THE EFFECTS OF PROLONGED SITTING ON ARTERIAL STIFFNESS AND
CIRCULATING ANGIOGENIC CELLS IN HEALTHY ADULTS

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ABSTRACT

William S. Evans: The Effects of Prolonged Sitting on Arterial Stiffness and Circulating Angiogenic Cells in Healthy Adults

To assess the effects of prolonged sitting on measures of arterial stiffness (AS) and circulating angiogenic cells (CAC), healthy but sedentary subjects completed 3 hours of sitting in a randomized crossover design with 10 calf raises every ten minutes (Calf) or a control condition (CON). Pulse wave velocity (PWV) and pulse wave analysis (PWA) and blood draws were performed pre and post to assess AS. CACs were isolated from venous blood for analysis. PWV significantly increased (Mean Difference [MD]0.30 m/s (0.46), p<0.01) and augmentation index (AIx) from PWA significantly decreased from pre-to post (MD=-9.14% (11.09), p<0.001). There was no significant change in CAC counts per 500,000 events or gene expression in either condition across time. In conclusion, aortic AS increases during sitting despite decreases in arterial wave reflection, and local changes observed previously with physical activity do not persist systemically.
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LIST OF ABBREVIATIONS

AIx – Augmentation Index

APL – Applied Physiology Laboratory

AS – Arterial Stiffness

BF – Blood Flow  CFU – Colony Forming Unit

CAC – Circulating Angiogenic Cells

CAD – Coronary Artery Disease

CD – Cluster of Differentiation

cDBP – Central Diastolic Blood Pressure

cSBP – Central Systolic Blood Pressure

CVD – Cardiovascular Disease

EC – Endothelial Cell

eNOS – Endothelial Nitric Oxide Synthase

EPC – Endothelial Progenitor Cell

FMD – Flow Mediated Dilation

HRV – Heart Rate Variability

HSC – Hematopoietic Stem Cells

MAP – Mean Arterial Pressure

NIRS – Near Infrared Spectroscopy

NO – Nitric Oxide

PBMC – Peripheral Blood Mononuclear Cells
**PI3K/Akt** – Phosphoinositide 3-kinase/Akt pathway

**PWA** – Pulse Wave Analysis

**PWV** – Aortic Pulse Wave Velocity

**RMSSD** – Root Mean Square of Standard Deviations

**ROS** – Reactive Oxidative Stress

**SCI** – Spinal Cord Injury

**VEGF** – Vascular Endothelial Growth Factor
CHAPTER I

Introduction

Emerging evidence suggests that sedentary behavior, including prolonged sitting, increases the risk for cardiovascular disease (CVD), the leading cause of death globally [1-3]. While chronic associations are known, the underlying mechanism are unclear. Evidence from acute bouts of sitting suggest that prolonged sitting decreases blood flow and shear stress (SS) resulting in local endothelial dysfunction of the lower extremity, but these changes may be rescued by intermittent physical activity, and the systemic effects remain unclear [4-6].

Optimally, assessing central changes in arterial stiffness (AS) such as aortic pulse wave velocity (PWV) and pulse wave analysis (PWA) would aid in understanding the adverse effects of sitting and the increased burden on the heart. While endothelial dysfunction increases during sitting, evidence also suggests that endothelial repair is attenuated in response to 10 days of physical inactivity as indicated by CFU-Hill counts[7]. CACs have paracrine function and contribute to repair of damaged vessels. However, the effects of acute inactivity on CAC number and function are not known. Therefore, to better understand functional changes in stiffness and the effects of sitting on vascular repair, PWV, PWA, and CAC count and function need to be examined before and after acute bouts of inactivity. Additionally, strategies to increase blood flow and shear stress need to be included to 1) provide additional mechanistic insight and 2) to develop other strategies to counteract the detrimental effects of sitting.
PWV is the gold standard approach for measuring aortic AS [8]. The aorta is directly proximal to the heart and its elasticity is responsible for dampening the speed and amplitude of retrograde pressure waves that increase the heart’s workload during systole [9]. Indeed, aortic PWV has particular relevance to CVD, with a 1 m/s increase in PWV corresponding to a 14% and 15% increase in total CV events and CV mortality respectively, independent of traditional risk factors [10]. Pulse wave analysis (PWA) is also commonly used in junction with PWV to assess arterial wave reflection and central blood pressure, which has been shown as a better predictor of CVD than brachial blood pressure [10, 11]. Together these measures provide valuable information relevant to cardiac burden and serve as clinically relevant markers of arterial stiffness.

Acutely, endothelial dysfunction and AS may occur in response to decreased blood flow and shear stress. Indeed, it has been repeatedly shown that one bout of sitting reduces the NO dependent measure FMD in the lower leg suggesting endothelial dysfunction while local heating, exercise, and fidgeting have been shown to restore vascular health via increased shear stress [6, 12, 13]. However, these changes only represent local changes in vascular health and do not provide central insight unlike PWV and augmentation index (AIx). Though changes in PWV and AIx are generally chronic and observed with aging, evidence does show a functional stiffening in response to NO antagonists [14]. In light of this evidence, simple perturbations such as fidgeting or greater stimuli such as calf raises should be generalizable to changes in central measures of AS such as PWV and AIx.

In addition to changes in AS, shear stress has also been shown to indirectly affect CACs which are important for the repair of damaged endothelial cells. Specifically, CACs home to ischemic sites following damage, and secrete various angiogenic cytokines[15]. CACs are
CD34+ hematopoietic stem cells that were initially termed endothelial progenitor cells. In response to increased blood flow and shear stress, CACs increase expression of endothelial nitric oxide (eNOS) and NO production which mediates function of important angiogenic cytokines such as VEGF[18]. Additionally, shear stress dose dependently increases surface protein expression of VEGFR-2, VE-cadherin and Tie2 which are important for angiogenic capabilities and maintaining a restrictive endothelial barrier[19]. Furthermore, shear stress has been shown to augment homing and engraftment of CACs via increased signaling of CXCR4/JAK-2 pathway while also increasing expressing of CXCR4 in-vitro[19]. By affecting the secretion of VEGF and CXCR-4, shear stress may impact the homing, engraftment and angiogenic properties of CACs on damaged endothelium. Therefore, assessment of VEGF and CXCR-4 as gene targets on CACs is important, as they are likely impacted by decreased shear stress.

Though blood flow and shear stress affect CACs, little is known regarding the effects of physical inactivity on CACs. Ten days of reduced physical inactivity reduced colony forming unit counts but did not affect circulating counts of CACs [7] but the acute effects of inactivity have yet to be determined. On the other hand, it is generally accepted that CAC counts and function increase with acute exercise [20-23]. If acute inactivity decreases CAC function, it begs the question if simple lower extremity muscular contractions (i.e. calf raises) may increase shear stress and CAC activity to offset the effects of sitting. Given their role in repair, regulation by blood flow and shear stress, and the paucity of evidence, it is important to establish the effects of sitting on both the quantity and quality of CACs.

In light of the current evidence on sitting and vascular health, there remains a need to need to translate these findings into clear public health message and to understand mechanistic underpinnings. As such, addressing PWV, AIx and CACs during sitting provide as meaningful
variables that yield clinical and mechanistic relevance. Therefore, the purpose of this study is to understand the balance between damage and repair during prolonged sitting through assessing 1) changes in PWV and PWA, 2) CAC counts and gene expression and 3) if intermittent calf raises during prolonged sitting attenuates these changes. If prolonged sitting does increase AS and calf raises can prevent these changes, current public health recommendations and guidelines can be updated to prevent the deleterious effects of sitting.

**Research Questions**

1. Does PWV and PWA increase with 3 hours of prolonged sitting?
2. Do CD34+ CAC counts decrease following 3 hours of prolonged sitting?
3. Does gene expression of CACs change following 3 hours of prolonged sitting?
4. Do intermittent calf raises prevent negative changes in AS or CACs?

**Research Hypothesis**

1. A 3-hour bout of prolonged sitting will increase PWV and AIx from PWA from pre to post testing.
2. A 3-hour bout of prolonged sitting will decrease CAC counts.
3. A 3-hour bout of prolonged sitting will decrease number of CD34+ CACs and expression of \( VEGF \) and \( CXCR-4 \).
4. Intermittent calf raises during a 3-hour bout of prolonged sitting will prevent negative changes in PWV, AIx, and CD34+ CAC counts and function.
Assumptions

2. Pre-assessment guidelines were adequate to control for baseline changes in outcomes.
3. All subjects answered the medical history questionnaire and physical activity readiness questionnaire truthfully.

Delimitations

1. When able, women’s menstrual cycles were recorded and controlled.
2. Repeated measures design was used to control for condition specific variability.
3. All subjects had similar dietary intake prior to and during testing.
4. All subjects were between the ages of 18-35.

Limitations

1. Blood flow induced shear stress was not directly measured during testing.
2. Magnetic isolation of CD34 CACs did not result in 100% purity.

Significance of Study

CACs have clinical significance given their relationship to the development of CVD. They function to both repair and potentially incorporate into damaged endothelium. Furthermore, they can decrease following sedentary behavior and with other risk factors such as smoking and hypertension. Their relationship to emerging risk factors such as central measures of AS may further explain the etiology of cardiovascular disease and the underlying mechanisms. Moreover, because prolonged sitting is a precursor to and is associated with increased risk of CVD, a better understanding of the balance between damage and repair will help with treatment and
prescription. Lastly, the results of this study may lead to simple public health initiatives during the work day, such as calf raises, to prevent CVD and lower the economic burden on health care.
Chapter II

Literature Review

This review is divided into the following sections: 1) Mechanisms of Inactivity, Sitting, and Vascular Physiology 2) AS and Inactivity 3) AS and Endothelial Dysfunction 4) Progenitor Cells and eNOS/NO 5) Physical Activity and CACs.

Section 1: Mechanisms of Inactivity, Sitting, and Vascular physiology

The effects of inactivity are well documented in current literature. Beginning with the London bus study, study of physical activity and inactivity, has progressed [24]. Through this evidence, sedentary behavior is now considered a risk factor for the development of cardiovascular disease (CVD) according to the American College of Sports Medicine [25]. However, the precise mechanisms governing these changes are not concrete, and recent evidence suggests the type of inactivity seems to matter.

