The longer this continues, the more blood pools. A study was conducted that showed blood pooling in the calves at 5.3 mL per 100 mL total blood after 5 hours. If blood continues accumulating in the calves, it means less blood is available to return to the heart. Decreased return of blood to the heart is synonymous with decreased preload. Preload is a determinant of stroke volume in the heart, and a decrease in preload results in a decreased stroke
volume. Stroke volume is also a determinant of cardiac output, meaning cardiac output declines proportionally. I hypothesize that a decrease in cardiac output results in two synergic pathways in the cerebral arteries occur during an acute bout of sitting.

The decrease in cardiac output causes a decrease in central blood pressure. The decrease in central blood pressure is detected by baroreceptors in the carotid artery, which then trigger a release of catecholamines throughout the body. These catecholamines constrict arteries throughout the body, including the cerebral arteries. Once constricted, the ability of the arteries to dampen pressure waves via the Windkessel effect is reduced. This means the cerebral arteries experience a greater pressure wave from the heart, which result in damage and remodeling. During arterial remodeling from an acute bout of sitting, it is possible that atherosclerotic changes are made that can accumulate over time.

Furthermore, the decrease in cardiac output simultaneously decreases the amount of blood flowing to the head. The decrease in brain blood flow likely leads to reduced shear stress on the cerebral arterial walls. The resulting reduction in shear stress would induce endothelial dysfunction, particularly at arterial bifurcations more proximal to the brain. In this way, endothelial damage and dysfunction are promoted by both arterial stiffening and reductions in brain blood flow, potentially leading to cerebral arterial atherogenesis.

**GAPS IN KNOWLEDGE**

While blood pooling during sitting has been observed previously, we aren’t sure of the impact of blood pooling on acute cerebrovascular function. There is evidence that blood pooling does have some effect on cerebral vascular function via changes in executive function, but the
rates and magnitudes of arterial stiffening and cerebral blood flow changes during an acute bout of sitting have not been explored in depth.

**WHY IS THIS STUDY NEEDED?**

Currently, it is unclear if blood pooling in the calves influences hemodynamic changes in the cerebral arteries. If so, treatment strategies targeting blood pooling would allow for the changes in cerebral hemodynamics to be reduced or eliminated. Furthermore, no study to date has simultaneously assessed each physiological step of the proposed mechanism by which these changes occur, including blood pooling, changes in cardiac output, arterial stiffening, and cerebral blood flow. These measurements are all steps in the proposed mechanism, and can be measured non-invasively. Establishment of a mechanism allows researchers a framework with which to conduct future studies on changes that happen throughout the body, and affect the cerebral vasculature.

**METHODOLOGICAL AND RIGOR CONSIDERATIONS**

**STUDY DESIGN CONSIDERATIONS**

A multitude of study designs were considered to assess the mechanism of this project. The study design needed to consider factors such as participant convenience and ability to participate, reduction of influence for potential covariates, and ability of investigators to administrate the procedure. Furthermore, the study would need to separate the effects of blood pooling from the effects of sitting. A cross-sectional study design would be convenient for participants and investigators, but would not allow the degree of investigation into the function of the mechanism that would be possible with variable manipulation. A randomized controlled trial (RCT) would address the need for manipulation well, and is considered the gold-standard for experimental physiological research. However, it would require a large sample size to
achieve results, and there are insufficient resources to perform one for this question. Lastly, a randomized crossover trial would allow for assessment of the mechanism under two conditions, allowing us to make conclusions based on within- and between-subject data. To differentiate between the effects of blood pooling and those of sitting alone, two randomized cross-over trials were settled on. One cross-over trial had participants lie down with and without cuffs, and the other had participants sit without and without cuffs. The two-trial design can inform investigators as to the effects of blood pooling in the calves alone, versus the effect of blood pooling combined with a bout of sitting.

For the sitting trial, it is worth considering what type of sitting is most ecologically valid. Most sitting occurs in a chair, with the knees and hips bent at approximately 90 degrees. Therefore, a standardized chair was used. Because a standard chair may not cause each participant to sit with the same joint angles, an adjustable set up was needed. An adjustable bed could be used that can fold up to form a back and seat in conjunction with an adjustable footrest to ensure all participants are sitting similarly to a standard seated posture. Furthermore, the adjustable bed could be folded down and used for the lying trial. For this posture, the requirement would be to ensure that the participant be lying flat, so as to eliminate any pooling due to gravity in any part of the body. The adjustable bed would make both postures achievable.

The randomized cross-over trial model allows each participant to be in a control and experimental group, allowing for within-subject comparisons that reduce the influence of potential covariates on the data. Measures of cerebral arterial stiffness, brain blood flow, and
cognitive function were assessed between Cuff and Non-Cuff visits for each participant, and mean values between cross-over trials were compared.

MEASUREMENT CONSIDERATIONS

As outlined in Figure 1, there are several steps proposed between venous pooling in the lower limbs and cerebrovascular outcomes. In order to provide evidence for the mechanism, as many of the proposed steps should be measured as is feasible. However, not all steps can feasibly be measured with non-invasive methods. Therefore, multiple measurement techniques are considered for each step below, based on the following mechanistic factors: blood pooling in the calves, cerebral arterial stiffening, cerebral blood flow, executive function and cognitive load, and cardiac output.

INDEPENDENT VARIABLE: BLOOD POOLING

Blood pooling can be assessed via multiple methods. Previous work has shown that calf circumference can be used to measure venous distension due to blood pooling\(^73\). Near-Infrared Spectroscopy (NIRS) can also be used. NIRS can allow for continuous assessment of oxygenated hemoglobin concentration, which can be used to assess blood pooling\(^74\). While a NIRS instrument is more expensive and complex, it would give continuous data compared to a manual measurement of calf circumference. NIRS is also a valid and reliable technique for venous pooling measurements\(^75\). However, NIRS probes can only be placed on one portion of the calf at a time, and do not allow analysis of the whole calf at once. Despite this, continuous data can help to show the rate of blood pooling over time, and would be helpful in providing clear evidence of a mechanism. Furthermore, the Cardiometabolic Lab (CML) has a NIRS device available to use,
so the expense of the device does not apply to this study. Lastly, calf circumference and NIRS would not interfere with each other, so they can also both be used. The use of calf circumference can serve as a secondary measurement, with a lower opportunity for failure, but no ability for continuous measurement. The device redundancy provided more methods for assessing changes in the mechanism.

**PRIMARY OUTCOME: CEREBRAL ARTERIAL STIFFENING**

Arterial stiffening is the primary outcome for this experiment, as it has been directly shown to be associated with atherosclerosis of the cerebral arteries. The gold-standard for measuring clinical arterial stiffness is Pulse Wave Velocity. Pulse wave velocity involves the measuring of the speed of the pulse pressure generated by the heart through a given arterial segment. Performing a measurement of pulse wave velocity on an arterial segment more local to the area of interest has been shown to be a better predictor of disease more systemic measurements. The MCA is particularly vulnerable to atherosclerotic development. Therefore, Heart-to-Middle Cerebral Artery pulse wave velocity (hmPWV) can be used to assess arterial stiffening in the cerebral arteries. This is a novel method that uses the entire path from the aorta, connected to the heart, through the carotid artery, to the middle cerebral artery (MCA), one of the most prominent arteries that supplies blood to the brain. hmPWV can be assessed using a Transcranial Doppler (TCD), and allowed for non-invasive monitoring of the MCA. hmPWV is a relatively novel measure, but has been used in one previous study to be a repeatable measure. hmPWV was developed to analyze the entire path blood takes to get to the cerebral vasculature, and may therefore offer more insight into hemodynamics leading to and including the cerebral arteries.
SECONDARY OUTCOME: CEREBRAL BLOOD FLOW (CBF)

Changes in cerebral blood flow (CBF) indicate the ability of the brain to respond to brain metabolic needs in the presence of hemodynamic disturbances, such as blood pooling. The brain is strongly reliant on CAR and NVC to supply enough oxygen and glucose-carrying blood to metabolically active regions of the brain. Where CAR functions at the level of the large arteries, including the common carotid artery, NVC functions at the level of the distal cerebral arteries and arterioles. Furthermore, both CAR and NVC are hypothesized to be reliant on healthy endothelial function in the cerebral arteries and arterioles. To measure the reactivity of both cerebrovascular systems, two related methods were required. The distinction between CAR and NVC in the different regions of the cerebral vasculature is illustrated in Figure 2 below.

![Figure 2. Relationship between cerebral processes and vasculature units responsible for function.](image)

Therefore, the methods of measurement should be able to discern the function of these two mechanisms to determine the extent of hemodynamic disruption. Doppler ultrasound and TCD present potential methods. Doppler ultrasound of the CCA can be used, and has been used in the medical field as an assessment of total CBF. The CCA is proximal to, but not within the cerebral vasculature, and both CCAs (left and right side) supply about 80% of the total blood the
brain receives. Changes in blood flow through the CCA then provide a good estimate for changes in total CBF. Changes in total CBF would help determine CAR functionality, but not NVC function. Using TCD to assess mean blood flow velocity through the middle cerebral artery (MCA BFV) is a validated method for assessing CBF, and has been used widely. While technically only a measure of velocity, rather than volumetric flow, there is evidence that MCA BFV varies linearly with volumetric flow with 0.93 agreement compared to magnetic resonance imaging (MRI), which can be used to determine volumetric flow, within-subjects over an intervention. Approximately 80% of the blood for the brain flows through the MCA, which means that changes in MCA BFV are representative of CBF through the cerebral circulation. TCD can also collect MCA BFV and hmPWV simultaneously, which makes taking both measures logistically simpler. In this experiment, both CCA CBF and MCA CBF can provide data both for CAR and NVC function respectively during the intervention. CCA CBF measurements can be taken before and after the intervention, and MCA CBF used as a continuous measure. The arterial pathway to the brain is shown below in Figure 3.
Figure 3. Depiction of the arterial pathway between the heart and brain.

MCA BFV can also be used with a more specific method for assessing NVC. Established methods for measuring changes in NVC, include functional Magnetic Resonance Imaging (fMRI) and functional NIRS (fNIRS). Both are used in “functional” neuroimaging, which involves monitoring changes in CBF during a task. fMRI can show blood flow through regions of the brain as well as neural activity in those regions. However, fMRI is expensive, and due to the magnetic field produced, excludes any other measurements from being performed on a participant. fNIRS is a more compact, less expensive technology that allows assessment of hemoglobin levels in various brain regions, and therefore oxygen perfusion. fNIRS uses near-
infrared light to determine hemoglobin levels in various regions of the cerebral circulation. It is differentiated from NIRS in that it is highly time-sensitive, making it useful for assessing rapidly changing cerebrovascular function.\textsuperscript{89} fNIRS is specific to a certain region of the brain at a time, and must be targeted to a desired section of the cerebral vasculature and cannot perform overall scans. Despite this, accurate data can be collected if one knows what regions are likely to undergo blood flow changes due to NVC. Because we are using performative tests of executive function in this experiment, fNIRS can be directed at the frontal lobe, which is responsible for most cognitive function.\textsuperscript{90} fNIRS’s compatibility with other measurements and lower cost can justify its use for assessment of NVC.

