THE ROLE OF THE AUTONOMIC NERVOUS SYSTEM ON VASCULAR FUNCTION DURING A PASSIVE LIMB MOVEMENT TEST

A Thesis
by
RACHEL SZEGHY

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APPROVED BY:

Rebecca M. Kappus, Ph.D
Chairperson, Thesis Committee

Matthew J. Rogatzki, Ph.D.
Member, Thesis Committee

Stephen M. Ratchford, Ph.D.
Member, Thesis Committee

Kelly J. Cole, Ph.D.
Chairperson, Department of Exercise Science

Marie Hoepfl, Ed.D.
Interim Dean, Cratis D. Williams School of Graduate Studies

Abstract

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Rachel Szeghy
B.S., Appalachian State University
M.S., Appalachian State University

Chairperson: Rebecca M. Kappus, Ph.D.

Background: Cardiovascular diseases (CVD) cause an estimated 17.9 million deaths globally every year, with over 83 million people in the United States having one or more forms of CVD. Vascular dysfunction is often a prerequisite in the development of CVD. The passive limb movement (PLM) test has recently been developed to assess vascular function and identify early signs of vascular dysfunction. However, the role of the autonomic nervous system, which can act on the vasculature to alter assessment outcomes, during the PLM test response is not known. **Purpose:** The purpose of this study was to elucidate the contribution of the autonomic nervous system, via muscle sympathetic nerve activity (MSNA), on vascular function measures during the PLM test. **Methods:** We assessed 5 (1M/4F) healthy, premenopausal adults aged 20±2yr. Hemodynamic responses via Doppler ultrasound, and MSNA responses via microneurography, to PLM in a room temperature condition and during a cold pressor test (CPT) were assessed in a randomly assigned order. A single PLM (sPLM) movement and a continuous PLM (cPLM) were performed in each condition. Stages

were defined as baseline, sPLM, and cPLM. **Results:** No significant differences were observed in MSNA across conditions (MSNA burst frequency, p=0.31; MSNA burst incidence, p=0.57) or stage (MSNA burst frequency, p=0.62; MSNA burst incidence, p=0.57). There was a main effect of stage for blood flow response, expressed as area under the curve for 60s (BF_{AUC60}), with baseline being different than cPLM (p=0.04). There was a main effect of stage for vascular conductance response (VC_{AUC60}), with baseline being different than cPLM (p=0.02). During the CPT, a strong correlation between $\Delta BF_{average}$ and ΔMSNA burst incidence was observed (r=-0.94; p=0.02). **Conclusion:** We were unable to detect differences in MSNA during the CPT, and therefore were unsuccessful in identifying the role of MSNA on CPT. However, it seems as though PLM may act independently of MSNA as we observed differences in vascular responses between conditions.

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List of Abbreviations and Symbols

aBF	anterograde blood flow
ADMA	asymmetric dimethyl-L-arginine
ANOVA	analysis of variance
AUC	area under the curve
BF	blood flow
BI	burst incidence
BL	baseline
cGMP	cyclic guanosine 3',5-monophosphate
cPLM	continuous passive limb movement
CO	cardiac output
COPD	chronic obstructive pulmonary disease
CPT	cold pressor test
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
eNOS	endothelial nitric oxide synthase
FMD	flow-mediated dilation
HF	heart failure
HFrEF	heart failure with reduced ejection fraction
HR	heart rate
LBNP	lower body negative pressure
LMMA	NG-monomethyl-L-arginine
MAP	mean arterial pressure
MSNA	muscle sympathetic nerve activity

NO	nitric oxide
MAP	mean arterial pressure
NE	norepinephrine
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
PLM	passive limb movement
rBF	retrograde blood flow
RH	reactive hyperemia
ROS	reactive oxygen species
SBP	systolic blood pressure
SNS	sympathetic nervous system
sPLM	single passive limb movement
SD	standard deviation
SR	shear rate
SV	stroke volume
VC	vascular conductance
VSMC	vascular smooth muscle cell
ΔPeak	change to peak