1.1 Animal Models

Mechanistically, studies of physical inactivity utilizing animal models have greatly added to our body of knowledge. These studies have shown that vascular health, assessed by ability to produce proteins or respond to drug and mechanical stimuli, relies on changes in blood flow. Specifically, animal models utilizing in-vitro flow testing of arterial tissue and rat hind-limb suspension provide evidence suggesting a role of increased blood flow (BF) and shear stress, the frictional force of blood. In the rat hindlimb-unloading model, the rat is suspended by the tail to inhibit use of the hindlimbs and promote use of the forelimbs. Results from this model show a
decrease in blood flow due to inactivity and the absence of gravity result in a decrease in the expression of eNOS mRNA as well as protein expression, suggesting a decreased ability to produce NO [26]. This model also showed a decrease in the ability to vasodilate in response to acetylcholine and differing flow patterns, functionally supporting the hypothesis that the absence of flow decreases NO availability and sensitivity. Further testing these mechanisms, studies used acetylcholine and NO, which induces vasodilation, and inhibitors of NO, thus decreasing vasodilation, on arteries from hind-limb unloaded rats. Similar to previous studies, these findings indicated a decreased vasodilatory response to acetylcholine in hindlimb unloaded rats [27]. Furthermore, administration of eNOS and NO inhibitors reduced acetylcholine vasodilation by 40% in control rats, but completely inhibited vasodilation in hind limb unloaded rats [28]. Together, these findings showed attenuated endothelium-dependent vasodilation with a particular emphasis on the NO pathways, thus providing evidence that shear stress modulates NO pathways and potentially vascular health. In addition to changes in vasodilatory function, vasoconstriction is also inhibited in response to hind-limb unloading [29]. In response to vasoconstrictors such as norepinephrine and KCl after hindlimb unloading, gastrocnemius arteries showed decreased ability to vasoconstrict and maintain myogenic tone at high pressures, indicating a loss of vascular sensitivity and strength. These studies also found that the changes in vasodilation and vasoconstriction may be a result of decreased thickness of smooth muscles and subsequently loss of motor strength.

To more specifically test mechanisms necessitating the presence of shear stress, soleus muscle feed arteries were isolated and introduced to various levels of flow [30]. Results from this study showed that arteries dose dependently produce eNOS in response to various levels of flow. Furthermore, pre-treatment with high-flow resulted in a significantly greater vasodilatory
response to acetylcholine than no-flow. Again, administration of NOS inhibitors abolished vasodilation. Together, these findings suggest that blood flow induced transmural pressure and shear stress are important for the maintenance of endothelial function, which depends on release of eNOS and NO and subsequently sensitivity to NO.

1.2 Human Models

In human models of decreased activity, spinal cord injury patients (SCI) and bed rest have been used. These studies compared SCI and healthy men for differences in endothelial function via flow mediated dilation (FMD), a test of endothelial dependent response to shear stress, and in response to nitroglycerin spray, a test of endothelial independent sensitivity to NO [31]. SCI showed a significantly enhanced FMD response in the superficial femoral artery, but no significant differences were found in the brachial artery. When correcting for the amount of shear rate stimulus (FMD is dose dependent), there was no significant difference between SFA arteries of SCI and control, but control had significantly greater increase in brachial artery FMD. The response to nitroglycerin spray was not significantly different between the conditions, suggesting that these patients had preserved vascular function, even in inactive legs. Similar to this study, 52 days of deconditioning by bed rest resulted in an increased sensitivity to nitric oxide in the SFA as measured by dilation in response to nitroglycerin and FMD [32]. This study did find a reduction in the diameter of conduit arteries. Further studies assessing 60 days of bed rest found decreases in plasma volume, increases in isovolumic relaxation time and myocardial performance index, suggesting a decrease in cardiac function [33]. These studies found decreases in anterior tibial artery intimal-medial thickness and a trend towards decreased orthostatic tolerance following 60 days of bed rest. Additionally, subjects in this study showed no
significant difference in response to sublingual nitroglycerin spray, and a significant increase in FMD of the anterior tibial artery. Although these seem different, it was proposed that the decrease in smooth muscle cross sectional area would increase the diffusion of NO into to the muscle to induce vasodilation, while the NO-sGC signal transduction cascade in smooth muscle (tested via nitroglycerin spray) was unlikely, as there is no systemic change in endothelium-independent NO dilation. Although these studies report somewhat surprising findings regarding the mechanisms behind changes in changes in endothelial function with inactivity, evidence remains that SCI and inactivity increases risk for CVD, suggesting confounding variables such as changes in smooth muscle volume may partially explain these findings.

Another possible explanation of these findings was proposed by a study evaluating autonomic nervous system function [34]. In this study, heart rate variability (HRV) and blood pressure variability were monitored in SCI and CON. Using spectral analysis of R to R interval, cross-spectral analyses of low-frequency spectra, this study showed that SCI had reduced sympathetic drive to the heart and vasculature, increased baroreflex delta in cervical SCI and reduced cardiac vagal tone in thoracic SCI subjects. Together, these findings were used to show that autonomic nervous system dysfunction may partially explain changes in cardiovascular health.

1.3 Prolonged Sitting

Although the effects of bed rest and SCI on vascular physiology is less congruent, the effects of physical inactivity such as sitting is clearer. In terms of risk, increased sitting time is associated with increased risk of cardiovascular disease and all-cause mortality. A 15 year prospective study included 17,000 participants and asked them to subjectively report their sitting time [35]. The findings of this study indicated that individuals who reported sitting almost none
of the time to almost all of the time were progressively more likely to die from cardiovascular disease even after adjustment for other variables such as smoking, age, sex, and body mass index. This study reported hazard ratios ranging from 1.00 when active to 1.54 when sitting almost all of the time [35]. Although this study clearly established the connection between sitting and cardiovascular disease. Sitting also significantly increases the risk of all-cause mortality (HR, 1.220), diabetes (HR, 1.910), cardiovascular disease incidents (HR, 1.143), and cancer incidence (HR, 1.130) independent of exercise [36].

In light of these findings, studies aimed at explaining the specific mechanisms were conducted. Because the role of blood flow induced shear stress regulated various measures of vascular health at the micro and macrovascular scale, models of sitting were developed to assess changes in blood flow, shear stress, and endothelial macro and microvascular function. Initially, these models aimed to assess hemodynamic changes in both the brachial and femoral arteries [37]. Similar to animal models, these studies assessed endothelial function via changes in Flow Mediated Dilation. In review, FMD is the gold standard for measuring endothelial function and is an indication of endothelial cells’ ability to autoregulate in response to stresses by releasing NO and other vasodilators to maintain blood flow. The first of these studies assessed changes in SFA and BA FMD and flow patterns over 3 hours of sitting [37]. This study showed that sitting resulted in impaired shear patterns associated with endothelial dysfunction in both the SFA and BA. However, the SFA showed decreases in FMD, but the BA did not. This study suggested that leg vascular function was primarily impaired during sitting as a result of changes in shear stress. A similar design and study was performed following this study; however, subjects walked at the 30 minute, 1 hour and 30 minute, and 2 hour and 30 minute time point [38]. Similar to the previous study, sitting reduced measures of FMD and mean shear rate and antegrade shear rate;
however, walking resulted in no significant declines in these measures. These findings illustrated that macrovascular function was clearly impaired during sitting and was likely regulating by changes in blood flow and shear stress.

To test changes in microvascular function, as the level of artery can differ in functional responses to shear stress, a similar design was used. Subjects again sat for 6 hours while measures of FMD and reactive hyperemia (a measure of microvascular reactivity) in the popliteal artery and BA were collected [5, 39]. This study found that sitting resulted in significant decreases in popliteal artery FMD and reactive hyperemia; conversely, BA FMD was unchanged after sitting while microvascular function was impaired. Additionally, walking post sitting restored measures of FMD and reactive hyperemia.

Several follow-up studies by the same condition used only 3 hours of sitting while using local heating or fidgeting one leg to assess the role of blood flow and shear stress [6, 13]. This condition found that local heating and fidgeting restored measures of blood flow, shear stress, and FMD, while the control leg showed significant decreases in FMD. These findings were consistent with hypotheses that sitting induced leg endothelial dysfunction via reductions in blood flow and shear stress, and that simple interventions could be used to mitigate these findings.

Section 2. AS and Inactivity

Although endothelial dysfunction plays a large role in the development of atherosclerosis, other vascular measures such as AS have associations with CVD [40]. Additionally, methods of measuring AS require less expertise for accurate results, and are highly reproducible. The specific mechanisms resulting in AS are still studied, but a link between characteristics of
endothelial dysfunction, such as decreased bioavailability of NO, and AS exist. Thus, this relationship may partially explain the underlying mechanisms by which sitting may result in AS and subsequently CVD.

AS is a measure of arterial compliance, aortic pulse wave velocity is the gold standard for measuring arterial compliance [8]. A recent study assessed aortic pulse wave velocity (PWV) in 2835 subjects who were apparently healthy [40]. Hazard ratios for development of coronary heart disease for subjects in the second and third tertile were 1.72 and 2.45, respectively. Additionally, these hazard ratios did not significantly differ after adjustment for cardiovascular risk factors. This study poses a strong prognostic value of PWV.

Additionally, a meta analyses including over 15,000 subjects found that increased PWV resulted in an increased risk clinical events including all-cause mortality, cardiovascular events, and cancer. [10] This increased in a step wise fashion. These studies established a clear relationship between increased PWV and disease.

Prolonged sitting and AS increases the risk for developing CVD and other non-communicable disease. A study of 1241 Brazilian adults 30 years of age showed a positive association between sedentary time measured by accelerometry and PWV [1]. Furthermore, a negative association was seen between individuals engaging in more PA and PWV. In summary, these findings indicate that AS could be altered by physical inactivity or vice versa.