Lastly, decreased blood flow can result in decreased shear stress in the cerebral arteries. Endothelial function is moderated by shear stress on the endothelial wall.\textsuperscript{40} While measuring blood flow through the CCA, shear rate in the artery can be determined using the equation $s^{-1} = 4 \times V_{\text{mean}}/d$, with $V_{\text{mean}}$ as mean velocity and $d$ as arterial diameter.\textsuperscript{91} This information is calculated automatically by the same software that monitors blood flow, so no extra work is needed. The CCA is upstream of the cerebral arteries in terms of blood flow, and changes in shear rate at the CCA during prolonged sitting are likely to reflect shear rate changes in cerebral vasculature.\textsuperscript{92} Furthermore, the CCA itself is vulnerable to arterial stiffening and atherogenesis, particularly at its bifurcations.\textsuperscript{92} Most importantly, decreases in cerebral artery shear stress is another proposed step in this mechanism.
To establish a connection between an acute bout of sitting and future cerebrovascular complications, a measurement must be made that shows acute cerebrovascular hemodynamic changes are associated with acute deficits in cognition. Evidence has shown a strong association between stiffening of the carotid and middle cerebral arteries and poorer scores on executive function (EF) tests in cross-sectional studies. Furthermore, EF is related to healthy NVC. However, there is less evidence for the relationship between acute cerebrovascular hemodynamic changes and acute cognition changes. To assess these changes, tests for cerebrovascular complications such as VaD would be most useful, as they could show the impact of altered cerebrovascular hemodynamics on the same areas that develop complications later. In particular, the Trail-Making Test (TMT) and Verbal Fluency Test (VFT) have been used to assess EF, and have been used as diagnostic criteria for VaD. The tests are also logistically simple and can be easily administered to participants with only a brief explanation. A test that can be quickly administered can lessen participant burden compared to a longer test. Furthermore, the TMT uses visual stimuli for assessment, and the VFT uses verbal stimuli. The TMT has participants connect circles with letters and numbers inside them by tapping a screen, and is scored for time, and the VFT involves articulating words related to a specific prompt for 1 minute, and is assessed by number of correct words produced. By combining the results of both tests, there would be minimal interference due to differences in perceptive ability or stimuli processing, better reflecting changes in EF. Ultimately, these tests offer a performative measurement that can show the effects of cerebrovascular disturbances on functioning brain tissue.
In combination with the TMT and VFT, eye tracking can be used to assess relative cognitive load. Cognitive load is the amount of working memory being occupied during a given task. A cognitive load measurement would measure the level of exertion by the brain for a given task. There is evidence that cognitive load increases during a given task as regional NVC becomes impaired. Therefore, an increase in cognitive load during a test of executive function could indicate functional NVC impairments. Saccade counts and blink rate are validated, logistically simple methods to determine relative cognitive load. Saccade counts are particularly useful during visual-spatial tasks, such as the TMT, and blink rate can be used during visual or auditory tests, including the TMT and VFT. Saccade counts are measured by frequency, with an increasing number indicating higher cognitive load. A higher blink rate would also indicate a higher cognitive load. While eye tracking devices are expensive, the CML already owns one, which eliminated cost concerns for this method. Eye tracking to determine relative cognitive load could add to the relationship between executive function and cerebral hemodynamic function during prolonged sitting.

STROKE VOLUME AND CARDIAC OUTPUT (CO)

Lastly, a central aspect of the proposed mechanism involves changes in cardiac output (CO). There are multiple methods available for CO measurement, including invasive measurements such as catheterization, and non-invasive methods, such as ultrasound monitoring of cardiac output and newer devices. While catheterization can be used and has a long history of use, it requires high operator skill and an area for sterile medical operations. Furthermore, its invasive nature could interfere with other measurements in the body. In contrast, ultrasound measurements of CO via stroke volume monitoring have been validated against...
catheterization. While ultrasound techniques are well-validated, they cannot obtain continuous data. Devices such as the Finapres® can be used to assess CO and stroke volume continuously. They acquire a value via the Model Flow method. This allows the analysis of stroke volume with every heartbeat, and a value for cardiac output when stroke volume is multiplied by heart rate per minute. While the Finapres® can obtain data continuously, the device has not been thoroughly validated. These methods are not exclusive, and can both be used simultaneously to generate data for the proposed mechanism. Also, the CML already has both Doppler ultrasound and trained operators, and owns a Finapres®. Having access to multiple methods justified the consideration of the use of these procedures to monitor changes in cardiac output.

<table>
<thead>
<tr>
<th>PRE-VISIT EXPERIMENTAL VISIT CONTROL</th>
</tr>
</thead>
</table>

Before testing, participants must ensure they adhere to the pre-assessment guidelines, which were as follows: 8 hours fasted except for water; no caffeine, alcohol, or drug use for 12 hours except prescribed medications; and 24 hours without vigorous exercise. Fasting for 8 hours is sufficient to decrease the influence of metabolic hormones on data quality. Caffeine is noted for having effects on blood pressure, which could confound results. Exercise is known to have effects on blood pressure for about 12 hours, so 24 hours was chosen to be certain it would not confound our results. Lastly, participants were asked about their sleep patterns. Acute sleep deprivation has been found to affect PWV and executive function measures. A summary of these effects is provided below in Table 1.
**Table 1. Pre-visit Testing Considerations**

<table>
<thead>
<tr>
<th>Consideration</th>
<th>Explanation</th>
<th>Control Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standardized time to begin experimental visits</td>
<td>Prevents influence of daily life on experimental data</td>
<td>All experimental visits began between 06:00-08:00</td>
</tr>
<tr>
<td>Prevention of vigorous physical activity before experimental sessions</td>
<td>Reduces the temporary effects of vigorous exercise and recovery on the cardiovascular and cerebrovascular system on data</td>
<td>Participants were sent a reminder email before their experimental visit reminding them to refrain from vigorous physical activity for 24 hours prior to testing</td>
</tr>
<tr>
<td>Prevention of caffeine consumption</td>
<td>Reduces the influence of caffeine on the cerebrovascular data. Caffeine is known to cause changes in arterial stiffening.</td>
<td>Participants were sent a reminder email before their experimental visit reminding them to refrain from caffeine or alcohol for 8 hours prior to testing</td>
</tr>
<tr>
<td>Prevention of glycemic changes</td>
<td>Reduces the influence of glycemic spikes during data collection</td>
<td>Participants were sent a reminder email before their visit reminding them to refrain from eating 8 hours prior to testing</td>
</tr>
<tr>
<td>Standardized amount of sleep between visits</td>
<td>Prevents differences in sleep duration from confounding PWV, cognitive data</td>
<td>Participants were asked about their previous night’s sleep duration</td>
</tr>
</tbody>
</table>

**INTERNAL VALIDITY**

Internal validity can be maintained by ensuring consistent measurement practice and experimental procedure. All testing took place in the CML, which is a controlled environment at a comfortable temperature. Curtains can be used to isolate the lab from surprising stimuli offer the participant a safe testing environment. Prior to testing, investigators calibrated equipment regularly to ensure quality measurements. During testing, all continuous measurements were kept on the participant throughout the procedure, and all baseline and post measurements were performed in the supine position for consistently reliable measurements. Lastly, the non-stimulating documentary that participants can watch was standardized, to ensure no variation due to mental stimulation. A summary of the described effects can be found below in Table 2.
Table 2. Controlled Variables.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Method</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient Temperature</td>
<td>Thermometer check prior to testing</td>
<td>Temperature could affect comfort level of participants</td>
</tr>
<tr>
<td>Freedom from Unintended Stimuli</td>
<td>Use of curtains during testing</td>
<td>Unintended stimuli or noise could startle participant, changing hemodynamic and performance test results</td>
</tr>
<tr>
<td>Standardized Documentary</td>
<td>Download and save videos to use for every participant</td>
<td>A non-stimulatory documentary can alleviate boredom for the participant, but not elicit hemodynamic changes</td>
</tr>
<tr>
<td>Participant Accurately Follows Pre-Assessment Guidelines</td>
<td>Screening interview</td>
<td>Participants were asked about conformity to pre-assessment guidelines before each visit</td>
</tr>
</tbody>
</table>

POPULATION/SAMPLING

The population of study for this experiment would be healthy, young adults. “Healthy” is defined as free of cardiovascular disease, and “young” is defined as 18-45 years old. The age range for the participant sample was chosen because after age 45 in men and 55 in women, the cardiovascular system changes. Therefore, this sample would minimize the influence of potential confounders on data due to age and cardiometabolic disease. At the conclusion of this research, specialized populations could be tested to determine the nuances of the physiological mechanism in that population. Specific inclusion and exclusion criteria are listed below in Tables 3 and 4 respectively.

Table 3. Eligibility Criteria.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Method</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 18-45 y</td>
<td>Screening interview</td>
<td>To ensure normal physiological responsiveness to research methods</td>
</tr>
</tbody>
</table>
Table 4. Exclusion Criteria.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Method</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Currently pregnant</td>
<td>Screening interview</td>
<td>Pregnancy may affect ordinary cardiovascular function</td>
</tr>
<tr>
<td>Current tobacco or nicotine user</td>
<td>Screening interview</td>
<td>Nicotine use affects the cardiovascular system</td>
</tr>
<tr>
<td>Diagnosis of a cardiometabolic disease</td>
<td>Screening interview</td>
<td>Participants must be free of disease that may affect data on the cardiovascular system</td>
</tr>
<tr>
<td>Currently taking medicine that affects cardiovascular function</td>
<td>Screening interview</td>
<td>Participants must be free of known alterations to cardiovascular function</td>
</tr>
</tbody>
</table>

SEX AS A BIOLOGICAL FACTOR

This study was not powered to assess sex differences. However, it could give a trend that would inform future experiments. Women who are experiencing or have experienced menopause were unable to participate in this study due to the related changes to their cardiovascular system. All women in either experimental trial underwent all testing during the follicular phase of their menstrual cycle, because estradiol levels were likely to be lowest. Estradiol is likely to be a covariate of PWV.