CHAPTER 1: INTRODUCTION

Background

Cardiovascular diseases (CVDs) cause an estimated 17.9 million deaths globally every year (1, 2), with over 83 million people in the United States having one or more forms of CVD (3). In the Unites States, the burden of CVDs, including medical expenses, loss of productivity, and death, is estimated to cost $378 billion per year (4). The prevalence and cost of CVD, globally, is expected to increase (2, 5) in the coming years, further elevating the burden to society and to the individual. Performing tests to evaluate vascular health to predict risk of, or identify, CVD is important for early treatment in order to improve health of the individual and reduce disease burden.

The vasculature and autonomic nervous system work in tandem to regulate blood flow and blood pressure (6, 7), which is important to maintain homeostasis and prevent disease. Vascular and/or autonomic dysfunction, therefore, are indicators of an increased risk for CVD (8-11). Many non-invasive assessment techniques exist to examine vascular structure and function. The passive limb movement (PLM) assesses microvascular function by demonstrating the blood flow (BF) response to the movement. This technique is thought to be >80% nitric oxide (NO) mediated in young, healthy adults (12), allowing PLM to assess NO bioavailability. Blunted response to the PLM is indicative of vascular dysfunction. For example, in patients with heart failure with reduced ejection fraction (HFrEF) (13), BF response is blunted, suggesting reduced NO bioavailability.

The PLM elicits an increase in blood flow, the mechanism behind which is hypothesized to be due to changes in peripheral artery tone (14). Alterations in vascular tone are regulated by the endothelium, more specifically by NO (12, 15, 16). When the limb is

moved, NO is released, resulting in vasodilation (12, 16, 17). When the PLM is performed after infusion of N^G- monomethyl-L-arginine (L-NMMA), a NO synthase inhibitor, PLM blood flow response is blunted. This suggests blood flow response during the PLM is up to 80% NO dependent (12, 16).

However, recent literature suggests the autonomic nervous system plays a role in the PLM response. *Venturelli* et al. utilized an arm crank exercise to increase autonomic activity before a PLM was performed. They observed muscle sympathetic nerve activity (MSNA) was inversely related to vascular conductance (VC) and BF area under the curve for 60 seconds (BF_{AUC60}). VC is BF set relative to mean arterial pressure (MAP) and is another indicator of CVD risk as reduced VC is correlated to increased MSNA, autonomic dysfunction which is common among disease states (12, 18, 19). The AUC of both BF and VC have been suggested to be indicators of overall NO bioavailability, therefore a greater AUC would be indicative of greater NO bioavailability and a greater response to PLM (12). These results suggest an autonomic component of skeletal muscle VC and BF regulation. However, a limitation to this study is the presence of exercising metabolites, which may have confounded PLM results (20).

Another method of increasing MSNA, without the presence of exercising metabolites, is the cold pressor test (CPT), which consists of the cutaneous application of ice water, such as by submerging a limb in ice water or placing an ice pack on the head. This test assesses sympathetic and hemodynamic responses to the cold stimulus (86, 87). CPT results in an increase in MSNA as the cold water is a noxious pain stimulus (94, 95). This test is well-established, as it is known to increase MSNA in both young and old individuals (30, 87, 114), as well as in those with heart failure and with coronary artery disease (115).

Statement of the Problem

The extent to which sympathetic nerve activity impacts measures of vascular health and function during the PLM assessment are unknown. Elucidating the role of sympathetic nerve activity, in addition to the role of NO, is important to ensure accurate interpretation of PLM results. Elucidating the impact of the sympathetic nervous system in PLM response is important to identify whether reduced PLM response is indicative of primarily vascular dysfunction in NO bioavailability, or if it is neural. This will aid in assessing CVD risk through identifying what part of the body is predominantly experiencing dysfunction, allowing for more comprehensive assessment of the participant (21).

Purpose Statement

The purpose of this study is to elucidate the contribution of MSNA on vascular function measures during the PLM.