Section 3. AS and Endothelial Dysfunction

A link between physical inactivity such as prolonged sitting and endothelial dysfunction is quite clear. Additionally, these changes are associated with hemodynamic changes. The results of these hemodynamic changes may be regulating changes in AS.
As aforementioned, endothelial dysfunction is characterized by a decrease in the release of NO. Decreases in the bioavailability of NO can be observed in response to decreases in blood flow and shear stress. Studies have assessed the role of NO and shear stress on AS, and the two appear to be interrelated[30, 41]. For example, Kinlay et al. injected various concentrations of Nitroglycerin, an exogenous form of NO, and L-NMMA an inhibitor of NO and measured changes in PWV[42]. L-NNMA resulted in increased measures of PWV, while nitroglycerin had the opposite effects. Another study administered concentrations of acetylcholine and glyceryl trinitrate, both characterized as NO donors, and observed significant decreases in PWV[43]. Furthermore, this study illustrated that co-injection of acetylcholine and L-NMMA, an NO inhibitor, resulted in no significant difference in elasticity. Of note, these changes occurred independent of changes in mean arterial pressure (MAP). This is significant given the fact that increases in vasoconstriction would increase MAP thus increasing PWV.

These studies were all local in nature, i.e. use direct arterial injection of NO directly into arteries where elasticity was measured. Systemic administration of NO and inhibitors has produced varying results. In healthy humans, radial arterial pulse pressure waves forms were monitored with a tonometer device to monitor reflected waves and compliance.[14] In this study, intravenous injection of NO agonist and L-NAME, a stereospecific inhibitor of NOS produced. In this study infusion of L-NAME decreased small artery compliance but had the opposite effect on systemic vascular resistance. Sublingual glycerol trinitrate decreased both large and small artery compliance. These studies were conducted in arteries primarily characterized as muscular arteries. With age, these arteries have a smaller decrease in arterial compliance as opposed to the elastic aorta.
In a similar design, this was tested. L-NMMA was introduced with norepinephrine and dobutamine to help control for potential changes in stiffness as a result of pressure [44]. Norepinephrine increases pressure through an increase in peripheral resistance, but dobutamine is a positive inotrope that vasodilates smooth muscle. Despite their differing mechanism of action, both of these drugs increase MAP. In this study, increases in PWV and AIx were observed during injection of L-NMMA, suggesting that inhibition of NO increases stiffness. However, these effects were similar to those observed during injection of norepinephrine and dobutamine. Therefore, this study suggested that changes in stiffness were not actually a result of compliance, but instead a result of changes in MAP.

Notably, elasticity in relation to NO production can change as a function of exercise. A rat study using 8-weeks of aerobic exercise illustrated increases in aortic rings vasorelaxation [45]. This study dissected thoracic aortic rings and tested NO production along with aortic tension in response to methacholine, an endothelium dependent agonist of NO. This study established that trained rats had increased production of NO and increased maximum relaxation, suggesting again that NO’s effect on vasomotor tone of smooth muscles can be modified by physical activity and is partially dependent on NO.

However, these studies primarily assessed changes in stiffness using either drug interventions or chronic training adaptation. Additionally, prior studies assessing stiffness with exercise or sedentary behavior have been cross sectional or longitudinal in nature [1, 10, 40]. However, it has been shown that NO dependent measures of endothelial function such as FMD are decreased following only an acute bout of sitting [5, 6, 12, 13, 37]. Therefore, it is possible that increases in stiffness, and thus cardiac afterload, could result following sitting. These increases in stiffness may likely occur as a result of decreased blood flow and shear stress as
blood pools in the legs and systemic flow decreases [4, 38]. Interestingly, some studies have proposed that stiffness may actually increase endothelial dysfunction, suggesting a cyclic relationship [46]. Although local changes have clearly been assessed, it is unclear whether central measures of elastic arteries such as the aorta decrease.

Section 4. Progenitor Cells and eNOS/NO

Endothelial Progenitor Cells (EPC) are a bone marrow derived stem-cell capable with angiogenic capabilities. EPC are now more broadly recognized as circulating angiogenic cells (CAC). Similar to conventional endothelial cells (EC), this cell type produces and is regulated by shear stress, eNOS and NO. Although much debate surrounds their specific function, origin, and phenotyping, these cells are important for repair and generation of endothelial cells, and counts and assays are clinically relevant in regard to the development of CVD and CV related events.

EPCs were initially identified by Asahara et al. because of prior evidence suggesting HSC circulated in the periphery. By staining for CD34+, an HSC marker and Flk-1, a receptor for vascular endothelial growth factor (VEGF) that is expressed by endothelial cells, this group was able to identify and isolate EPCs from peripheral blood. Further analyses showed that these cells uptake acetylated low density lipo-protein, which is specifically uptaken by endothelial cells [47]. Additionally, fluorescent labeling with the dye DiI, showed specifically which cells were capable of differentiation and producing spindle shaped cells in-vitro. Lastly, this group was able to show that specifically through various markers and prior uptake of DiI labeled acetylated low density lipo-protein that these cells could incorporate into ischemic damaged tissue to produce blood vessels. Additionally, these cells showed positive immunoblotting for eNOS and the endothelial marker CD31.
However, this cell population has some drawbacks which have been extensively covered in a review by Yoder 2013. [48] Briefly, EPCs characterized by various clinical are most likely HSCs at various stages of development during their isolation. Additionally, these cells may also be monocytes and macrophages that release proangiogenic cytokines, thus contributing to the angiogenic properties. Therefore, these cells are more recently termed Circulating Angiogenic Cells (CAC) to describe a heterogenous cell population labeled with traditional markers, such as different combinations of CD34, VEGFR2, and CD133, capable of promoting angiogenesis and neovascularization either directly or indirectly.

Specifically, the use of mouse models where the SFA is cut, thus inhibiting blood flow, have been used to test their neovascular capabilities. [49] Asahara et al. also included a plethora of other tests utilizing a mouse model and staining for EPCs to show that EPCs appear to recruit to capillaries among skeletal myocytes after ischemic damage post myocardial infarction [16]. Additionally, it was shown that EPC help reestablish blood flow after cutting of the SFA.

Similar models assessing their regenerative capabilities expounded on these findings with a specific emphasis on the role of eNOS and NO. NO is produced in response to shear stress via the Akt pathway by endothelial cells, and CACs as they express similar membrane proteins [19, 41]. NO is anti-atherosclerotic in nature as it prevents leukocyte adhesion and migration into the sub endothelial space, the proliferation of smooth muscle cells, and NO operate as an anti-oxidant and prevent platelet aggregation[50-54]. Mice deficient of the eNOS creating gene showed an inability to mobilize in response to VEGF, an autocrine factor released by EPCs, additionally, these mice showed increased mortality after myelosuppression. However, when EPCs from wild-type mice were administered, the neovascular effects were restored in a rat
hind-limb ischemia model. Bone marrow transplantation did not have these effects suggesting that eNOS plays a role in proper recruitment and development of EPCs during hematopoiesis.

Furthermore, CACs migration is regulated by eNOS/NO. In healthy subjects, the ability of CACs to migrate in response to VEGF was impaired by NOS inhibitors, while addition of NO donors resulted in increased movement and migration in response to VEGF[55]. Additionally, CAD patients showed no expression of eNOS in comparison to healthy participants, and CACs from CAD patients showed impaired response to VEGF that was restored when NO donors were administered. Patients with CAD also showed decreased measures of NO dependent FMD and concentration of plasma NO. Together these findings showed that CACs function is dependent on proper expression of eNOS and subsequently NO. Furthermore, patients with CAD have impaired CAC responses and eNOS expression suggesting that absence of NO may not only increase development of atherosclerosis but may also impair repair of endothelial cells. These results are not surprising given the association between presence of CVD and decreased count and migratory ability of CACs [56].

Lastly, a study utilizing a viral vector, AVE9488, to overexpress the eNOS protein, showed significant promise [57]. This study illustrated that after MI, rats treated with AVE9488 showed various improvements in heart function and molecular changes. Mice overexpressing eNOS prevented the decreases in seen in CACs post MI, improved LV function, improved FMD results, increased eNOS protein expression, and decreased presence of the superoxide anion. Clearly, these results suggest a significance of eNOS post infarction, and these changes may possibly be through the function and maintenance of CACs.
Section 5. Physical Activity and CACs

Multiple relationships exist between inactivity, endothelial dysfunction and disease. Additionally, similar links exist between CACs, endothelial dysfunction and disease. Therefore, some evidence exists relating changes in CACs to both physical activity and inactivity.

CACs do respond to physical activity. Volunteer subjects (n=22) performed exercise to max intensity, and pre and post blood draws were compared. The markers used in this study were VE-Cadherin and CD133. The number of CACs and EPCs increased immediately post exercise by over 2.5-fold in CACs and 4-fold in EPCs. However, secretion of angiogenic cytokines VEGF, GM-CSF, HGH, and G-CDF were not significantly different in pre to post measures from CACs.

However, a more recent study using a more comprehensive anti-body panel did not see as robust changes. This study was designed to assess the kinetics of CAC and EPC, so multiple time points during exercise were examined [58]. CD34+ CACs were significantly increased at 20 minutes and 40 minutes of training compared to pre-test measures. Additionally, HSPCs and HSCs labeled as CD34+/CD45low and CD34+/CD45low/CD38-, respectively, were significantly increased at 20 minutes during exercise compared to 60 minutes post exercise, suggesting a biphasic response. This data indicated slightly different results most likely due to the use of different markers, and different intensities.

Interestingly, exercise training appears elicit increases in EPCs and CACs, and these changes can even occur in diseased populations such as CAD[59]. CAD patients showed decreased migratory capacity at baseline but was improved post exercise. Additionally, these patients showed improved FMD measures along with a 77% increase in migratory capacity of CACs. Interestingly, changes in CACs from pre to post exercise were not observed post training,
suggesting that training may reduce sensitivity of CACs to stress. Lastly, EPC counts were significantly greater post training in CAD patients. These findings suggest that repeated bouts of exercise may initially increase measures of CAC to induce repair, which is subsequently reflected by changes in FMD and improved functionality of endothelial cells.

In addition to the effects of exercise training, studies assessing the effects of training status and detraining on behavior of CACs has provided insight into their responses [60]. Comparisons between highly active endurance trained men and healthy low-active men showed no significant difference between baseline values of CACs or EPC. However, following 10 days of detraining, highly active men showed significant decreases in CACs, and a positive correlation between the change EPCs and reactive hyperemia. Additionally, the percent change in EPCs and EPC senescence was correlated with changes in total oxidative capacity. These results introduced new and interesting perspectives with regards to inactivity and the possible underlying mechanism, i.e. oxidative stress and nitric oxide.