ETNICITY/RACE

Participants were not excluded or analyzed based on race or ethnicity. Furthermore, while health disparities in cerebrovascular diseases is documented, it is highly unlikely that this study has adequate power to ascertain racial and ethnic differences in the proposed mechanism.

EXTERNAL VALIDITY / GENERALIZABILITY

External validity can be maintained by constructing a sample that represents the population of interest. In this case, we were interested in assessing adults at the University of North Carolina at Chapel Hill. To do this, investigators sampled from students across the
university, so that the sample can match the school demographics as closely as possible. All studies balance external and internal validity measures, and this study is included. This study focuses on internal validity to establish consistent results of a mechanism, the function of which can then be explored in diverse or vulnerable populations.

**STATISTICAL CONSIDERATIONS**

The question for this experiment is: “Does 1) hmPWV and 2) CBF change during an acute bout of prolonged sitting?” To answer this question, multiple statistical tests were considered. Paired t-tests or mixed-design Analysis of Variance (ANOVA) could have been used to compare values between pre and post treatment. T-tests would have been inadequate, as participants undergo multiple conditions with before and after measurements. A mixed-design ANOVA could have accommodate for multiple conditions, but would not have allowed for control of covariates, and not allowed for the full use of most of the measurements, most of which are continuous. A linear mixed model can accommodate for adjustment for covariates and can be used to analyze continuous data. It can also adjust for random and fixed effects. In this study, fixed effects included within-subject fixed variables such as sex differences or differences due to height or body weight. Random effects could include the degree of arterial stiffening that is due to factors outside of the scope of the experiment, such as increased arterial stiffening not due to sitting that was occurring. The linear mixed model was used to observe differences in the primary outcome (hmPWV) and secondary outcomes (CBF, executive function assessment) between timepoints (pre/post) and between visits (CUFF or CONTROL) for each participant. Pearson’s correlation coefficients were be used to determine the relationships between each outcome in the study. Furthermore, Cohen’s d, a measure of effect size,\textsuperscript{115} was used to offer a
comparison of difference in means relative to sample variance. Mechanistic outcomes were used to guide discussion of the mechanism.

**POTENTIAL CHALLENGES & ALTERNATIVE STRATEGIES**

There were several potential challenges that could have arisen during the experiment. Specifically, there were numerous potential challenges that could have arisen due to the ongoing COVID-19 pandemic. For instance, human subject research could have been canceled at any time, indefinitely. To meet these potential challenges, some contingency plans were considered. A two-tiered strategy for recruitment would have helped address these challenges. The lying trial was planned to recruit six participants, and the prolonged sitting trial requires a minimum of 12 participants to be adequately powered for conclusions of hmPWV. This number was inflated to 16 to provide a buffer in the event of poor data quality or participant lost to follow up. The lying trial was conducted first due to the lower participant requirement. In the event of research cancellation during the sit trial, the results of the lay trial can stand alone as a pilot trial to inform future studies. Whereas beginning the sit trial first may have lead to under-powered results for the trial, and an inability to separate the effects of blood pooling from prolonged sitting. Once the lying trial is complete, the sit trial began. If the trial can reach 12 full participant data sets, then it would be adequately powered to make conclusions about arterial stiffening due to blood pooling. However, 16 participants would potentially increase data quality and external validity.

Furthermore, the COVID-19 cancellation of human subject research made pilot testing impossible for a time. Depending on the size of the participant’s head, it may be difficult or impossible to fit the fNIRS and eye tracking glasses on simultaneously, and this could have been
considered in pilot testing. Once pilot testing was possible, testing to determine the feasibility of frontal lobe fNIRS and eye tracking was performed. While fNIRS monitoring of the occipital lobe would have been useful to track activity during the TMT, which is a visual task,\textsuperscript{101} it is unlikely to produce useful data during the VFT, which is a test with auditory stimuli. However, it would potentially have offered some useful data for executive function.

There could have also been recruitment challenges related to external validity. If there was an abundance of research volunteers from a single sex, then participant selection would have been limited from that sex to include an even number of males and females. This would have maintained the external validity of the experiment. If no participants of one sex can be found, then an increase in participants from the opposite sex would have been considered for the study to be adequately powered.

During data collection, equipment could have presented challenges also. It is possible that equipment could have failed or become unavailable. However, this experiment had several outcomes that could still inform the hypothesis if others failed. For example, while NIRS would show the most data for blood pooling in the calves, it is possible the machine could malfunction. For this reason, investigators took measurements of calf circumference pre/post sitting. Similarly, several outcomes were proposed that could show changes in the primary outcome (arterial stiffening) at varying levels of detail.
### SUMMARY

#### WHY IS THIS STUDY NEEDED?

- Acute bouts of prolonged sitting may contribute to arterial stiffening, and thereby atherosclerotic changes of the cerebral arteries.

- The mechanism by which cerebral arterial stiffness occurs during prolonged sitting is unknown.

- If the mechanism can be elucidated, the knowledge can be used to create treatment strategies that reduce arterial stiffening during bouts of prolonged sitting.

#### WHAT IS KNOWN

- Cerebral arteries become more stiff during a bout of prolonged sitting.
• Arterial stiffness causes dysfunction and damage to the vascular endothelium, resulting in remodeling.

• During a bout of prolonged sitting, blood pools in the calf, causing local endothelial dysfunction and central hemodynamic changes.

WHAT IS NOT KNOWN

• It is unclear what is causing cerebral hemodynamic changes during a bout of prolonged sitting, and if and how much of the effect is related to blood pooling in the lower limbs.

• The mechanism for changes in cerebrovascular function from prolonged sitting has not been established. This mechanism would include evidence for all contributing or related physiological steps between blood pooling and cerebrovascular changes.

CRITICAL NEED

• Evidence for influence of blood pooling on cerebral endothelial dysfunction would help design treatment strategies aimed at changing blood pooling behavior to reduce damages that to the cerebral arterial endothelium.

• These could reduce the rate at which atherosclerotic changes occur.

WHAT THIS STUDY ADDS

• This study is the first to provide evidence of every feasibly measured step of the proposed mechanism. It is also the first to test multiple outcomes simultaneously, such as PWV and CBF, which reflect changes in arterial endothelial function, and executive function outcomes, which can show impairment of neurovascular coupling and offer a performative outcome related to cerebrovascular pathology.

• Implications for future use:
- Future studies can investigate the role of this mechanism in specific populations, such as older adults or populations with cerebrovascular disease risks.
- Treatment strategies for highly sedentary young people can potentially alter their long-term health outcomes.
CHAPTER III: METHODOLOGY

This study is reported in accordance with CONSORT (Consolidated Standards of Reporting Trials) guidelines. Ethical approval was given by the Internal Review Board (IRB) of the University of North Carolina at Chapel Hill.

PARTICIPANTS

For the first cross-over trial, 6 participants of any gender were recruited. For the second, 6 participants were recruited. To be included in the study, participants must have been aged 18-45 years old. This age range was chosen to specifically observe changes related to the physiological mechanism in this study and minimize the influence of potential covariates on measurements. Participants were excluded for the following criteria: currently pregnant, current smoker or tobacco user, currently taking medicine that alters cardiovascular function, or diagnosis of any cardiometabolic disease.

EXPERIMENTAL DESIGN

The study consisted of two randomized cross-over trials, with two conditions each. One trial had prolonged sitting (SIT) or prolonged lying (LAY) to discern the effects of blood pooling versus the effects of an acute bout of sitting alone. In the SIT trial, participants sat in an upright position with knees bent for two hours for the Non-cuff condition; and prolonged sitting with a
venous pooling intervention (Cuff). Both conditions were held on an adjustable table that replicates a standard chair (Tiger Medical, TIGER#TM83695). The LAY trial had a Cuff and Non-cuff condition as well, but both were assessed while the participant was lying down. In the Cuff condition, participants sat in the same upright position, but have a standard blood pressure cuff (Hokansen, NJ, USA) inflated to sub-diastolic pressure proximal to the knee joint. Placing the cuff above the knee allowed for occlusion of the entire calf. Two hours of sitting time was selected because evidence indicates that at least an hour of prolonged sitting is needed to elicit significant cardiovascular disruptions.\cite{116,117} All visits started between 05:30 and 09:00 to reduce the influence of daily activities on the data. For each participant, the second condition visit was scheduled to begin within 30 minutes of the first condition visit, to control for diurnal variation (for a 09:00 first condition visit start time, a start time from 08:30 to 09:30 would be acceptable).

**Figures 4 and 5** depict the order of measurements and interventions for each visit in each trial.

**Figure 4.** Measurement order for supine blood pooling randomized cross-over trial.
VISIT 1: FAMILIARIZATION

Participants interested in study participation were met in the Applied Physiology Lab (APL) to discuss the study. Participants who remained interested in participation after initial discussion were asked to complete a general medical questionnaire of health habits and medical history for the purpose of inclusion/exclusion criteria screening. Once screened, those eligible to were provided an informed consent form. The form included descriptions of all experimental devices and their use in the protocol, as well as how and when they were affixed to the participant for data collection. Participants were informed and asked to verbally confirm understanding of pre-testing guidelines. Following that, anthropometric data (height, weight, age, arterial path lengths, etc.) was collected by a member of the research team. Finally, the first study condition visit was scheduled, and the participant would leave for the day. The next visit was scheduled within 72 hours.

Figure 5. Measurement order for seated blood pooling randomized cross-over trial.
VISITS 2 & 3: EXPERIMENTAL VISITS

Investigators met with the participant in the APL and confirmed that he or she followed pre-assessment guidelines. If the participant had not followed the guidelines, investigators would reschedule the visit with the participant. If the participant had followed the guidelines, he or she was escorted to the study area.

Both protocols began with 20 minutes of rest in a supine position. While rested, the research team affixed NIRS probes to the skin superficial to the medial gastrocnemius. Doppler ultrasound was used to confirm correct placement of the probe according to manufacturer guidelines. The Finapres® NIBP device was placed on the participant’s non-dominant wrist. The Finapres® would provide continuous data throughout the procedure. Lastly, Doppler ultrasound was used to find the aortic notch, which was used for USCOM cardiac output monitoring. The devices would provide continuous data during the procedure. Then, the tourniquet leg cuff would be attached immediately superior to the knee joint, but not inflated. Transcranial Doppler (TCD) ultrasound data would be collected immediately to establish a baseline. Once the data was verified from each device, the tourniquet cuff was inflated to a sub-diastolic pressure of 80 mmHg based on pilot data. This pressure was well tolerated by participants during pilot testing. Immediately after the supine rest time is complete and measurements are completed, participants were either left supine or moved to an upright seated position for 120 minutes. The measurement order for the supine and seated trials was randomized, and is visualized in Figures 3 and 4 respectively, below. Participants would have the option to watch a low-stimulation nature documentary for the remainder of the sitting or supine period to minimize mental stimulation and discomfort but maintain wakefulness.
At the conclusion of the sitting time for either visit, the participant was laid supine if seated, and rested for 5 minutes. Then, TCD and CBF measurements were performed. Upon completion, the tourniquet cuffs were deflated and removed. After the cuffs were deflated, participants were administered the tests of executive function. During the tests, eye-tracking data was taken. At the conclusion of the experimental session, the research team scheduled the follow-up visit with the participant. The second visit was within 72 hours of the first visit. Figure 6 shows the order of experimental conditions for each trial.