Hypotheses

We hypothesize during the CPT, MSNA and hemodynamic measures will be increased, which will result in a blunted blood flow and vascular conductance response to PLM, when compared with control/room temperature conditions.

Delimitations

- Male and female participants were utilized to represent the population.
- Post-menopausal females were not be utilized in an effort to eliminate the effects of menopause on study outcomes.

- Individuals with cardiovascular, renal, metabolic, or pulmonary diseases, were not included in the study to minimize the confounding effects of pathophysiology on study outcomes.
- Participants fasted for >4 hr to minimize the effect of metabolism.
- Participants refrained from caffeine for >12 hr, and from alcohol, and exercise for >24 hr to reduce effects on the vasculature.
- Menstrual cycle was controlled for by having participants schedule visits around the onset of menstruation to reduce effects of fluctuating hormones.
- Participants with a history of fainting from needles were not used to reduce risks to the participant.
- Pregnant females were not be utilized to reduce risks to the fetus.

Limitations

- Males and females were included and were not be split into two groups. Any potential sex differences are unknown.
- Participants were from the same geographic region.
- All participants were premenopausal and between the ages of 18 and 60 years, thus prohibiting inferences of study findings to children and older adults.

Definition of Terms

For the purpose of the study, the following definitions will be utilized:

Cardiac output (CO): HR · SV

Cardiovascular disease (CVD): diseases of the heart and/or blood vessels (1)

Cold pressor test (CPT): a test where a participant submerges a body part in cold water for an extended period of time. This test is designed to increase sympathetic activity (22)

Flow-mediated dilation (FMD): a vascular function assessment, typically performed at the brachial artery, characterized by baseline measures, followed by a cuff occlusion, then reperfusion (23)

Microneurography: technique used to examine sympathetic activity. Briefly, a needle is inserted into a peripheral nerve and recordings of sympathetic activity are taken (24)

Muscle sympathetic nerve activity (MSNA): efferent nerve signals identified most commonly through microneurography

Passive limb movement (PLM): a vascular function assessment where a research team member moves the participant's leg for them, without muscle contraction, as central hemodynamic and blood flow responses to the movement are recorded (14)

Reactive hyperemia (RH): the exaggerated reperfusion following a period of ischemia (25)

Sympathetic activity: part of the autonomic nervous system, sympathetic nerves send out nerve signals which result in alterations to the cardiovascular system (26)

CHAPTER 2: REVIEW OF LITERATURE

Cardiovascular Disease

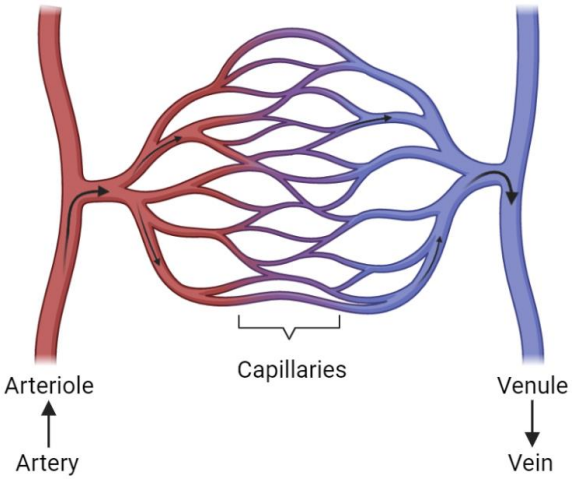
Globally, the leading cause of death are CVDs, causing an estimated 17.9 million deaths every year (1). Comprised of diseases of the heart and blood vessels, such as heart failure and coronary artery disease, CVDs are more prevalent in elderly populations (27). In the United States the burden of heart disease, including medical expenses, loss of productivity, and death, is estimated to cost $378 billion per year (4) and is projected to keep increasing (27, 28). Risks for developing CVD include inadequate levels of physical activity, unhealthy diet, smoking, hypertension, high blood glucose, high blood cholesterol, and increasing age (27, 29-34). Coinciding with these risk factors are decrements to the vascular and autonomic systems. Vascular dysfunction, such as development of atherosclerosis, increased inflammation, and endothelial dysfunction, are often prerequisites for CVD; these factors are often concurrent with the presence of autonomic dysfunction, as indicated by increased sympathetic outflow (10, 35-37). Based on this, several non-invasive tests have been created to examine vascular and autonomic health in order to identify possible dysfunction, and therefore risk of CVD. Vascular structure and function are examined through pulse wave velocity, FMD, and carotid stiffness testing, whereas microneurography measures MSNA, which is indicative of autonomic function. Each test examines slightly different aspects of vascular health and have different mechanisms driving the responses observed during testing.