A follow-up study using very similar methods further assessed the role of NO and oxidative stress[20]. This study utilized a similar design in apparently healthy men and evaluated changes in CAC counts, CFU-CACs, intracellular NO, related gene expression of NO and oxidative stress. This study found significant decreases in the number of CFU CACs along with production of intracellular NO. However, this study did not observe any significant decreases in genes related to NO or oxidative stress. Additionally, this study did not observe any significant differences in CD34+ CACs. In light of previous findings, this study suggested that changes in CACs is population dependent.

Finally, work by Dr. Landers-Ramos has further evaluated the effects of training and detraining on various subpopulations of CACs, primarily CD34+ and CD34-/CD31+ cells [61].
In these studies, apparently healthy active, inactive, and endurance trained subjects were assessed. Again, no differences in redox status was found in any group across either cell type. Media from CD34+ and CD34-/CD31+ CACs of endurance trained individuals and inactive individuals’ cells was added to human umbilical vein endothelial cells. In inactive participants, endothelial cells produced vessels that were shorter and less complex. Furthermore, the changes in tube formation was attributed to two specific proteins and their respective ratios.

These findings establish relationships between CACs and physical activity. Although the role of NO and other proteins appears insignificant, it is likely that NO still plays a role in their function with activity. These studies have been observed either due to training status or prolonged physical inactivity, with only one study reporting sedentary time trending towards a significant difference [7]. Yet, significant changes in hemodynamics and NO dependent FMD are observed after only one bout of prolonged sitting, while walking, fidgeting, and prior aerobic exercise were able to mitigate these findings altogether [12, 38, 62]. Therefore, we propose that changes in hemodynamics associated with entering the lab or prior training status may have played a role in inconclusive findings. Regardless, the effects of an acute bout of sitting may decrease counts or function of CAC, and these changes should be evaluated to better understand and treat the effects of prolonged sitting.
Chapter III

Methods

Subjects

Five males and 15 females, ages 18-35 were recruited. All subjects were sedentary, according to the American College of Sports Medicine Guidelines. Exclusion criteria included: any known cardio-metabolic disorders, pregnant women, smoking, physically active more than 150 minutes of moderate physical activity per week, and any medication known to affect cardiovascular health. Because fluctuations in estrogen or testosterone can affect cardiovascular measures, women were tested within 1-10 days of their menstrual cycle. The study was approved by the University of North Carolina at Chapel Hill’s institutional review board. Subjects signed an informed consent form prior to participate in the study.

Study Design and Pilot Testing

The design was a randomized crossover design with two experimental conditions (control [CON] and CALF raises [CALF]) preceded by a familiarization session (Figure 1). All testing took place in the Applied Physiology Laboratory at the University of North Carolina. Prior to arrival for testing, participants filled out a medical history questionnaire, physical activity readiness questionnaire (PAR-Q) and informed consent. Using online randomization software (www.randomizer.org) participants were randomized into either CON or CALF intervention for first visit.
Because calf raises have not been previously shown to restore leg vascular function, pilot testing was performed. To verify that calf raises were sufficient to increase blood flow and shear stress, a one hour sitting protocol with single leg calf raises was administered. Subjects rested in the supine position for 20 minutes and were then seated for one hour. During sitting subjects performed 10 calf raises on one leg every 10 minutes. An ultrasound probe was placed on the femoral artery to measure blood flow, antegrade shear stress, retrograde shear stress and oscillating shear stress on both legs. Measurements were collected for five minutes at 5, 15, 25, 35, 45, and 55 minutes on each leg.

**Figure 1.** Example of subject visits in randomized crossover design. Subjects were familiarized and randomized into either CALF or CON on two subsequent visits. All testing was completed within two weeks of visit 1.
**Familiarization**

Subjects reported to the laboratory to review documentation and to sign the informed consent. Participants were placed in a chair and chair height was selected to place feet flat on ground and thigh to calf angle at approximately 90 degrees to standardize within and between trials. During this time, participants practiced several sets of 10 calf raises. After reviewing pre-test guidelines, subjects were given a supplement bar (Pure Protein, Bohemia, NY, USA) to consume 2 hours before their first visit to prevent risk of hypoglycemia.

**Visit 1 & 2**

Subjects arrived after an overnight fast between 0600 and 1000 and one to seven days following the familiarization visit. After reviewing pretest guidelines, participants were fitted for an accelerometer (wActiSleep +; ActiGraph LLC, Fort Walton Beach, FL) on their right ankle to covary for spontaneous movement, and a 3-lead electrocardiogram for HRV analysis (HRV Add-On For LabChart, PowerLab 4/26; ADInstruments, Colorado Springs, CO, USA). Subjects then rested in the supine position for 20 minutes for accurate resting baseline measures. PWV and PWA were collected in triplicate followed by a venous blood draw.

Following the blood draw, subjects were then transferred to a seated upright position in the same chair with settings recorded from familiarization. Subjects were given 500 mL of water and began sitting while watching home improvement show. In both conditions, CALF and CON, subjects were notified at 10 minute intervals, but only CALF performed 10 calf raises to a metronome (Pro Metronome Xiao Yixiang ©2016 EUMLab, Xanin Tech) set to 20 beats/minute at 10 minute intervals to activate the muscle pump. To understand changes in wave reflection during sitting, PWA was collected at 10, 90, and 170 minutes of sitting.
After 180 minutes, subjects were carefully moved back to the supine position and rested for 20 minutes followed by measures of PWV, PWA and a final blood draw (36 mL). Blood could not be collected for pre to post CAC comparison on two subjects, and there was insufficient blood volume from four subjects to complete CAC gene expression.

Arterial Stiffness

During rest, a blood pressure cuff was placed on the non-dominant leg for calculation of PWV and on the non-dominant arm for PWA. To determine PWV, a path length from the carotid artery to the femoral artery is needed. Path length for PWV was measured by palpating for the carotid artery and marking the neck. The carotid notch was identified superior to the meeting of the clavicles and sternum. A cuff was then placed on the non-dominant leg so the top of cuff aligned near the femoral artery. Distance from the carotid artery to sternal notch, the top of cuff to sternal notch and from the top of cuff to femoral crease was recorded and entered into Sphygmocor software for PWV calculation. The top edge of the cuff was marked to ensure identical placement during post sitting measures. A tonometer was placed on the neck, and PWV was collected in triplicate when wave form and quality control were recognized by the Sphygmocor.

For PWA, a blood pressure cuff was placed on the arm and Sphygmocor device to calculate central pressures and arterial wave reflection. PWA was recorded in triplicate on the non-dominant arm.
HRV

HRV was collected to give indices of the autonomic nervous system, as it has been shown to affect vascular parameters [34, 63]. For analysis, 5-minute of ECG data was pooled and ectopic beats were excluded. Spectral analysis of the R-R interval was used to assess contribution of the parasympathetic nervous system. The root mean square of the standard deviation of R to R intervals was also recorded as an indication of autonomic nervous system function.

Peripheral Blood Mononuclear Cells (PBMC) Isolation and Immunofluorescence Labeling

Immediately following the final draw, peripheral bound mononuclear cells (PBMCs) were isolated using SepMate™-50 (Stemcell, Vancouver, BC Canada) as specified by the manufacture. CAC cell phenotyping was determined using direct immunofluorescence labeling of cell surface markers with mouse anti-human monoclonal antibodies. Cells were stained with Live-Dead Zombie Dye (Green) and incubated at 4°C for 15 minutes in the dark. Cells were then incubated with pre-titrated antibodies [CD3 (APC-Cy7); CD45 (PerCP-Cy5.5); VEGFR-2 (PE); CD34 (BV421); CD31 (APC); Biolegend, San Diego, CA, USA] and FCr blocker (Miltenyl Biotec, Auburn, CA, USA) in a total volume of 100µL of cell staining buffer (Biolegend, San Diego, CA, USA). Cells were washed to remove excess antibody and fixed in 2% paraformaldehyde prior to being suspended in 200 µL of cell staining buffer for flow cytometry analysis. Appropriate isotype and fluorescence minus one (FMO) controls were run. Cells were analyzed using an Attune NXt flow cytometer (BD Biosciences, CA USA) and FCS Express (De Novo Software, Glendale, CA).
CD34+ Isolation and Gene Analysis

Remaining PBMCs were resuspended at a density of 2*10^8 cells/mL (RoboSep Buffer, Stemcell, Vancouver, BC Canada). Anti-CD34 antibodies were added at a concentration of 100µL/mL and incubated at room temperature for 15 minutes. After incubation, magnetic particles were added at a concentration of 50µL/mL and incubated for 10 more minutes at room temperature. Cells were then placed in the EasySep™ Magnet (Stemcell, Vancouver, BC Canada) and incubated for five minutes at RT. The negative fraction was decanted and the remaining positive fraction was washed with 1mL of RoboSep Buffer and topped up to 2.5mL, and this was repeated four more times. Pilot testing was performed prior to starting the experiments to determine the optimal balance between sufficient cell yield and purity (refer to appendix?). Following washes, cells were resuspended in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and placed in -80°C freezer until further analysis.

Gene analysis was performed according to previously published results [64]. Briefly, RNA was quantified using a spectrophotometer (BioTek H1 Synergy Hybrid Reader, BioTek Instruments Inc.) and reverse transcriptase was used to generate cDNA (Life Technologies, Grand Island, N.Y., USA). Quantitative RT-PCR was completed using a Applied BioSystems 7300 Real-Time PCR System. Purchased from IDT (Coralville, Iowa, USA), primers were optimized for concentrations producing efficacy of >90%. Reactions were performed in duplicate on a 96-well plate and contained iTaq Universal Probes Supermix (Bio-rad, Hercules, Calif., USA), respective primer probe, and the cDNA template. The PCR conditions used were as follows: 95 °C for 3 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 45 s.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used as control to verify cycle thresholds (CTs) were not different across time in the present study. mRNA expression
values are presented as $2^{-\Delta CT}$, where $\Delta CT$ is the CT of the target gene minus GAPDH control for each condition

**Power Calculation**

Sample size calculations were based on the primary central vascular health outcome, aortic pulse wave velocity (PWV). While the effects of prolonged sitting on central vascular health have not been investigated, previous studies have reported that prolonged sitting reduces leg vascular health (endothelial function) between 57-80%[6]. For the current study, we opted to sample based on a conservative change score of 1 m/s. We also opted to use a conservative typical error of 1 m/s. Using magnitude-based inference, to estimate the sample size required to detect the smallest detrimental (or beneficial) effect in a cross-over study, with the maximum chances of a type 1 and 2 error set at 5% (i.e. very unlikely), approximately twelve participants were required. Oversampling was performed to account for potential incomplete cases and the exploratory nature of sitting on CAC number and function.