![Diagram of potential randomization combinations for both randomized cross-over trials.](image)

*Figure 6. Diagram of potential randomization combinations for both randomized cross-over trials.*
Acute arterial stiffening is a primary risk for atherosclerotic development. Therefore, an appropriate measure was needed to determine arterial stiffening. While some studies have associated aortic stiffness directly with changes to cerebral artery stiffness,\textsuperscript{15} other researchers have noted that this is not appropriate because the approach lacks specificity to the cerebral arteries.\textsuperscript{118} A method for assessment of cerebral arterial stiffness would need to be specific to the arteries that deliver blood to the brain.

Heart-MCA pulse wave velocity (hmPWV) was measured using a continuous bilateral TCD (ST3, Spencer Technologies). The data was acquired continuously using an analog to digital convertor (Powerlab 30, ADInstruments). It is calculated as the time between the peak of the QRS complex on an electrocardiogram (ECG) and the peak of pulse velocity in the middle cerebral artery (MCA) divided by the pulse transit time of those two points. hmPWV is a novel technique for measuring cerebral arterial stiffness, so a clinically meaningful change in this parameter has not been established. Methods to assess cerebral arterial stiffness are new, with one of the first clinical trials to establish cerebrovascular disease risk being registered within the past year.\textsuperscript{119}

MCA cerebral blood flow (CBF) was measured using Transcranial Doppler (ST3, Spencer Technologies). The data was acquired continuously using an analog to digital convertor (Powerlab 30, ADInstruments). An average value for blood flow velocity through the middle cerebral artery (MCA BFV) was used to assess changes in CBF continuously in both CUFF and
CONTROL conditions. For CCA CBF, pre and post measurements were taken using Doppler ultrasound, which was used to record CCA CBF (LOGIQ P6; GE Healthcare, Wauwatosa, USA) in both the CUFF and CONTROL conditions. At each measurement time, three (3) ten second videos of the CCA were recorded. The videos were processed using an imaging software (FMD Studio®, QUIPU, Italy) that generated data for both volumetric blood flow and shear rate on the arterial wall. Continuous results were compared over the duration of the sitting or supine period, and pre and post measurements were compared among time points and conditions (CUFF or CONTROL) in the SIT or LAY trials (4 measurements total per participant).

Functional Near-Infrared Spectroscopy (fNIRS) was used to monitor regional changes in blood flow. fNIRS works by monitoring changes in oxygen metabolism. fNIRS can detect hemoglobin changes in the cerebral vasculature specifically, and can be used to determine cerebral perfusion rates for oxygen at different regions in the brain. Cerebral perfusion rates allow insight into healthy or impaired neurovascular coupling. A healthy brain can send a greater flow of oxygen-rich blood to active areas of the brain. To use fNIRS, sensors are placed on the surfaces of the scalp. In this experiment, the frontal lobe is an area of interest, particularly during cognitive testing, as it is responsible for most of the executive function in the brain. To analyze the frontal lobe, sensors were placed just below or on the hairline.

**SECONDARY OUTCOME: EXECUTIVE FUNCTION & COGNITIVE LOAD**

Executive function (EF) was tested through the use of the Trail Making Test (TMT) and the Verbal Fluency Test (VFT). The Trail-Making Test was divided into two parts: Part A and Part B. Only Part B was used in this study, as it is a better indicator of executive function.
Part B, participants were presented with numbers and letters placed semi-randomly. The participant would then connect 25 numbers and numbers, alternating between numbers and letters in numerical or alphabetical order. For example, a participant would connect “1” to “A”, then connect “A” to “2”, and “2” to “B” until 25 symbols have been connected. Results from Part B were reported in “seconds to completion.”

The VFT includes 2 parts as well: a test of semantic fluency and a test of phonemic fluency. For semantic fluency, participants were given 1 minute to name as many items as fit in a given category. For phonemic fluency, participants are given a letter, and must have stated as many words as they can that start with that letter in 1 minute. Both sections were scored in correct number of words stated. Times for the TMT were recorded by the software used and recorded to data sheets during testing, but VFT words were recorded during testing and analyzed after testing. The results of the VFT and the TMT were combined to make a value of EF.

During both tests, relative cognitive load can be assessed with the use of eye-tracking goggles. Number of saccades and blink rate were tracked to determine the relative cognitive load of each test. Both the use of saccade counts and blink rate are validated methods to determine relative cognitive load of participants.101,102 Changes in cognitive load indicators during the cognitive tests could indicate a reduced ability to direct blood to active areas of the brain, and impaired NVC.
INDEPENDENT VARIABLE: BLOOD POOLING

Multiple methods were used to determine blood pooling. Calf circumference is a simple method to assess venous pooling in the calves, and NIRS offers continuous assessment via monitoring of hemoglobin concentration. Calf circumference measurements assess venous distension due to blood pooling. The measurement is based on the assumption that the volume of the calf tissue changed proportionally to the volume of blood pooling within the calf, and that the volume of calf tissue change was proportional to the change in calf circumference. Calf circumference was measured by assessing the total circumference change in the lower limb at the mid-gastrocnemius, which is often the widest part of the calf. A measuring tape was used to determine circumference, and the value recorded. After the baseline measurement, a marker was used to mark the measurement site. This was to allow exact replication for the measurement after sitting. Results were compared pre and post in the SIT or LAY trials, and for both the CUFF and CONTROL conditions (4 measurements total per participant).

Near-Infrared Spectroscopy (NIRS) was used on the calf to determine oxygen metabolism rates. The NIRS instrument (Portalite, Artinis) uses non-ionizing light to detect oxygenated and deoxygenated hemoglobin in the vasculature, and can be used to determine perfusion rates for oxygen. The measurement of NIRS on the calf allowed us to determine venous pooling in the calf. Venous pooling can be observed when there is an increase in total hemoglobin in the tissue. Over time, a greater portion of the hemoglobin becomes deoxygenated hemoglobin, indicating that the tissue is absorbing oxygen, but not enough blood is leaving the tissue to carry away all the deoxygenated hemoglobin.
MECHANISTIC OUTCOME: STROKE VOLUME AND CARDIAC OUTPUT

A central factor of the proposed mechanism is a change in cardiac output. This can be detected non-invasively using the Finapres®, which can determine heart rate, stroke volume, and cardiac output using the Model method.105 Results can be acquired continuously, and can be measured throughout each testing session. Comparison of cardiac output over time helps to establish cardiac output as one of the changes that connect blood pooling in the calves with arterial stiffening changes in the cerebral arteries. While the Finapres® is validated and reliable for blood pressure monitoring,123 less work has been done to determine the validity and reliability of the device for testing cardiac output.

Furthermore, stroke volume, and therefore cardiac output, can be measured using Doppler ultrasound as well. Cardiac output monitoring using the USCOM 1 is a validated, non-invasive method for measuring cardiac output.104 In this method, a Doppler ultrasound probe is placed on the chest, anterior to the aortic valve. The USCOM 1 software uses the detected flow velocity and cross-sectional area of the aortic valve to create a value for cardiac output. However, this method cannot be used continuously, and so was used during pre and post measurements for each condition in each crossover trial.

RANDOMIZATION

Randomization for the experiment was performed using a public website (www.randomizer.org), which was used to assign groups to the Control or Cuff session first. The randomization protocol was performed by a member of the research team who was not a principal investigator. Due to the nature of the experiment, it was impossible to blind the
participant or the researcher to the ongoing experimental protocol. A research assistant would inform the principal investigator which condition is to be completed immediately prior to testing.

**SAMPLE SIZE**

Sample size calculations were performed using G*Power 3.1 (Heinrich-Heine-Universität Düsseldorf, Germany). A conservative estimate for the primary outcome, hmPWV, using an α-level of 0.05, effect size of 0.25 (moderate effect), and power (1-β) of 0.8, generated a minimum sample size of 12 participants. For the SIT trial, the number was inflated to 16 to account for potential data quality problems and loss-to-follow up while retaining adequate power.

**QUALITY CONTROL**

For a given outcome all measurement and analysis was conducted by a single observer. At the start of the study, the first three data sets were checked by an independent observer. A quality grading score was part of this over-reading so that protocol deviations could be detected.

The TCD measurement of pulse wave velocity is continuous, and a minimum of 20 seconds of data was collected at each measurement interval before and after treatments. During this time, care was taken to ensure minimal noise in the collected data. Similarly, all data for primary outcomes was assessed in triplicate to reduce intra-observer error.

At the conclusion of the study, a random selection of 10% of the data sets (e.g., all data from 3 participants) were re-scored by an independent observer and used to calculate inter-observer reliability.
DATA MANAGEMENT AND STATISTICAL ANALYSIS

During testing, data collection, and data analysis, participant information was tied to a unique identifier pertaining to the experiment and was anonymized to the individual participant. The identifier used the related experiment code and participant number (e.g. First participant would be VP01, etc.). Data management and analysis was completed using multiple software programs. Raw data collection was performed using Powerlab. Microsoft Excel (Excel, IN., Redmond, WA, USA) was used for data aggregation and organization. Analyses were performed using Jamovi v0.9. The \( \alpha \)-level for the data was set a priori at \( \alpha=0.05 \). Effect sizes were calculated using Cohen’s \( d \), with 0-0.19 considered a trivial effect, 0.20-0.50 considered a small effect, 0.50-0.8 considered a moderate effect, and above 0.80 as a large effect. Missing data was filled in using mean substitution where appropriate or discarded.

A mixed linear model was used to compare participants between experimental conditions and between time points. This method is well-suited for accommodating variance among multiple variables into a relationship and accounting for their effects. It can be used to assess relationships between variables while also adjusting for fixed or random effects. The mixed linear model can also help to show which variables have a moderating effect in a relationship. In this experiment, all measures of PWV were adjusted by blood pressure and age, which have been identified as a mediators.\textsuperscript{124}
CHAPTER IV: RESULTS

PARTICIPANTS

Recruitment and testing of 6 participants in the SIT trial took place between February and April 2021. Out of the 6 participants recruited, 1 dropped out due to a vasovagal reaction (Figure 7). Five participants (23.6 ± 5.3 years, 40% female, 23.1 ± 3.2 kg/m²) completed all experimental conditions in this trial.
Recruitment and testing of 6 participants in the LAY trial took place between January and March 2021. All 6 participants (26.5 ± 7.6 years, 4 females, 22.5 ± 2.3 kg/m²) completed all experimental conditions (Figure 7).
HARMS

One participant in the SIT trial had a vasovagal reaction while undergoing the CUFF condition in the SIT trial. The participant was dropped from the study.