Vascular System

The vascular system is composed of vessels through which blood is moved throughout the body (Figure 1). Broken into two parts, the vascular system is composed of the macro

vasculature (arteries and veins) and the microvasculature (arterioles, capillaries, and venules). Arteries are the largest blood vessels and are highly compliant, as they have a thick tunica media comprised of elastic connective tissue and smooth muscle. Veins, which range from 0.5 mm to 3 cm in diameter, have a thick tunica externa, comprised of collagen and elastic fibers. Arterioles are microscopic arteries, ranging from 15-300 μm in diameter. They regulate the blood flowing from arteries to capillaries by altering resistance. Capillaries are the smallest blood vessels, ranging from 5-10 μm in diameter. Composed of primarily thin layers of the endothelium and a basement membrane, these vessels are responsible for the exchange of substances between blood and interstitial fluid. Finally, venules are thin blood vessels ranging from 20-200 μm in diameter which begin movement of blood back to the heart and are highly distensible, not readily maintaining their shape (6).

Figure 1: Blood Flow in the Vascular System



Blood vessels are lined with endothelium, a single layer of endothelial cells which function to maintain the wall of the blood vessel and to maintain circulatory function. Under

basal conditions, endothelial cells release vasoactive substances, like NO, which inhibit platelet aggregation and cause vasodilation. This mechanism is most active in arteries and least active in veins (38, 39). Within the endothelial cells, eNOS synthesizes NO from L-arginine in the presence of cofactors, such as tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate, generating L-citrulline as a byproduct. After it is synthesized, NO diffuses into vascular smooth muscle cells (VSMCs), activating guanylate cyclase which leads to an increase in intracellular cyclic guanosine 3',5-monophosphate (cGMP) and cGMP-mediated vasodilation through smooth muscle cell relaxation (**Figure 2**) (40-42). Stretching of the blood vessel wall and flow sheer stress, such as is experienced during exercise, activates eNOS stimulating NO synthesis (39, 43, 44). Endothelial dysfunction (Figure 3) is a precursor in many CVDs, such as atherosclerosis, thrombosis, chronic venous insufficiency, atherogenesis, coronary artery disease, hypercholesterolemia, hypertension, heart failure, and other inflammatory syndromes (38, 39, 43, 45).

Figure 2: L-arginine nitric oxide synthesis pathway. NO, nitric oxide; NOS, nitric oxide synthase, GC, guanylate cyclase; cGMP, cyclic guanosine-3',5-monophosphate; GTP, guanosine triphosphate (41)