**Statistical Analyses**

Data collected was analyzed with SPSS Statistics version 21.0 (SPSS, IN., Chicaogo, IL USA). The $\alpha$ level was set *a priori* for all statistical procedures at $\alpha=0.05$. Descriptive statistics will be used to summarize subject characteristics. Changes in PWA were compared in the supine and seated position respectively and changes over time are represented as mean difference and standard deviation (MD(SD)). The effect size (ES) is represented as partial eta squared and was calculated by SPSS software during ANOVA analyses. A one-way, repeated measures ANOVA was used to compare results from CAC counts, PWV, PWA and gene expression between baseline and post testing after exercise, respectively. An effect size of $<0.2$ was deemed trivial, 0.2-0.4 small, 0.4-0.6 moderate, and $> 0.6$ large.
Chapter IV

Results

Participants Information

Seventy percent of the recruited participants were women and 21.7 (2.9) years old. The average BMI was 25.7 (5.3) kg/m².

Arterial Stiffness

There was no significant interaction for PWV or PWA. PWV significantly increased from pre to post [0.30 m/s (0.46), p<0.01 ES=0.532] following sitting (Figure 2A). During sitting AIx significantly decreased for both conditions from 10 to 90 and stayed depressed at 180 [-9.64 % (11.78), p<0.01] and [-8.26 % (13.91), p=0.01] (Figure 2B). In the supine position, AIx from PWA significantly decreased from pre to post in both conditions [-9.14 % (11.09), p<0.001].
HRV

Heart rate significantly decreased from pre to post in the supine position (Table 2, p=0.012) but did not significantly change during sitting (Table 3). RMSSD used to calculate HRV significantly increased during sitting from 10 to 180 minutes [10.3 (25.19), p=0.014 ES=0.236]. The low frequency (LF) range of HRV tended to change across time (p=0.056) with a trend for LF to increase from 10 to 90 minutes [7.4 (12.18), p=0.057] and the high frequency range (HF) of HRV significantly decreased during sitting (p<0.035) (Table 4).

Blood Pressure

SBP, cSBP, cDBP, DBP, and MAP were not significantly different in either condition during or after sitting (Table 2 and 3).

CD34+ CAC

There was no significant interaction for CACs as a percentage of the parent population, and CACs per 500,000 events, nor was there a significant main effect of time or condition (Figure 3).
Expression of the angiogenic cytokine VEGF and the chemokine receptor CXCR4 relative to GADP expression did not significantly change between conditions or across time for immunomagnetically separated CD34⁺ CACs (Figure 4).

**Figure 3.** Graph illustrating a) Percent change in CACs in CON vs. CALF and b) changes in CAC counts/500,000 from pre to post in CON and CALF. There were no significant condition, time or interaction effects. Error bars are represented as SD. CAC circulating angiogenic cells, CON control group, CALF calf raise group, SD standard deviation.

**CD34⁺ CAC mRNA expression**

Expression of the angiogenic cytokine VEGF and the chemokine receptor CXCR4 relative to GADP expression did not significantly change between conditions or across time for immunomagnetically separated CD34⁺ CACs (Figure 4).
Figure 4. Gene expression of A) the angiogenic cytokine VEGF and B) the chemokine receptor CXCR4 did not change with sitting or not change with sitting or calf raises. mRNA is expressed without units relative to GADPH expression. There were no significant group, time or interaction effects. Error bars are represented as SD. VEGF vascular endothelial growth factor, CXCR4 CXC chemokine receptor-4, CAC circulating angiogenic cells, CON control group, CALF calf raise group, SD standard deviation.
<table>
<thead>
<tr>
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<th>CON</th>
<th>EXP</th>
<th>P value</th>
<th>ES</th>
</tr>
</thead>
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<tr>
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<td>84 (7)</td>
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<td>-0.015</td>
</tr>
<tr>
<td>HR</td>
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<td>64 (10)</td>
<td>0.140</td>
<td>0.267</td>
</tr>
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<td>-0.101</td>
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Mean (SD). Exp experiemental, Con control, SBP systolic blood pressure, DBP diastolic blood pressure, MAP mean arterial pressure, HR heart rate, cSBP central systolic blood pressure, cDBP central diastolic blood pressure, AIX augmentation index, Pf pressure wave forwards, Pb pressure wave backwards
Table 2. Supine PWA measures at baseline vs. post 3-hours of sitting with and without calf raises on central hemodynamics

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Pre</th>
<th>Post</th>
<th>Interaction</th>
<th>Time</th>
<th>Leg</th>
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<td></td>
<td></td>
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<td>ES</td>
<td>P</td>
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<td>72 (9)</td>
<td>0.947</td>
<td>&lt;0.001</td>
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<tr>
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<td>70 (8)</td>
<td>72 (8)</td>
<td>0.947</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>0.601</td>
<td>0.015</td>
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<td>Calf</td>
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<td>85 (10)</td>
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</tr>
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<td>63 (10)</td>
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<td>Pf</td>
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<td>26 (3)</td>
<td>0.733</td>
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<td>Pb</td>
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<td>11 (2)</td>
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Mean (SD), SBP systolic blood pressure, DBP diastolic blood pressure, MAP mean arterial pressure, HR heart rate, cSBP central systolic blood pressure, cDBP central diastolic blood pressure, AIx augmentation index, Pf pressure wave forwards, Pb pressure wave backwards
### Table 3. PWA and HRV throughout a 3-hour bout of sitting with or without calf raises

<table>
<thead>
<tr>
<th>Measure</th>
<th>Time (min)</th>
<th>Interaction</th>
<th>Time</th>
<th>Condition</th>
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<td></td>
<td>10</td>
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<td>SBP</td>
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Mean (SD), Con control SBP systolic blood pressure, DBP diastolic blood pressure, MAP mean arterial pressure, HR heart rate, cSBP central systolic blood pressure, cDBP central diastolic blood pressure, AIx augmentation index, Pf pressure wave forwards, Pb pressure wave backwards.
Chapter V

Discussion

This study shows for the first time that an acute bout of 3 hours of sitting increases aortic AS and burden on the heart as indicated by increases in PWV. Despite increases in PWV, A1x decreased during sitting while RMSSD from HRV increased. Contrary to our hypothesis, sitting did not affect absolute or relative CAC counts. Additionally, intermittent calf raises were insufficient to prevent the changes in AS observed with acute inactivity and did not affect CACs. Cumulatively, these findings suggest: 1) a complex central hemodynamic response with different responses in AS versus arterial wave reflection, 2) that CD34+ CAC mobilization is not altered during 3 hours of sitting, and 3) that calf raises every 10 minutes do not appear to be a strong enough stimulus to alter central hemodynamics or circulating factors, contrary to previous work showing alterations at the local level.

Arterial Stiffness

This is the first study to our knowledge measuring the effects of an acute bout of prolonged sitting on PWV. We observed that PWV increased by 0.35m/s following 3 hours of sitting, which was statistically significant but falls below the 1.0 m/s that has been deemed clinically significant, at least chronically [10]. The chronic effects sitting are well demonstrated on CV risk and mortality, but their effects on AS are less prevalent. The best available comparison to our knowledge comes from data in older men showing that after adjustment for physical activity, each additional hour/week spent sitting over a 5-year period is associated with a faster increase in PWV (0.007 m/s [95% CI 0.001, 0.013]) [65]. The finding that chronic sitting
increases PWV along with acute increases in PWV during one bout of sitting implies that increased burden on the heart may contribute to increased risk of CVD. The aorta is the most proximal elastic tube that the heart “feels” during each beat. Therefore, repeated bouts of sitting resulting in increased aortic AS suggest repeated burden on the heart.

Despite the increase in PWV, we found ~9% decrease in AIx during sitting which is indicative of decreased arterial wave reflection and also AS. The conflicting results of PWV and PWA may be a result of different theory and methodology behind PWV and PWA, which must be discussed to determine potential explanatory mechanisms. The expert consensus document on AS suggests that the methods of PWV and PWA incorporate differing levels of the arterial tree to different degrees [8]. PWV is a direct measurement of aortic stiffness and is considered the gold standard that corresponds to the accepted propagative model of the arterial system and is highly dependent on the elasticity of the aorta. Meanwhile, PWA is an indirect measure of AS calculated from forward and reflected pressure waves. The origin of these reflected pressure waves is a result of forward and reflected waves that incorporate many conduit arteries. These conduit arteries have greater smooth muscle content and vasomotor tone, which does impact AIx independently of PWV [66]. As such, the nervous system provides as one potential mediator.

There was a trend for increasing RMSSD during sitting which suggests an increase in parasympathetic nervous system function. However, the peripheral vasculature is primarily innervated by the sympathetic nervous system. Both LF and HF frequency decreased, so the sympathetic branch likely had decreased innervation, which would be in agreement with the general convention that resting decreases sympathetic outflow. Furthermore, the forward and backward pressure waves, which are related to the contractility of the heart and the location of reflected waves, respectively, both decreased during sitting. Together these findings suggest that
the decreased AIx may resonate from increased vasodilation which may be occurring as a result of decreased sympathetic outflow during sitting. Lastly, a 10 bpm increase in heart rate has been demonstrated to decrease absolute AIx by 4%, but there were no significant changes in HR during sitting thus eliminating this possibility [67]. In summary, these findings suggest that sitting promotes a complex central hemodynamic response that incorporates many heterogeneous regions of the arterial tree that are variably affected during sitting and potentially regulated by the ANS.