MANIPULATED OUTCOME: BLOOD POOLING

Blood pooling, as measured by calf circumference, had a significant interaction effect between time and condition ($\beta = -1.26$, 95% CI: -1.50 to -1.02, ES = 2.27; Figure 8). In the Non-Cuff condition, calf circumference increased by 0.25% ($\beta = 0.088$, 95% CI: -0.08 to 0.26, ES = 0.19), whereas it increased by 3.69% in the Cuff condition ($\beta = 1.34$, 95% CI: 1.15 to 1.51, ES = 3.12).

![Figure 8. SIT trial calf circumference. Calf circumference is in units of centimeters.](image-url)
Data from NIRS showed no significant interaction (p = 0.733), time, (p = 0.691) or condition effect (p = 0.230) in tHb. HHb had a nonsignificant interaction effect (p = 0.463) and nonsignificant time effect (p = 0.839), but did have a significant condition effect (β = -18.35, 95% CI: -33.07 to -3.634, ES = 0.43, p = 0.023).

**LAY TRIAL**

In the LAY trial, there was a nonsignificant interaction (p = 0.279) and time effect (p = 0.143) and nonsignificant condition effect (p = 0.423) for calf circumference (Supplemental Figure 1).

Using NIRS, there was a nonsignificant interaction (p = 0.643) and time effect (p = 0.576) and a nonsignificant condition effect (p = 0.330) for tHb. HHb was found to have a nonsignificant interaction (p = 0.279) and time effect (p = 0.831) but did have a significant condition effect (β = -12.72, 95% CI: -21.58 to -3.854, ES = 0.65, p = 0.010).

**PRIMARY OUTCOME: Heart-MCA PWV (hmPWV)**

**SIT TRIAL**

In both the Cuff and Non-Cuff conditions, hmPWV sharply decreased over time. There was a nonsignificant interaction effect (p = 0.856), and a large, significant main effect for time (β = -97.17, 95% CI: -125.1 to -69.28, ES = 1.55) as well as a non-significant main effect for condition (p = 0.636; Figure 9). Over time, hmPWV decreased by 62.97%.
In both the Cuff and Non-Cuff conditions, hmPWV decreased over time. There was a nonsignificant interaction effect (\( p = 0.177 \)), and a moderate, significant main effect for time (\( \beta = -64.55, 95\% \text{ CI: } -110.9 \text{ to } -18.24, \text{ ES} = 0.39 \)) as well as a non-significant main effect for condition (\( p = 0.670; \) Figure 10). Over time, hmPWV decreased by 42.36%.

**Figure 9.** hmPWV results in the SIT trial. hmPWV, heart-middle cerebral artery pulse wave velocity (units of cm/s), presented as means with standard error.
Figure 10. hmPWV results in the LAY trial. hmPWV, heart-middle cerebral artery pulse wave velocity (units of cm/s), presented as means with standard error.

SECONDARY OUTCOME: Common Carotid Artery Cerebral Blood Flow (CCA-CBF)

SIT TRIAL

There was a large, significant interaction effect between time and condition ($\beta = 35.91$, 95% CI: 4.612 to 67.21, ES = 1.41; Figure 11) for CCA-CBF. Between conditions, CCA-CBF decreased 3.71% in the Cuff condition ($\beta = -5.68$, 95% CI: -29.75 to 18.4, ES = 0.14), and
increased 19.50% in the Non-Cuff condition ($\beta = 30.23$, 95% CI: 6.16 to 54.3, ES = 0.76).

**Figure 11.** CCA-CBF results in the SIT Trial. BFMean, equal to CCA-CBF (units of ml/min), presented as means with standard error.

### LAY TRIAL

There was not a significant interaction ($p = 0.962$), time ($p = 0.681$), nor condition effect ($p = 0.891$) for CCA-CBF (Supplemental Figure 2).

### SECONDARY OUTCOME: EXECUTIVE FUNCTION TESTING AND EYE-TRACKING

#### SIT TRIAL

For each trial, cognitive tests were performed at the end of the familiarization visit for a baseline reading, and at the end of each experimental visit to obtain scores. For the Trail-Making Test (TMT), there were no significant differences between conditions (Base and Cuff, $p = 0.652$; Base and Non-Cuff, $p = 0.741$; **Figure 12**). However, the Verbal Fluency Test (VFT) showed a significant difference between the Base and the Non-Cuff condition ($\beta = 6.250$, 95% CI: 1.533 to

**Cuff:** $\beta = -5.68$, 95% CI: -29.75 to 18.4  
**Non-Cuff:** $\beta = 30.23$, 95% CI: 6.16 to 54.3
10.97, ES = 0.62, p = 0.041). Between the Base and Non-Cuff condition, this was a 15.78% increase. There was no significant difference between the Base and Cuff condition scores for the VFT (p = 0.842).

Figure 12. Trail-Making Test (TMT) scores across conditions, presented as means with standard error.

Figure 13. Verbal Fluency Test (VFT) scores across conditions, presented as means with standard error.

During each cognitive test, blink rate and pupil diameter were measured as well. However, no significant differences in blink rate or pupil diameter were observed across conditions for any test (Tables 6 and 7).
Table 6. Statistical data for blink rate measures in the SIT trial.

<table>
<thead>
<tr>
<th></th>
<th>Base to Cuff</th>
<th>Base to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trail-Making Test</strong></td>
<td>p 0.28</td>
<td>0.275</td>
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<tr>
<td><strong>Verbal Fluency Test - Part 1</strong></td>
<td>β 2.41</td>
<td>2.44</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>p 0.356</td>
<td>0.767</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>β 6.4</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 7. Statistical data for pupil diameter measures in the SIT trial.

<table>
<thead>
<tr>
<th></th>
<th>Base to Cuff</th>
<th>Base to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trail-Making Test</strong></td>
<td>p 0.998</td>
<td>0.566</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 1</strong></td>
<td>β 0.006</td>
<td>-1.631</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>p 0.996</td>
<td>0.720</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>β 0.054</td>
<td>-3.943</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>p 0.825</td>
<td>0.962</td>
</tr>
<tr>
<td><strong>Verbal Fluency Test - Part 2</strong></td>
<td>β 2.410</td>
<td>-1.080</td>
</tr>
</tbody>
</table>

LAY TRIAL

For the TMT, there were no significant differences between conditions at the \( p \geq 0.05 \) level (Base and Cuff, \( p = 0.138 \); Base and Non-Cuff, \( p = 0.098 \); Supplemental Figure 3). There were also no significant differences between conditions of the VFT at the \( p \geq 0.05 \) level (Base and Cuff, \( p = 0.138 \); Base and Non-Cuff, \( p = 0.098 \); Supplemental Figure 4).

In terms of eye-tracking, no significant differences were detected in blink rate across any of the cognitive tests. There was a large, significant difference in pupil diameter between the Base and Cuff condition (\( \beta = 10.82 \), 95% CI: 5.60 to 16.03, ES = 0.91, \( p = 0.011 \)) during the second half of the VFT, which was a 42.19% increase from the Base condition to Non-Cuff.
There was no significant difference between the Base and Non-Cuff condition *(Supplemental Figure 5).*

**MECHANISTIC OUTCOME: fNIRS**

**SIT TRIAL**

To assess changes in hemoglobin content across the frontal lobe during the experimental condition, changes in tHb and TSI% were assessed. There was a nonsignificant interaction (\(p = 0.448\)) time (\(p = 0.107\)), and condition effect (\(p = 0.553\)) for tHb *(Supplemental Figure 6).* Similarly, there was a nonsignificant interaction (\(p = 0.379\)), time (\(p = 0.418\)), and condition effect (\(p = 0.802\)) for TSI%.

**LAY TRIAL**

There were nonsignificant interaction (\(p = 0.576\)) and time effects (\(p = 0.563\)), but there was a moderate, significant condition effect on tHb (\(\beta = 3.005, \text{95\% CI: 0.192 to 5.818, ES = 0.72, } p = 0.045; \text{Supplemental Figure 7.}\)) For TSI%, there were nonsignificant interaction (\(p = 0.340\)) and time effects (\(p = 0.268\)) for TSI%, and a nonsignificant condition effect (\(p = 0.051\)).

**MECHANISTIC OUTCOME: CARDIAC OUTPUT**

**SIT TRIAL**

Cardiac output, as measured by the USCOM device, had an interaction effect that trended toward significance, but did not quite reach the \(p \leq 0.05\) level (\(p = 0.057, \text{ES = 0.34}\)). There was a large time effect (\(\beta = -0.395, \text{95\% CI: -0.622 to -0.168, ES = 0.76; Figure 13}\)) and a nonsignificant condition effect (\(p = 0.180\)). Over time, cardiac output decreased by 8.01%.
Figure 14. Cardiac Output results from the SIT trial, presented as means with standard error. CO, cardiac output (units of L/min).

**LAY TRIAL**

There was a nonsignificant interaction effect ($p = 0.146$), a moderate, significant time effect ($\beta = -0.475, 95\% CI: -0.766 \text{ to } -0.173, ES = 0.44$), and a nonsignificant condition effect ($p = 0.199$; **Figure 14**). Over time, cardiac output decreased by 6.13%.
MECHANISTIC OUTCOME: STROKE VOLUME

SIT TRIAL

Stroke volume, a component of cardiac output, was measured by the USCOM device.

There was no significant interaction (p = 0.293), time (p = 0.981), or condition effect (p = 0.677).

LAY TRIAL

There were no significant interaction (p = 0.903), time (p = 0.981), or condition effects (p = 0.677) for stroke volume in the LAY trial.

Figure 15. Cardiac Output results from the LAY trial, presented as means with standard error. CO, cardiac output (units of L/min).
MECHANISTIC OUTCOME: HEART RATE

SIT TRIAL

Heart rate, a component of cardiac output, had a significant interaction effect between time and condition ($\beta = -10.06$, 95% CI: -17.41 to -2.70, ES = 0.58; Figure 15). In the Cuff condition, heart rate decreased by only 4.46% ($\beta = -3.00$, 95% CI: -8.51 to 2.51, ES = 0.24), whereas in the Non-Cuff condition, it decreased by 18.93% ($\beta = -13.06$, 95% CI: -18.92 to -7.19, ES = 0.97).