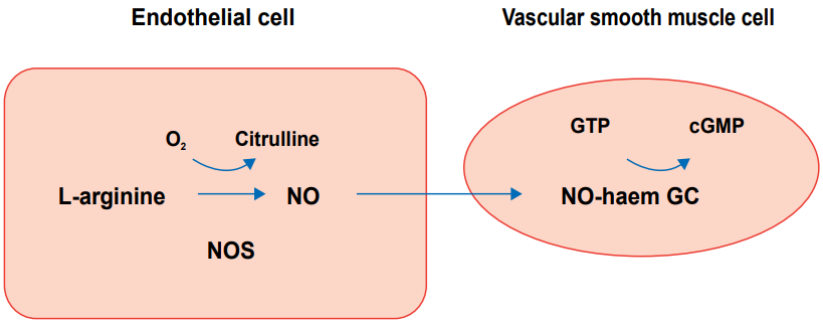
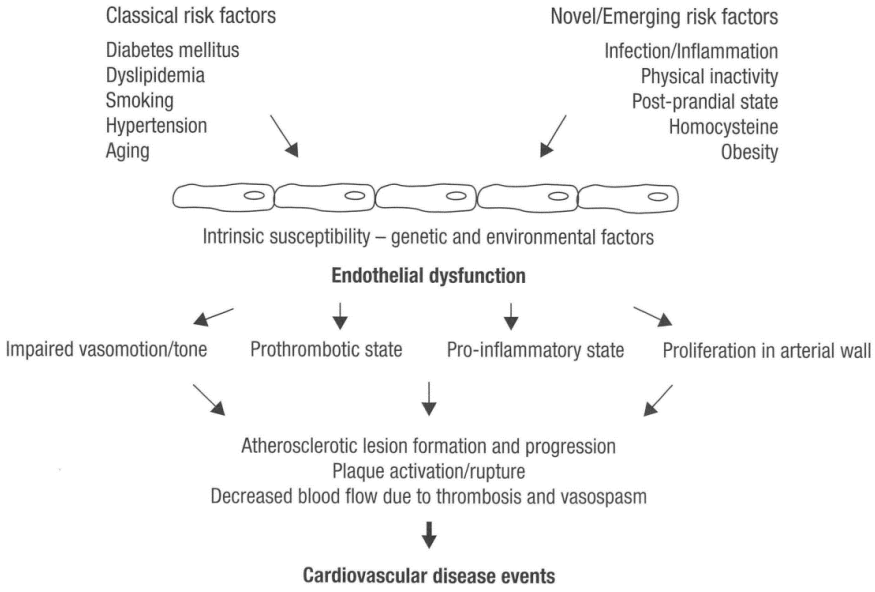


Figure 3: Risk factors resulting in endothelial dysfunction in turn increases cardiovascular disease prevalence (39).



Endothelial dysfunction can result from several alterations to local regulatory factors, most notably, attenuation of NO bioavailability and deficiencies in NO production (46). NO is a reactive species with an unpaired electron. Therefore, reactive oxygen species (ROS) react with NO forming peroxynitrite ($ONOO^-$), a reactive nitrogen species which increases oxidative stress, causes death of endothelial cells, and reduces NO bioavailability (46, 47). ROS is a term used to describe the molecules which are formed during the incomplete reduction of oxygen, such as the molecules containing the superoxide anion, hydrogen peroxide, and the hydroxyl radical (48). These free radicals have unpaired electrons and are highly unstable, causing damage to intra-/inter-cellular components. Increased production of ROS leads to oxidative stress, which is a common risk factor for cardiovascular diseases, such as atherosclerosis, hypertension, and diabetes (49, 50). ROS also plays a role in apoptosis, inflammatory responses, cellular growth, and alterations in vascular tone (51). However, ROS

are necessary in living organisms as they maintain cellular homeostasis and promotion of antioxidant synthesis to defend against future ROS, where the oxidative stress caused by ROS is balanced out by antioxidant reduction of these free radicals (52, 53).

Uncoupling of eNOS will reduce NO bioavailability and can occur in several different ways. First, eNOS needs dimerization in the presence of heme and BH_4 to move an electron to L-arginine which allows for NO synthesis. If this process is disrupted, NO is not synthesized and ROS are produced, increasing oxidative stress and endothelial cell death (46, 47). Further, reductions in BH_4 bioavailability seems to be a primary contributor of eNOS uncoupling within the vasculature (47, 54).

Another mechanism of eNOS uncoupling is lack of bioavailability of L-arginine or elevated amounts of L-arginine inhibitor, asymmetric dimethyl-L-arginine (ADMA) (46, 47). ADMA is suggested to be a primary contributor of eNOS uncoupling within pulmonary artery endothelial cells (55). Further, in elderly participants with atherosclerosis, serum ADMA levels were inversely related to vascular NO bioavailability and elevated levels of ADMA were associated with eNOS uncoupling (56).