**Circulating Angiogenic Cells**

Moderate and vigorous intensity exercise mobilize CD34+ CACs in an intensity dependent manner, but the precise mechanisms are not clear [68] [69]. We hypothesized that increased shear stress during exercise was one potential mediator, the decrease in shear stress during sitting would elicit the opposite effect [23, 68, 69]. Interestingly, despite very different populations, baseline CAC counts are similar (after unit conversion) to reports in young active males with VO$_{2\text{max}}$ >50ml/kg/min in which counts were ~250 CD34+ events/1mL [58]. However, CAC mobilization do not appear to be affected by prolonged sitting as there was no change in absolute or relative CD34$^+$ CAC counts. This was contrary to our hypothesis that sitting would decrease the release of CACs. Because this is the first study to our knowledge to assess the effects of an acute bout of sitting on CACs, comparisons to previous work are challenging. The closest comparison comes from a study on ten days of reduced physical activity which decreased CFU-CACs, but did not change CAC counts, which is agreement with our findings[7].
While exercise increases CAC counts and this change may be due to increases in shear stress, the decreases associated with sitting were likely not as large of a magnitude, albeit in the opposite direction. Blood flow and shear stress changes with exercise are sustained throughout the entirety of the bout. In our pilot data, calf raises did elicit considerable increased in shear stress, but these changes were transient and returned to resting values rapidly (within 1-2 minutes). Additionally, sitting decrease blood flow in the legs, but the same has not been found systemically. It is likely that the changes in systemic flow after sitting or during calf raises were not strong enough to alter CAC mobilization[5]. However, activity or health status could be affecting baseline counts as others have reported a relationship between risk of CVD and decreasing CAC counts [70]. Therefore, the possibility of low CAC counts to begin with may have overshadowed the potential decrease observed from sitting.

Functionally, mRNA analyses did not show any significant differences in expression of the chemokine receptor CXCR4 or the angiogenic cytokine VEGF, which are both expressed by CACs. Given the lack of change in counts, there may also be additional mechanisms that were not sufficiently targeted with this intervention. Specifically, hypoxia-inducible factor 1 (HIF-1) is a transcription factor expressed by bone marrow and CACs that is rapidly degraded under normoxic conditions[71]. On the contrary, under hypoxic conditions such as exercise, HIF-1 is not degraded and acts as a master regulator for genes related to proliferation, survival and mobilization such as stromal derived factor-1 (SDF-1), the chemokine receptor for CXCR4, VEGF, and erythropoietin[15, 72, 73]. Calf raises were unlikely to induce a large change in oxidative state in bone marrow or CACs; thus providing a possible explanation for the lack of changes in CAC count and function.
Calf Raises

Previous evidence suggests that simple perturbations during sitting such as walking, fidgeting or standing can improve endothelial health in the popliteal or superficial femoral artery [12, 13, 38], however, these studies did not report changes in the arm, which is the generally accepted measure of endothelial health. In this study, we attempted to address this gap by utilizing regular muscular contractions (e.g. calf raises) to activate the muscle pump and increase venous return while examining clinically relevant measures of vascular health, albeit at a different segment of the arterial tree. Data suggests that despite the decrease in blood pooling, calf raises do not acutely affect aortic AS. Despite evidence demonstrating the benefits of fidgeting and walking on leg endothelial function, similar perturbations such as calf raises do not alter AS during sitting. This may be as a result of the fact that FMD is highly dependent on shear stress whereas, AS is only partially dependent on it and this varies depending on the location within the arterial tree [74]. Additionally, the changes from fidgeting and brief exercise were only seen in the legs and not the arms, which may mean that the change in blood flow and shear stress are not sufficient to affect the arterial tree overall, rather the legs are may be more sensitive to small increases in shear stress and that only monitoring the legs may lead to misleading conclusions. Here we show that while fidgeting may be sufficient to prevent decreases in leg health, such simple perturbations appear to be inadequate for inducing changes in variables related to overall CV health.

Sitting Recommendations

In terms of general guidelines for sitting, chronic sitters may prevent the long term detrimental effects of sitting by performing 60-75 minutes of moderate to vigorous activity per week [62]. While these guidelines should be heeded to prevent the effects of sitting, ACSM
guidelines recommending 150 minutes of moderate to vigorous physical activity should also be recommended to prevent other consequences of sedentary lifestyles [75]. Lastly, preliminary evidence on the acute effects of sitting suggest that interventions such as fidgeting, local heating, and intermittent exercise can prevent the detrimental changes in local blood flow associated with sitting, but these changes may not be generalized to systemic vascular health such as PWV.

Limitations

While we present several novel findings that expand our knowledge on the effects of an acute bout of sitting, there are limitations. This study included majority women (n=15) currently using birth control. The effects of estrogen levels on vascular health have mixed results [76, 77]. Use of contraception such as birth control was not a delimitation of this study, and the majority of women is this study reported either use of contraceptives that inhibited their ability to report their menstrual cycle. Although men and women differ in sex hormone levels which may affect basal counts of CACs, we did test the effects of sitting on men vs. women, on CAC counts and did not find any significant differences (data not shown). Additionally, when participants were moved from seated to the supine position, a hemodynamic shift and changes in blood flow likely occurred that may have affected results. To limit these changes, participants were manually moved from position to position in 5-8 seconds to prevent an orthostatic challenge. Yet, it must be noted that because PWV and PWA were performed in the supine position, these hemodynamic shifts may have affected our results. Circulating CAC cell number and frequency were assessed from systemic changes and may not reflect events at endothelial tissue. Lastly, CACs are a rare cell subtype that resulted in very low mRNA yield, as a result, only two targets could be assessed.
Conclusions

Prolonged sitting is still an emerging risk factor for the development of CVD. However, the precise mechanism explaining the effects of sitting remained in question, with many clinically relevant targets still unexamined. Here we show that sitting in may increase central burden on the heart, and that calf raises do not appear to clinically alter these changes. Furthermore, sitting does not induce changes in CAC counts or function. Because sitting confers increased risk of mortality, these findings suggest that increased burden on the heart due to increased aortic AS may be a contributing factor. Cumulatively, these findings suggest that sitting produces a detrimental vascular response from a systemic but not stem cell level that intermittent calf raises do not seem to effect, and that the nervous system likely plays a compensatory mechanistic role.

Clinical Implications

Practically, these results add to the body of evidence regarding the effects of sitting. In terms of lifestyle interventions, ACSM guidelines of 150 minutes of moderate to vigorous physical activity are recommended for optimal health benefits; if these can’t be met current at least 60-75 minutes of moderate to vigorous activity can prevent the increased risks associated with sitting [62, 75]. Interventions while sitting lack longitudinal clinical evidence, but fidgeting, local heating, and intermittent exercise while sitting can prevent the detrimental changes in blood flow associated with sitting.
Figure S1. Gating Strategy and CD34 Purity

A)
**Fig S1.** A) Gating strategy for CACs and purity of magnetic separation. Live cells were gated first. Singlets were gated, followed by all mononuclear cells and a lymphocyte gate to capture CD34+ PBMCs. B) Flow cytometry data collected from magnetic isolation and subsequent staining with anti-CD34\(^+\) antibodies. Data represents positive fraction, negative fraction, and unseparated PBMCs from the same participant.
Figure S2. Example titration data for CD34

A) B)

C) D) E)

Fig S2. Example titration data for CD34. Concentration with greatest signal to noise ratio was selected as optimal concentration. A-D) Samples were prepared using step wise increasing concentrations of anti-body stains from 0.5 to 1.0 to 2.5 to 5.0 µL. E) Overlaid histograms with 0.5 in plum, 1.0 in green, 2.5 in blue, and 5.0 in gray.
Appendices

Appendix A

Department of Exercise and Sport Science

Medical History Questionnaire Screening

Subject:________________________________ Telephone:______________

Address:________________________________________________________________

Email:___________________________________ Age:______________________

Patient History

1. How would you describe your general health at present? YES NO

2. Excellent______ Good_______ Fair_____ Poor______

3. Do you have any health problems at the present time? _____ _____

4. If yes, please describe:______________________________________________

____________________________________________________________________

5. Have you ever been told you have heart trouble? _____ _____

6. If yes, please describe:______________________________________________

____________________________________________________________________

7. Do you ever get pain in your chest? _____ _____

8. Do you ever feel light-headed or have you ever fainted? _____ _____

9. If yes, please describe:______________________________________________

____________________________________________________________________

10. Have you ever been told that you have high blood pressure? _____ _____

11. If yes, please describe:______________________________________________
12. Have you ever had difficulty breathing at rest or with exertion?  
   13. If yes, please describe:__________________________________________________________

14. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year?  
   15. If yes, name the disease:______________________________________________________

16. Have you ever been treated for or told you might have diabetes?  
17. Have you ever been treated for low blood sugar?  
18. Have you ever experienced heat stroke or heat exhaustion?  
   19. If yes, when?______________________________________________________________

20. Are you now taking any pills, medications, or supplements?  
   21. If yes, please list:____________________________________________________________

22. Have you had any recent (within 1 year) difficulties with your:  
   a. Feet  
   b. Legs  
   c. Back  

Menstrual Cycle
23. What was the start date of your most recent menstrual cycle? ____________

Family History
24. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
Bone and Joint History

25. Have you ever been treated for Osgood-Schlatter’s disease? _____ _____

26. Have you ever had any injury to your neck involving nerves or vertebrae? _____ _____

27. Do you experience pain in your back? _____ _____

28. Have you ever had an injury to your back? _____ _____

29. If yes, did you seek the advice of a doctor? _____ _____

30. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____

31. Do you think you have a trick knee? _____ _____

32. Do you have a pin, screw, or plate anywhere in your body as the result of bone or joint surgery that presently limits your physical capacity? _____ _____

33. If yes, indicate where:

Activity History

34. During your early childhood (to age 12) would you say you were:

   Very active _____ Quite active _____ Moderately active _____ Seldom active _____

35. During your adolescent years (age 13-18) would you say you were:
36. Did you participate in:
   - Intramural high school sports? _____ _____
   - Community sponsored sports? _____ _____
   - Varsity high school sports? _____ _____
   - Active family recreation? _____ _____