Cuff: $\beta = -3.00$, 95% CI: -8.51 to 2.51
Non-Cuff: $\beta = -13.06$, 95% CI: -18.92 to -7.19

Figure 16. Heart Rate results from the SIT trial, presented as means with standard error. HR, heart rate (units of beats/min).

LAY TRIAL

Heart rate, a component of cardiac output, did not have significant interaction ($p = 0.060$), time ($p = 0.115$), nor condition effects ($p = 0.112$; Supplemental Figure 8).
CHAPTER V: DISCUSSION

The primary aim of this study was to determine the role of venous pooling on cerebral arterial stiffening response during sitting. In the SIT trial, venous pooling was observed, particularly in the Cuff condition, which aligned with the hypothesis. There was a sharp decrease in hmPWV across all conditions in both trials, which ran counter to the hypothesis. Following that, secondary aims were to assess hemodynamic changes via CBF and functional changes in cognition that may accompany the arterial stiffening response. The Cuff condition was related to a significant decrease in CCA-CBF relative to the Non-Cuff condition. In addition, we found significant differences in scores for the VFT in the Cuff condition relative to the Non-Cuff condition in the SIT trial. Lastly, CO did change significantly with venous pooling, but appears to have been driven by changes in heart rate, rather than changes in stroke volume, as hypothesized. The main findings for the study were that hmPWV decreased over time, CCA-CBF decreased with venous pooling, and a decreased CO did occur.

LIMITATIONS AND STRENGTHS

The limitations and strengths of the current study should be considered in evaluating the results. First, both trials (SIT and LAY) were only powered to detect changes in hmPWV. Some results that were close to, but not statistically significant at the p $\geq 0.05$ level may have been affected, and further research with a greater sample size may detect different results. Second, the generalizability of this study may be limited due to a relatively homogenous population of young, healthy adults. With that in mind, this was deliberately chosen to minimize confounding
due to factors such as age and cardiometabolic disease. Third, there may have been a learning effect for both the TMT and VFT for executive function. While the order of conditions was randomized, the same type of test was used at each visit for consistency and could have influenced the results.

Despite some limitations, this study has several strengths as well, such as novelty. This study is one of the first to use hmPWV to assess cerebral arterial stiffening. The use of hmPWV will provide a new measure to study the cerebral arteries non-invasively. Furthermore, several potential confounders were controlled for in this study, such as sleep, diet, and physical activity, that give this study robust internal validity. Lastly, the variety of measures used in data collection help show the multitude of physiological changes occurring in response to blood pooling at multiple timepoints in each condition.

<table>
<thead>
<tr>
<th>COMPARISON TO LITERATURE</th>
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<tr>
<td>hmPWV</td>
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Pulse wave velocity (PWV) is the gold standard non-invasive method for measuring arterial stiffness.\textsuperscript{125,126} While PWV techniques to measure central and peripheral arterial stiffness are common in the literature, techniques to study stiffness in the cerebral arteries are fairly novel, and currently being explored. In this study, hmPWV, which measures stiffness of the arterial segment between the aorta and the middle cerebral artery, decreased over all conditions, but had a relatively greater decrease while participants were seated rather than laying (Time effects: SIT: ES = 1.55; LAY: ES = 0.39). While there is sparse evidence for comparable results available, one study, using one of the closest examples of PWV techniques for evaluating cerebral arterial stiffness called carotid-cerebral PWV (ccPWV), noted that patients with cerebral arterial atherosclerosis (likely indicative of stiffer arteries) had a higher mean ccPWV value than those without.\textsuperscript{127} The responsiveness of hmPWV to different postural conditions in this experiment
may indicate that it is useful for future studies that seek to monitor differences in cerebral hemodynamic function.

In contrast to these results, one of the few other studies to use hmPWV (Blackwell et al. 2020), found a non-significant change in hmPWV in middle age adults after a 2 hour sitting bout. While the conditions between this study’s Non-Cuff condition and the sitting bout in Blackwell et al. were comparable, the study sample was different, as Blackwell et al. focused on middle-aged adults. The results could indicate that age is a factor that influences hemodynamic responses during sedentary behavior.

### Executive Function

Results for a change in executive function were mixed. The only significant difference seen was between Base and Control conditions for the VFT in the SIT trial, and there were no significant differences seen in the TMT. It is possible these results show a potential learning effect for the VFT, that was eliminated in the Cuff condition. This would indicate that the Cuff condition (increased venous pooling) played a role in reducing an increase, that is, reduced scores relative to the Non-Cuff (control) condition. One study, using an attentional cognitive task assessment (SART test), showed a decrease in score associated with an increase in calf circumference (indicating venous pooling) after a bout of prolonged sitting, which agrees with these results. Furthermore, these results could have been related to the decrease in CCA-CBF that occurred in the Cuff condition of the SIT trial in this study.

The proposed mechanism for this experiment stated that an increase in venous pooling would cause a decrease in cardiac output by way of decreased stroke volume, which would then increase hmPWV. While the results of this experiment did not show a relationship between
venous pooling and hmPWV, there was a drop in cardiac output with increased venous pooling. However, these changes appeared to be driven by changes in heart rate, rather than changes in stroke volume. While decreasing cardiac output has been reported in other studies that assess prolonged sitting, the mechanism for these changes is different from the proposed mechanism.

A previous study (Stoner et al.) has shown no significant difference in a test of executive function (Stroop task) over the course of a sitting bout. While these studies differ in test methodology (Stroop vs. Trail-Making Test and Verbal Fluency Test), all three tests are valid in assessing different aspects of executive function. In particular, the TMT and Stroop tests rely on visual stimuli, whereas the VFT relies on auditory stimuli, which could have contributed to some of the observed differences. In our study and Stoner et al., there were minimal differences, as both studies used standardized sets of instructions was used at each test for consistency. However, both studies used healthy, young adults as a study population. There may have been minimal significant differences between conditions due to insufficient challenge to participants in the form of a ceiling effect. In normative data of participants performing the TMT, participants between the ages of 18-54 had faster scores than participants older than 54, followed by a generally linear increase in completion time with age. While the TMT is useful to diagnose dementias in older adults, participants younger than 54 may have completion times too low to observe the same changes even in the presence of altered neurovascular hemodynamics. Future studies may need to consider more complex cognitive tasks to elicit noticeable changes in a younger population.
IMPLICATIONS

In both the SIT and LAY trials, venous pooling was increased in the respective Cuff condition. This study shows that occlusive cuffs can be used to effectively manipulate venous pooling. Increased venous pooling was not found to be related to decreases in hmPWV. However, changes in cognition as shown with the VFT, and changes in CBF, may be related to increased venous pooling. In populations such as older adults, who may be sensitive to acute reductions in CBF, strategies to acutely reduce venous pooling may help to mitigate effects on executive function in these populations. Reduced CBF can also potentially contribute to acute endothelial dysfunction, which may contribute to atherosclerosis with chronic exposure.

Furthermore, future research could investigate the interaction between central and cerebral PWV measures over time. A paper by Yu et al. on the arterial stiffness gradient has shown that a combination of increasing central PWV and slight to no increases in peripheral measures of PWV (such as femoral-ankle PWV) in a sitting bout may be a useful indicator for pathogenic conditions. Increasing central PWV would indicate that pressure waves during systole are transferred farther, to more distal arteries and arterioles, exposing these blood vessels to greater pressure waves. If this phenomenon applies to the cerebral arteries, then an increasing central PWV combined with a decreased hmPWV as seen in this study could expose the cerebral arteries to high pressures waves. Exposure to higher pressure waves can be pathogenic to cerebral arteries. Since hmPWV decreased over time in this study, future studies could investigate potential PWV gradients between central and cerebral PWV measures over a sitting bout.
Table 8. Knowledge Summary

<table>
<thead>
<tr>
<th>What did we know?</th>
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<tbody>
<tr>
<td>• Cerebral arterial stiffening has been reported with exposure to prolonged sitting.</td>
</tr>
<tr>
<td>• Acute arterial stiffening exposes small arteries and arterioles to greater pressure waves,</td>
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<tr>
<td>which can be potentially damaging.</td>
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<table>
<thead>
<tr>
<th>What did we not know?</th>
</tr>
</thead>
<tbody>
<tr>
<td>• The role of venous pooling on cerebral hemodynamic parameters such as arterial stiffening are not understood.</td>
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</tbody>
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<table>
<thead>
<tr>
<th>What have we learned?</th>
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<tbody>
<tr>
<td>• Cerebral artery stiffness at the middle cerebral artery does not seem to be related to venous pooling, but does seem to be related to changes in cardiac output.</td>
</tr>
<tr>
<td>• Prolonged sitting, with additional venous pooling, seems to affect verbal executive function, which may be related to changes in CBF.</td>
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<tr>
<th>Why is this new information useful?</th>
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<tbody>
<tr>
<td>• Venous pooling could be a factor that decreases in CCA-CBF and verbal executive function, which means interruptions to prolonged sitting that focus on reducing venous pooling may help to preserve blood flow.</td>
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<tr>
<th>What do we need to know next?</th>
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<tbody>
<tr>
<td>• More information is needed on the aspects of prolonged sitting that affect cerebral arterial stiffness.</td>
</tr>
<tr>
<td>• Future research could investigate the potential for relationships between cerebral arterial stiffness and central arterial stiffness.</td>
</tr>
<tr>
<td>• Strategies to reduce venous pooling, and therefore reduce acute decreases in executive function and CBF, should be investigated for efficacy.</td>
</tr>
<tr>
<td>• The findings in this study are likely generalizable to young, healthy adults, but can point to future research in specific populations at risk for acute changes in CCA-CBF (older adults, cardio-metabolically diseased, etc.).</td>
</tr>
</tbody>
</table>

CONCLUSIONS

This study aimed to assess the role of venous pooling on cerebral hemodynamic status, which has been hypothesized to contribute to hemodynamic changes throughout the body, including the cerebral vasculature. To do this, we observed changes in hmPWV, CCA-CBF, and hemodynamic measures such as cardiac output to determine a potential mechanism for changes during a bout of prolonged sitting or laying. In doing so, we found that hmPWV may be a useful measure for looking at cerebral arterial stiffness. Separately, the VFT indicated potential cognitive changes related to venous pooling, that may be explained by a simultaneous decrease
in CCA-CBF in the Cuff condition of the SIT trial. These data indicate that our proposed mechanism is incomplete, and more study is needed regarding the relationship between prolonged sitting and cerebral arterial stiffness.
APPENDIX A. CONSENT

University of North Carolina at Chapel Hill
Consent to Participate in a Research Study
Adult Participants

Consent Form Version Date: __________
IRB Study # 20-3403
Title of Study: The Role Of Blood Pooling In The Legs During Prolonged Sitting On Cardiovascular And Cerebrovascular Outcomes: A Mechanistic Study
Principal Investigator: Alexander Pomeroy
Principal Investigator Department: Exercise and Sport Science
Principal Investigator Phone number: (919) 962-0396
Principal Investigator Email Address: alexpom@live.unc.edu
Faculty Advisor: Lee Stoner
Faculty Advisor Contact Information: (919) 962-0534

CONCISE SUMMARY

We are looking to examine the vascular, cerebrovascular, and executive function response to blood pooling in the veins of the lower limbs, a common side effect of prolonged sitting, with and without exposure to prolonged sitting itself. It is currently unknown whether (1) venous pooling has a role in systemic vascular health effects seen during prolonged sitting (2) whether venous pooling has a role in changes in blood flow to the brain and perfusion of the prefrontal cortex (3) whether venous pooling and the corresponding cerebrovascular changes affect executive function, and (4) what specific physiological pathways explain these changes. The devices used in this study are non-invasive and no known adverse events have occurred with use of the stated devices. The findings from this study may result in better understanding of how changes during prolonged sitting occur, and help elucidate potential strategies for reducing acute vascular, cerebrovascular, and cognitive dysfunction during prolonged sitting. The purpose of the study is to measure the changes in the heart, vasculature, brain perfusion and cognitive function caused by venous pooling in the legs from prolonged sitting.