37. Since leaving high school, how active have you been?
   Very active ____ Quite active____ Active____ Inactive____

38. Have you previous participated in strength training _____ _____

39. Do you participate in any moderate to vigorous activity at present? _____ _____

40. If yes, please list:

<table>
<thead>
<tr>
<th>Activity</th>
<th>Frequency</th>
<th>Duration</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

41. Whom shall we notify in case of emergency?
   Name:____________________________________________________
   Phone: (Home)__________________________ (Work)____________________

Signature:_________________________________________ Date:________________
## Appendix B

<table>
<thead>
<tr>
<th>NAME</th>
<th>ADDRESS</th>
<th>TELEPHONE: HOME/CELL / E-MAIL ADDRESS</th>
<th>OCCUPATION/EMPLOYER / BUSINESS PHONE</th>
<th>MARITAL STATUS: (check one) SINGLE ☐ MARRIED ☐ DIVORCED ☐ WIDOWED ☐</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td>GENDER: M / F</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>ADDRESS</th>
<th>GENDER: M / F</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gender: M / F</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Address:</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TODAY'S DATE</th>
<th>DATE OF BIRTH</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Date of birth</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reason for last doctor visit?</th>
<th>Date of last physical exam:</th>
<th>Have you ever had any other exercise stress test?</th>
<th>YES ☐ NO ☐ DATE &amp; LOCATION OF TEST:</th>
</tr>
</thead>
<tbody>
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<td></td>
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</table>

<table>
<thead>
<tr>
<th>Have you ever had any cardiovascular tests?</th>
<th>YES ☐ NO ☐ DATE &amp; LOCATION:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Date of last physical exam:</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Person to contact in case of an emergency</th>
<th>Phone (relationship)</th>
<th></th>
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<tbody>
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</table>

Please provide responses (YES or NO) to the following concerning family history, your own history, and any symptoms you have had:

### FAMILY HISTORY

<table>
<thead>
<tr>
<th>Had a:</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart attack</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary stent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac catheterization</td>
<td></td>
<td></td>
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<tr>
<td>Congenital heart defect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other chronic disease:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### PERSONAL HISTORY

<table>
<thead>
<tr>
<th>Have you ever had:</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>High blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
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<td></td>
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<tr>
<td>Any heart problems</td>
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<tr>
<td>Disease of arteries</td>
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<tr>
<td>Thyroid disease</td>
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<tr>
<td>Lung disease</td>
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<tr>
<td>Asthma</td>
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<tr>
<td>Cancer</td>
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<tr>
<td>Kidney disease</td>
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<tr>
<td>Hepatitis</td>
<td></td>
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<tr>
<td>Other</td>
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</tbody>
</table>

### SYMPTOMS

<table>
<thead>
<tr>
<th>Have you ever had:</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chest pain</td>
<td></td>
<td></td>
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<tr>
<td>Shortness of breath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart palpitations</td>
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<td></td>
</tr>
<tr>
<td>Skipped heartbeats</td>
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<td></td>
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<tr>
<td>Heart murmur</td>
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<td></td>
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<tr>
<td>Intermittent leg pain</td>
<td></td>
<td></td>
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<tr>
<td>Dizziness or fainting</td>
<td></td>
<td></td>
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<tr>
<td>Fatigue -- usual activities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snoring</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Back pain</td>
<td></td>
<td></td>
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<tr>
<td>Orthopedic problems</td>
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<td></td>
</tr>
</tbody>
</table>

### STAFF COMMENTS:

<table>
<thead>
<tr>
<th>Have you ever had your cholesterol measured?</th>
<th>YES ☐ NO ☐ If yes, value</th>
<th>Where:</th>
</tr>
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<tbody>
<tr>
<td></td>
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<table>
<thead>
<tr>
<th>Are you taking any prescription (include birth control pills) or nonprescription medications?</th>
<th>YES ☐ NO ☐</th>
</tr>
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<tbody>
<tr>
<td>For each of your current medications, provide the following information:</td>
<td></td>
</tr>
<tr>
<td>MEDICATION</td>
<td>Dosage—times/day</td>
</tr>
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<td>------------</td>
<td>-----------------</td>
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</tbody>
</table>
HOSPITALIZATIONS: Please list recent hospitalizations (Women: do not list normal pregnancies)

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Reason</th>
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</table>

Any other medical problems/concerns not already identified? □ Yes □ No □ If so, please list: ______

________________________

LIFESTYLE HABITS

Do you ever have an uncomfortable shortness of breath during exercise or when doing activities?
□ Yes □ No

Do you ever have chest discomfort during exercise? □ Yes □ No
□ If so, does it go away with rest? □ Yes □ No

Do you currently smoke? □ Yes □ No □ If so, what? Cigarettes □ Cigars □ Pipe □
□ How long have you smoked? ________ years
□ How much per day: □ < ½ pack □ ½ to 1 pack □ 1 to 1 ½ packs □ 1 ½ to 2 packs □ > 2 packs □
□ Have you ever quit smoking? □ Yes □ No □ When? ________
□ How many years and how much did you smoke? _____________

Do you drink any alcoholic beverages? □ Yes □ No □ If yes, how much in 1 week? (indicate below)
□ Beer ____ (cans) □ Wine ____ (glasses) □ Hard liquor ____ (drinks)
□ Do you drink any caffeinated beverages? □ Yes □ No □ If yes, how much in 1 week? (indicate below)
□ Coffee ____ (cups) □ Tea ____ (glasses) □ Soft drinks ____ (cans)

Are you currently following a weight reduction diet plan? □ Yes □ No
□ If so, how long have you been dieting? ______ months
□ Is the plan prescribed by your doctor? □ Yes □ No

Have you used weight reduction diets in the past? □ Yes □ No □ If yes, how often and what type? ______

ACTIVITY LEVEL EVALUATION

What is your occupational activity level? □ Sedentary □ Light □ Moderate □ Heavy □

Do you currently engage in vigorous physical activity on a regular basis? □ Yes □ No
□ If so, what type(s)? __________________________ How many days per week? ______
□ How much time per day? □ < 15 min □ 15-30 min □ 31-60 min □ > 60 min □
□ How long have you engaged in this type of activity? □ < 3 months □ 3-12 months □ > 1 year □

Do you engage in any recreational or leisure-time physical activities on a regular basis? □ Yes □ No
□ If so, what activities? __________________________
□ On average: How often? ______ times/week; for how long? _____________ time/session
□ How long have you engaged in this type of activity? □ < 3 months □ 3-12 months □ > 1 year □

Your fitness goals and objectives are: __________________________

________________________

STAFF COMMENTS: ________________________________

________________________
Appendix C

University of North Carolina at Chapel Hill
Consent for Storing Biological Specimens Without Identifying Information

Consent Form Version Date: ______________
IRB Study #: 16-3051
Title of Study: Effects of Prolonged Sitting on Cerebral Perfusion and Executive Function
Principal Investigator: Quentin Willey
Principal Investigator Department: Exercise and Sport Science
Principal Investigator Phone number: (919) 962-0396
Principal Investigator Email Address: qwilley@live.unc.edu
Co-Investigators: Erik Hanson, Claudio Battaglini, William Evans

Faculty Advisor: Lee Stoner
Faculty Advisor Contact Information: (919) 962-0534

What are some general things you should know about research?
Research is designed to gain scientific information that may help other people in the future. You may not receive any direct benefit from participating. There also may be risks.

You may refuse to take part in research. If you are a patient with an illness, you do not have to be in research in order to receive treatment.

Details are discussed below. It is important that you understand this information so that you can make an informed choice. 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 specimen repository or “biobank?”
Research with blood, tissue or body fluids (specimens) can help researchers understand how the human body works. Research can also answer other questions by using specimens. Researchers may develop new tests to find diseases, or new ways to treat diseases. In the future, research may help to develop new products, such as drugs. Specimens are commonly used for genetic research. Sometimes researchers collect and store many specimens together and use them for different kinds of research, or share them with other scientists; this is called a specimen repository or “biobank.”
The purpose of this particular repository or biobank is to better understand the mechanisms altering vascular repair during sitting. This will include blood plasma or whole blood. Future research may be assessment of mRNA with sitting to assess potential changes in protein development during sitting.

**How will the specimens be collected?**

- Provide specific details about how the specimen will be collected, OR
- if specimen already exists from previous clinical sources or research studies, inform subjects.

**What will happen to the specimens?**
Address specific areas about how the sample will be used and stored:

- Provide a clear description of the operation of the specimen repository
- Where will the specimen be stored?
- When will the specimens be destroyed?
- Inform subjects of conditions under which data and specimens will be released to other investigators.

**What are Genome Wide Association Studies (GWAS)?**
Delete if this does not apply to your study and you know data will never be submitted to GWAS.
The National Institutes of Health (NIH) has established a national database that will hold information from many individuals across the country, including medical information and genetic information. Your blood and tissues contain genes which are made of DNA that is unique to you. Access to this national database will be controlled and limited to other researchers.

**What are the possible benefits to you?**
Benefits to you are unlikely. Studies that use specimens from this repository may provide additional information that will be helpful in understanding the effects of prolonged sitting.

**What are the possible risks or discomforts involved with the use of your specimens?**
Describe immediate and long-term social, physical, and psychological risks/discomforts related to the specimen collection and storage. Address all risks that are applicable.

- Unknown risk: Subjects should be informed that there may be risks that at this time are unknown.
- Physical risks: If new samples are being collected include the physical risk associated with the sample collection for research purposes.
- In addition, use the following.

Sometimes there are concerns, even if hypothetical, that people may find out things about you (for example, that your genes make you susceptible to a certain disease). These concerns are minimized with this repository, because the specimens will not be able to be linked to your identity, so it will be impossible for anyone to know which sample came from you.
**Will there be any cost to you for storage of the specimens?**
There will be no cost to you for the storage and use of the specimens for research purposes.

**Will you receive anything for the use of your specimens?**
You will not be receiving compensation for taking part in this study.

**Who owns the specimens?**
Insert any contract, grant or agreement language related to specimen ownership or modify the following boilerplate.

Any blood, body fluids, or tissue specimens obtained for this purpose become the exclusive property of the University of North Carolina at Chapel Hill. This organization may retain, preserve or dispose of these specimens and may use these specimens for research that may result in commercial applications. There are no plans to compensate you for any future commercial use of these specimens.

**How will information about you be protected?**
The researchers will not have any identifying information about you so there is no risk to your privacy.

**Will you receive study results of future research involving your specimens?**
Most research with your specimens is not expected to yield new information that would be meaningful to share with you personally. In this case that would be impossible, because the researchers have no information that identifies you.

**Can you withdraw the specimen from this repository?**
You may not withdraw your specimen in the future because there are no identifiers on the specimen and the researchers will not know which specimen is yours.

**What will happen if you are injured by this research?**
Omit this section if the specimens have already been collected.
All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from having your specimen collected. If such problems occur, the researchers will help you get medical care, but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. However, by signing this form, you do not give up any of your legal rights.

**Who is sponsoring this research?**
This research is funded by a Junior Faculty Grant from the University of North Carolina. This means that the research team is being paid by the sponsor for doing the study. The researchers
do not, however, have a direct financial interest with the sponsor or in the final results of the study.

**What if you have questions about this research?**
You have the right to ask, and have answered, any questions you may have about this research. If you have questions, you should contact the researchers listed on the first page of this form.

**What if you have questions about your rights as a research subject?**
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 you may contact, anonymously if you wish, the Institutional Review Board at 919-966-3113 or by e-mail to IRB_subjects@unc.edu.

---

**Subject’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. I agree to my specimen(s) being stored without identifying code(s).

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Appendix D

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

Consent Form Version Date: _____________
IRB Study # 16-3051
Title of Study: Effects of Prolonged Sitting on Cerebral Perfusion and Executive Function
Principal Investigator: Quentin Willey
Principal Investigator Department: Exercise and Sport Science
Principal Investigator Phone number: (919) 962-0396
Principal Investigator Email Address: qwilley@live.unc.edu
Co-Investigators: Erik Hanson, Claudio Battaglini, William Evans

Faculty Advisor: Lee Stoner
Faculty Advisor Contact Information: (919) 962-0534

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 the current study is to examine the acute effects of prolonged sitting on
cardiovascular health and cognition. Findings from this study may identify a simple strategy for
offsetting the negative consequences of sitting, and may contribute to public health policy
pertaining to sedentary behavior.
You are being asked to be in this study because you are between the ages of 18-35, and are not engaging in 90 minutes of moderate intensity activity or 30 minutes of vigorous activity per week.

**Are there any reasons you should not be in this study?**
You should not be in this study if you are/have:

- Diabetes
- Heart Disease
- Atherosclerosis
- Arrhythmias
- Taking medications known to affect cardiovascular function
- Smoking cigarettes
- Pregnant

**How many people will take part in this study?**
There will be approximately 20 people in this research study at UNC-Chapel Hill.

**How long will your part in this study last?**
Should you wish to participate in the study, you will be required to attend Fetzer Hall for three visits. These visits will include an initial 30 minute visit to familiarize you, followed by two additional 4 hour visits.

**What will happen if you take part in the study?**
During visit one, participants will report to the UNC EXSS Laboratory where we will discuss the study with you. You will be screened for participation in the study which will include a medical history questionnaire. You will be fitted for a small probe and a chair. You will also take a cognitive test 7 to 10 times or until your scores normalize. Lastly, you will sit while measurements are taken during which you perform several sets of 10 calf raises.

During visits two and three a cannula, similar to an IV, will be inserted. You will lie for 20 minutes. After this, vascular health measures will be measured and 30mL of blood will be drawn. You will then be transferred to a sitting position, where you will remain for 180 minutes while watching a low-stimulus nature documentary. A probe will non-invasively measure brain blood flow continuously during this time, and after 10, 90 and 170 minutes of sitting, non-invasive vascular function and executive function will be measured. At 180 minutes, you will then be transferred to the supine position. Following 10 minutes of quiet rest, vascular health will be non-invasively measured again. Although you will use the restroom prior to sitting, if you need to use the restroom, this will be recorded, and you will be asked to repeat this movement for the subsequent visit. Visit two and three will be the same procedure, but you will be doing 10 calf raises to a metronome every 10 minutes in one of these visits.

**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?**
While in this study, blood will be collected. This requires an initial needle stick which may be uncomfortable and could cause bruising.

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

A Urine Pregnancy test provided by the study will be obtained for all women of child-bearing potential.

**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.

**How will information about you be protected?**
Your identity will be confidential and protected through the use of identification numbers. Additionally, all measurements will be collected in a private setting with access to the laboratory behind several secure doors. Identification numbers will be assigned to attached data and stored in a locked filing cabinet in the EORL, which only Quentin Willey and William Evans will have access to. Your identification number and associated data will only be accessible to the research team. All information uploaded to an external hard drive will be encrypted.

Participants will not be identified in any report or publication about this study. 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.

**What will happen if you are injured by this research?**
In the occurrence of a rare adverse event, all members of the research team are CPR/AED certified so that they can provide the proper care to the Participant. A member of the research team will be with the Participant the whole time while in the neuromuscular research lab and 1-2 members of the research team will be present during each exercise test. If deemed necessary, emergency medical services will be contacted.

All research involves a chance that something bad might happen to you. This may include the risk of personal injury. In spite of all safety measures, you might develop a reaction or injury from being in this study. If such problems occur, the researchers will help you get medical care,
but any costs for the medical care will be billed to you and/or your insurance company. The University of North Carolina at Chapel Hill has not set aside funds to pay you for any such reactions or injuries, or for the related medical care. You do not give up any of your legal rights by signing this form.

**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.

**Will you receive anything for being in this study?**

You will be receiving measures of cardiovascular health reports for taking part in this study. Otherwise, there will be no compensation for study participation.

**Will it cost you anything to be in this study?**

If you enroll in this study, you will not have any associated costs.

**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
Appendix E

Subject ID _____ Date & Time____________ Fasted: Y or N

Time of Protein Ingestion: _________ Stress level 0 to 10 ____

Last Exercise Session: _________ Did you walk/run/bike to campus? _________

Previous NIRS measures

Upward distance (cm):____ Distance to the right of previous dot (cm):____

Calf

Leg used: L or R

Dot on Tibia, Dot on medial malleolus

Length from malleolus to bottom of stencil: ______
Lines used (i.e. 1 or 10): ______

Side of line: toward high OR low

---

**Electrocardiogram (ECG) and HRV**

Resting HR: ______

---

**Metronome**

Set Metronome to 20 BPM every 10 minutes with buzzer

Start time: ______

10__ 20__ 30__ 40__ 50__ 60__ 70__ 80__ 90__ 100__ 110__ 120__

130__ 140__ 150__ 160__ 170__
**Water**

Y   N

---

**PWA**

Arm Side:  R  L

**PWV**

Carotid to Notch: __________cm  Notch to Top of Cuff: __________cm

Femoral to cuff distance: __________ cm
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### Blood Draw

**Pre time:** _____  **Post time:** _____
Appendix F

SitR Familiarization

Subject ID: ____________________ Date: _____________  Group: _________

Sex _____ Height (in/cm): _____/_____  Weight (lbs/kg): __________/_________

DOB: _____/_______/_______  Age: __________

Time: _________

Urine Pregnancy Test:    Y    N

Calculate and Give Protein Bars:   Y    N

Screening, Consent and Medical Health History?    Y    N

Randomization for Trial 1:  Raise    OR    Sit

Chair Settings and Foot Placement

69
Height _____ (cm)  Foot Placement _____ (cm)

NIRS placement: Head

Head Btwn eyes to occipital protuberance(cm): _____  10% of distance upwards (cm):____

Remeasure circumference ______  5% of circum to Right of previous dot (cm):____

Calf

Malleolus to bottom line Tibia Length (cm): ____

Lines used (i.e. 1 or 10) :_______

Side of line: toward high   OR   low

Calf Practice

Set Metronome to 20 BPM    Number of Practice sets _____
**Stroop Practice**

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<tr>
<th>Variable</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Trial 4</th>
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Appendix G

NIRS Optode Positions

Cerebral:

- **Positioned at FP1 and FP2.**
- Place elastic band on head.
- Measure Nz to Lz (approx. 36cm).
- Mark distance of 10% upwards for Nz and Lz, these are Fpz and lz.
- Move elastic band onto 10% line.
- Measure circumference at 10% line (Fpz to lz, approx. 56 cm).
- Measure and mark 5% of total circumference to left and right of Fpz and mark.
- These are Fp1 (LEFT) and Fp2 (RIGHT).

*Position probe 1 on Fp1 and probe 2 on Fp2.

Gastrocnemius:

- **Positioned bilaterally on the medial gastrocnemius belly.**
- Ask patient to stand against bed and move on to tip toes (if possible)
- Identify outer edge of muscle. Identify muscle belly and mark with dot.
  (This is just a preliminary identification to help with initial template placement.)

- Ask participant to sit on a bench or table high enough that their leg is relaxed and suspended with approximately 90° between calf and thigh.
- Next, find the joint line between the femur and the tibia on the medial side. Follow that joint line by palpation laterally towards the patella. Mark the point at which the joint line and the patella first intersect with one dot.
- Then, palpate the medial malleolus and find an approximate center and mark another dot.
- Between the two dots described above, use a flexible meter stick to mark a straight line at the level of the gastrocnemius that if continued would intersect with each dot (Image A).
- The line just drawn will be used as a base for the Calf ROI Stencil. Place the edge of the stencil on the line and make sure the slots of the stencil are parallel to the leg and on top of the medial gastrocnemius muscle belly (Image B).
- Mark the bottom edge of the stencil by making a perpendicular line with the line already marked on the participant’s leg. Then, measure and record the distance (cm) between the line at the bottom of the stencil to the dot placed in the center of the medial malleolus. This distance will be used on this participant in future visits.
- Finally, there will be 10 slots labeled on the stencil to mark where the NIRS probe will be secured. Two or three slots will need to be marked on the first visit in order to find the best NIRS placement. The best placement will be a relatively flat surface on the medial gastrocnemius.
- Note which slots were marked, which slot mark will be used, and on which side of the mark the NIRS will be placed (+ = towards higher number or - towards lower number).
- Remove any slot markings not being used from skin.

*Position probe 3 on left and probe 4 on right.*

A.  B.  C.
REFERENCES