We seek healthy adults 18-45 years of age, free of cardiometabolic disease, and who do not smoke nor vape. Pregnant women and those who take medications known to alter cardiovascular function are not eligible. A total time commitment of 305 min is required: the study consists of three visits, the first being a familiarization visit (45 min duration) and two experimental visits (130 min duration each).

No significant risks will occur should you take place in this study. Your participation will benefit the scientific body on the changes that occur from prolonged sitting and venous pooling. There is no benefit to you for completing this study, however, we are happy to provide a summary of your results, including blood pressure, cardiac output, arterial stiffness, carotid blood flow, and cognitive measures, in comparison to group means after the completion of the study.

What are some general things you should know about research studies?
You are being asked to take part in a research study. To join the study is voluntary.
You may choose not to participate, or you may withdraw your consent to be in the study, for any reason, without penalty.

Research studies are designed to obtain new knowledge. This new information may help people in the future. You may not receive any direct benefit from being in the research study. There also may be risks to being in research studies. Deciding not to be in the study or leaving the study before it is done will not affect your relationship with the researcher, your health care provider, or the University of North Carolina-Chapel Hill. If you are a patient with an illness, you do not have to be in the research study in order to receive health care.

Details about this study are discussed below. It is important that you understand this information so that you can make an informed choice about being in this research study.

You will be given a copy of this consent form. You should ask the researchers named above, or staff members who may assist them, any questions you have about this study at any time.

**What is the purpose of this study?**
The purpose of this research study is to (1) Explore the cardiovascular, cerebrovascular, and cognitive effects of venous pooling in the lower limbs during a bout of prolonged sitting, and (2) Explore the effects of venous pooling independently of prolonged sitting on cardiovascular function.

**Are there any reasons you should not be in this study?**
You should not be in this study if you have known cardiovascular or metabolic diseases (e.g. Congestive heart failure, peripheral artery disease, type I and II diabetes, etc.), you use tobacco or nicotine, take medications known to affect cardiovascular function (e.g. beta-blockers, ACE inhibitors) or you are pregnant.

**How many people will take part in this study?**
Approximately 22 people at UNC-Chapel Hill will take part in this study.

**How long will your part in this study last?**
Should you wish to participate in the study, you will be required to attend the Applied Physiology Laboratory at University of North Carolina at Chapel Hill on three occasions. The first visit will last approximately 45 minutes, and the CONTROL and CUFF visits last approximately 130 minutes.
**What will happen if you take part in the study?**

If you would like to take part in the study, you would be required to visit the Applied Physiology Laboratory at UNC, Chapel Hill on three occasions. See below for overall study design:

**Visit 1** - The first visit will be a familiarization session during which all experimental procedures will be described to you in full. You will provide informed consent before the study begins, then complete a brief questionnaire on your medical history to ensure you are eligible for this study. Pregnancy tests will be done on all females who might be able to get pregnant at the start of the study. The research team will pay for these pregnancy tests. If you meet the requirements, we will then show you how each device is prepared for this study, how it functions and where it will be placed on the body for data collection. At the conclusion of the visit, we will take your baseline cognitive assessment for the study. The following devices will be used for study purposes:

- Transcranial Doppler (TCD) – A headset snugly placed on top of the head
- VICORDER® – Non-invasive device using blood pressure cuffs to assess arterial health
- Ultrasound Probe – Small probe lightly placed over several arteries running up the neck to assess blood flow to the brain
- Near-Infrared Spectroscopy (NIRS) – Small probe (about 1 x 3 inches in size) placed on the calf
- Functional Near-Infrared Spectroscopy (fNIRS) – set of 8 probes (each about 2 centimeters in diameter) mounted in a neoprene headcap
- Non-invasive Blood Pressure cuff (NIBP) – Device wrapped around the wrist with small cuffs encircling the middle and index fingers
- Equivital – Chest-worn device and strap that is placed on the skin under a shirt
- USCOM – small, specialized doppler ultrasound that is pressed just above the suprasternal notch (approximately where the neck meets the sternum)
- Pupil Core – eye-tracking device worn like eyeglasses

This visit should take approximately 45 minutes.

**Visit 2 & 3** - During the experimental visits (CONTROL and CUFF), you will be required to rest quietly for a period of 10 minutes in a supine (lying) position. After, measures of cardiovascular function will be taken by the VICORDER® and Ultrasound devices. For the CUFF and CONTROL conditions, you will be asked to sit still and quietly for 2 hours while watching a non-stimulating documentary. Then, if seated, you will be passively shifted to a supine (lying) position where the VICORDER® and Ultrasound measurements will be taken again. At the end of the cardiovascular measurements in each experimental condition we will conduct a battery of cognitive tests.

The cognitive tests involved in these two visits are called the Trail-Making Test (TMT) and the Verbal Fluency Test. In the TMT, you will complete two short puzzles on an iPad with your finger. You will be presented with numbers and letters placed semi-randomly. You will then connect 25 numbers and numbers, alternating between numbers and letters in numerical or alphabetical order. For example, one would connect “1” to “A”, then connect “A” to “2”, and “2” to “B” until 25 symbols have been connected. The VFT is a verbal test that also consists of two parts. In Part A, you will be given a category and instructed to name as many items in the categories as you can within 60 seconds. In Part B, you will be given a letter and will be asked to name as many words that begin with the letter as you can within 60 seconds.
Prior to attending the Lab for visits 2 & 3, you will have to perform the following pre-assessment guidelines:

- Fasted (> 12 hours), consuming only water.
- No caffeine consumption 12 hours prior to testing
- No vigorous exercise 24 hours prior to testing.
- No alcohol consumption 24 hours prior to testing.

The total time commitment that will be required from you is approximately 305 minutes. Following the analysis of your data, we will happily provide a summary of your results in comparison to the group means.

**What are the possible benefits from being in this study?**
Research is designed to benefit society by gaining new knowledge. You will not benefit personally from being in this research study.

**What are the possible risks or discomforts involved from being in this study?**
The devices used in this study are non-invasive and there are no accounts of severe injury due to exposure to the stated devices. Physical harm due to participation in this study is likely very minimal:

**VICORDER®** - The system requires the placement of pressure cuffs over several arteries for the collection of PWV/A data. Pressure cuffs will only be inflated underneath a level of 65 mmHg.

Physical harm or discomfort is unlikely and include, but are not limited to:

- **Risk 1:** Discomfort/unease: Infrequent (1 – 10%) – Application of a slight pressure over the carotid artery may impose a sense of unease for the participant. However, the light pressure used for this experimental protocol will in no way significantly damage cardiovascular structure or place the participant in danger. Investigators will make certain that communication on the procedures during testing session are clearly conveyed to the participant for comfort and safety.

**Near-infrared spectroscopy (NIRS):** Risk of injury or discomfort is extremely low due to this device. Possible physical harms are, but not limited to:

- **Risk 1:** Eye damage/irritation: Rare (<1%) – Please do not, at any point, stare into the light emitted from the NIRS probe.
- **Risk 2:** Skin heating and irritation: Rare (<1%) – Wearing the NIRS probe for extended periods of time at once can theoretically lead to a warm feeling at the area where the probe is placed. However, this risk is minimal because the light emitted from this probe is not powerful enough to heat the skin. If you let the investigators know of any discomfort due to the probe, we will follow manufacturer guidelines to ensure the device is functioning correctly.

**Functional Near-infrared Spectroscopy (fNIRS):** Risk of injury or discomfort is extremely low due to this device. Possible physical harms are, but not limited to:

- **Risk 1:** Mild headache: Infrequent (1 – 10%) – High quality data from this device requires the placement of the probes in a snug headcap over the forehead, superficial to the prefrontal cortex. The slight pressure applied to the area may be slightly discomforting and unusual.
- **Risk 2:** Eye damage/irritation: Rare (<1%) – Please do not, at any point, stare into the light emitted from the fNIRS probe.
- **Risk 3:** Skin heating and irritation: Rare (<1%) – Wearing the fNIRS probe for extended periods of time at once can theoretically lead to a warm feeling at the area where the probe is placed. However, this risk is minimal because the light emitted from this probe is not powerful enough...
to heat the skin. If you let the investigators know of any discomfort due to the probe, we will follow manufacturer guidelines to ensure the device is functioning correctly.

**Transcranial Doppler (TCD):** Data collection from this system requires the affixation of a headpiece to the participant. Risk of injury due to this device is extremely low. Possible harms may include, but are not limited to:

**Risk 1:** Mild headache: Infrequent (1 – 10%) – High quality data from this device requires the placement of the probe over the middle cerebral artery (MCA) and posterior cerebral artery (PCA). The slight pressure applied to the area may be slightly discomforting and unusual for the participant.

There may be uncommon or previously unknown risks. You should report any problems to the researcher.

**What if we learn about new findings or information during the study?**
You will be given any new information gained during the course of the study that might affect your willingness to continue your participation.

The imaging we are using in this research study is not the same quality as imaging that you may have as part of your health care. The images will not be reviewed by a doctor who normally reads such images (such as a radiologist). As a result, you may not be informed of any unexpected findings. The results will not be placed in your medical record. Occasionally the technologist or principal investigator may notice something abnormal on the imaging. If this does occur, the images will be reviewed by a qualified doctor to determine if there is anything of clinical importance. If something is found to be important then you, and/or your primary care provider will be notified. Any further follow up and costs associated with the incidental finding will be your responsibility. There may be benefits to learning such results (such as early detection and treatment of a medical condition), but there are risks as well (such as problems with getting insurance or a job, or feeling worried about a finding for which no treatment is required or appropriate).

Do you wish to be informed in case of clinical/relevant unexpected findings? Please initial in the box below if you do not wish to be notified of clinical/relevant unexpected findings. If you do not initial in the box, you will be notified of any findings.

_____ I do not wish to be notified.

**Will I receive any other clinical results?**
There are no other clinically relevant results of this research that will be communicated with you.

**How will information about you be protected?**
The data generated from this study will be used for the purpose of scholarly publication and potentially for research presentation. Your personal data will not be identifiable.

However, there is an inherent risk for a breach of confidentiality due to the sharing of personal information with the research team for research purposes.

Breach of confidentiality will be minimized by limiting the number of research team members in the laboratory during any testing session. By needing key card access to the laboratory, we are
limiting the number of individuals not on the research team who have access to the lab. Those who do have key card access are exercise physiology professors, PhD candidates, and Master’s candidates, and selected undergraduate students who are directly associated with the study and have performed all necessary trainings regarding sample handling, laboratory procedures, and confidentiality. All participants within the study are coded with an individual ID and no names will be identified in any document besides a master key document. This master key document will be kept in a locked drawer in the Cardiometabolic Laboratory within the Applied Physiology Laboratory. Participants will not be identified in any report or publication about this study. We may use de-identified data from this study in future research without additional consent. Although every effort will be made to keep research records private, there may be times when federal or state law requires the disclosure of such records, including personal information. This is very unlikely, but if disclosure is ever required, UNC-Chapel Hill will take steps allowable by law to protect the privacy of personal information. In some cases, your information in this research study could be reviewed by representatives of the University, research sponsors, or government agencies (for example, the FDA) for purposes such as quality control or safety. Recordings will be taken of verbal responses during the cognitive assessment to clarify scores for data analysis. Once data is successfully recorded into the data spreadsheet, the audio recording will be destroyed. Audio recording files will be kept less than 1 week in total. Audio recordings are not required, but will help researchers to better record cognitive data. Recordings of the space in front of you will be made using Pupil Core. These videos may include parts of your body such as your hands, if held up close to the face. These videos are a requirement of the study. Check the line that best matches your choice:

_____ OK to record me during the study

_____ Not OK to record me during the study

What will happen if you are injured by this research?
All research involves a chance that something bad might happen to you. If you are hurt, become sick, or develop a reaction from something that was done as part of this study, the researcher will help you get medical care, but the University of North Carolina at Chapel Hill has not set aside funds to pay you for any such injuries, illnesses or reactions, or for the related medical care. Any costs for medical expenses will be billed to you or your insurance company. You may be responsible for any co-payments and your insurance may not cover the costs of study related injuries. If you think you have been injured from taking part in this study, call the Principal Investigator at the phone number provided on this consent form. They will let you know what you should do. By signing this form, you do not give up your right to seek payment or other rights if you are harmed as a result of being in this study.

What if you want to stop before your part in the study is complete?
You can withdraw from this study at any time, without penalty. The investigators also have the right to stop your participation at any time. This could be because you have had an unexpected reaction, or have failed to follow instructions, or because the entire study has been stopped. If you withdraw or are withdrawn from this study all data collected up until the point of
withdrawal will be retained, however no additional information will be collected unless you provide additional written permission for further data collection at the time of your withdrawal.  

**Will you receive anything for being in this study?**  
You will not receive anything for taking part in this study.

**Will it cost you anything to be in this study?**  
It will not cost you anything to be in this study.  

**What if you are a UNC student?**  
You may choose not to be in the study or to stop being in the study before it is over at any time. This will not affect your class standing or grades at UNC-Chapel Hill. You will not be offered or receive any special consideration if you take part in this research.  

**What if you are a UNC employee?**  
Taking part in this research is not a part of your University duties, and refusing will not affect your job. You will not be offered or receive any special job-related consideration if you take part in this research.  

**What if you have questions about this study?**  
You have the right to ask, and have answered, any questions you may have about this research. If you have questions about the study (including payments), complaints, concerns, or if a research-related injury occurs, you should contact the researchers listed on the first page of this form.

**What if you have questions about your rights as a research participant?**  
All research on human volunteers is reviewed by a committee that works to protect your rights and welfare. If you have questions or concerns about your rights as a research subject, or if you would like to obtain information or offer input, you may contact the Institutional Review Board at 919-966-3113 or by email to IRB_subjects@unc.edu.

**Participant’s Agreement:**  
I have read the information provided above. I have asked all the questions I have at this time. I voluntarily agree to participate in this research study.

______________________________________________________  
Signature of Research Participant

______________________________________________________  
Date

______________________________________________________  
Printed Name of Research Participant

______________________________________________________  
Signature of Research Team Member Obtaining Consent

______________________________________________________  
Date

______________________________________________________  
Printed Name of Research Team Member Obtaining Consent
Signature of Witness if applicable; e.g. literacy issues, visually impaired, physically unable to sign, witness/interpreter for non-English speaking participants using the short form)

Date

Printed Name of Witness
APPENDIX B. SUBJECT PRE-ASSESSMENT INSTRUCTIONS

Hello,

This is a reminder email of your appointment at the Cardiometabolic Lab for “The Role of Blood Pooling In The Legs During Prolonged Sitting On Cardiovascular and Cerebrovascular Outcomes: A Mechanistic Study,” on _____ at_____. The following pre-visit criteria must be met prior to your familiarization/experimental visit:

For familiarization visits (Visit 1):

- Abstain from alcohol 12 hours prior
- Abstain from caffeine 12 hours prior
- Wear comfortable clothes to the visit, such that your calves can be exposed easily
- If applicable, wear a sports bra for comfort and assess for ECG and skin temperature readings

The familiarization visit is expected to last approximately 45 minutes.

Please fill out the COVID-19 screening survey prior to each visit, either by scanning the QR code on the lab door upon arrival or filling out this link here: https://unc.az1.qualtrics.com/jfe/form/SV_9FxzSKmV2ezrU69

The Cardiometabolic Lab is part of the Applied Physiology Lab, located in the basement of Fetzer Hall. From the front entrance, go straight past Gym A and down the stairs on the right. From the bottom of the stairs, move down the hall in the direction of the Student Recreation Center (SRC). Before moving up the ramp to the SRC, turn left and look for the sign for Applied Physiology Lab on the right. Make this right turn and you will find a small lobby to the lab on the left, where you will be greeted by a member of the Cardiometabolic Lab.

If you have trouble finding the location, please call or text Katie at 919-360-7515 or Alex at 585-545-5563 and we will gladly assist you!
APPENDIX C. SUPPLEMENTAL DATA

INTRODUCTION TO SUPPLEMENT

This supplement contains data from both trials performed for further observation. The LAY trial was an exploratory trial, and therefore figures from this trial not depicting data directly related to the hypotheses were moved to the figure supplement for brevity in Chapters III and IV.
Supplemental Figure 1. LAY trial calf circumference by condition, presented as means with standard error. Calf circumference is in units of cm.

Abbreviations: Pre, experimental timepoint before prolonged sitting; Post, experimental timepoint after 120 minutes of prolonged sitting; cuff, occlusive cuff condition; non-cuff, control condition.

Interpretation: There were no significant interaction, time, or condition effects for calf circumference over the course of the LAY trial.
Supplemental Figure 2. CCA-CBF in the LAY trial, presented as means with standard error (units of ml/min).

Abbreviations: BFMean, equivalent to CCA-CBF; 0, experimental timepoint before prolonged sitting; 120, experimental timepoint after 120 minutes of prolonged sitting; cuff, occlusive cuff condition; non-cuff, control condition.

Interpretation: There were no significant interaction, time, or condition effects for CCA-CBF over the course of the LAY trial.
Supplemental Figure 3. Trail-Making Test results in the LAY trial.

**Abbreviations:** Base, initial measurement taken immediately after familiarization; cuff, occlusive cuff condition; non-cuff, control condition.

**Interpretation:** There were no significant differences between results from the Trail-Making Test (TMT) over the course of the LAY trial.
Supplemental Figure 4. Verbal Fluency Test in the LAY trial.

**Abbreviations:** Base, initial measurement taken immediately after familiarization; cuff, occlusive cuff condition; non-cuff, control condition.

**Interpretation:** There were no significant differences between results from the Verbal Fluency Test (TMT) over the course of the LAY trial.
Supplemental Figure 5. Pupil diameter during LAY Verbal Fluency Test part 2 (VFT2), presented as means with standard error.

Abbreviations: Base, initial measurement taken immediately after familiarization; cuff, occlusive cuff condition; non-cuff, control condition.

Interpretation: Pupil diameter significantly increased between the base and cuff conditions in the LAY trial. Greater pupil diameter could be related to a higher relative cognitive load, that is, increased requirement for resources in the brain relative to task difficulty.
Supplemental Figure 6. fNIRS results in the SIT trial, presented as means with standard error.

**Abbreviations:** tHb, total hemoglobin; TSI%, tissue saturation index; 0-10, change between baseline and 10 minutes which was used as baseline measure; 0-120, change between baseline and 120 minutes; 0-60, change between baseline and 60 minutes; cuff, occlusive cuff condition; non-cuff, control condition.

**Interpretation:** There were no significant interaction, time, or condition effects for tHB over the SIT trial. There were also no significant interaction, time, or condition effects for TSI% in the SIT trial.

Supplemental Figure 7. fNIRS results in the LAY trial, presented as means with standard error.

**Abbreviations:** tHb, total hemoglobin; TSI%, tissue saturation index; 10, change between baseline and 10 minutes which was used as baseline measure; 60, change between baseline and 60 minutes; 120, change between baseline and 120 minutes; cuff, occlusive cuff condition; non-cuff, control condition.

**Interpretation:** There were no significant interaction or time effects for tHb in the LAY trial, but there was a significant condition effect ($\beta = 3.005, 95\% \text{CI}: 0.192$ to $5.818$, ES = 0.72, $p = 0.045$) for tHB over the LAY trial. However, there were no significant interaction, time or condition effects for TSI% in the LAY trial.
Supplemental Figure 8. Heart rate data from the LAY trial, presented as means with standard error.

Abbreviations: HR, heart rate; Pre, experimental timepoint before prolonged sitting; Post, experimental timepoint after 120 minutes of prolonged sitting; cuff, occlusive cuff condition; non-cuff, control condition.

Interpretation: There were no significant interaction, time, or condition effects for heart rate in the LAY trial.
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