Blood flow through blood vessels

In the nineteenth century, Jean Léonard Marie Poiseuille performed research on the cardiovascular system, specifically trying to identify the relationship between flow, pressure gradients, and the dimensions of the vessel. Now known as the Poiseuille's equation, he found flow is related most strongly to the fourth power of the vessel's radius, expressed as:

$$Q = \frac{\pi P r^4}{8\eta l}$$

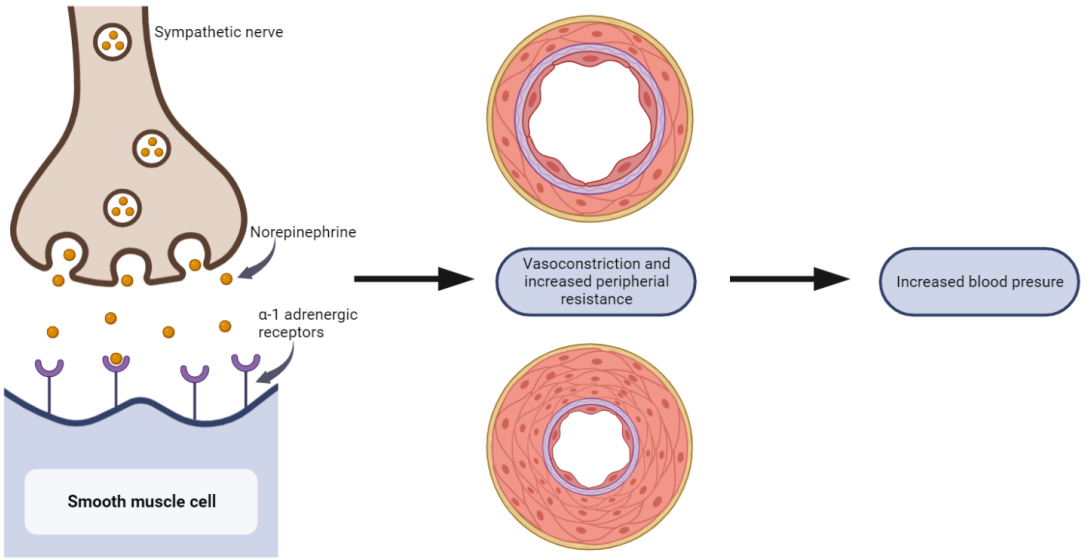
Where Q is flow rate (cardiac output), P is pressure, r is the radius of the vessel, η is fluid viscosity, and l is the vessel length. Poiseuille's experiments also confirmed the greatest controller of peripheral resistance is the smallest blood vessels, the arterioles. Applied to circulation, Poiseuille's equation assumes the following: 1) blood is homogeneous and its viscosity is the same regardless of shear rate, 2) blood does not slip along the vessel wall, 3) flow is laminar, 4) flow rate/velocity is constant, 5) the vessel is longer than the area being studied, 6) the vessel is cylindrical, therefore the cross-sectional view is circular, 7) vessel diameter does not vary with internal pressure (39).

Autonomic nervous system

The autonomic nervous system also plays an integral role in blood vessel function. The autonomic nervous system is comprised of the sympathetic (SNS), parasympathetic, and enteric nervous systems, which regulate responses to stress in order to maintain physiologic homeostasis (57). However, only the SNS innervates blood vessels and plays a large role in regulating the cardiovascular system (7).

In a healthy, non-diseased person, the SNS is responsible for the "fight or flight response", which is augmented in times of stress and attenuated in times of calm. During this stress response, norepinephrine (NE) is released from sympathetic neurons and binds to β−1 adrenergic receptors on the heart, increasing heart rate (7, 58). NE also binds to α-1 adrenergic receptors in smooth muscle cells in blood vessels, inducing vasoconstriction, ultimately increasing peripheral vascular resistance and blood pressure (Figure 4)(58).

Figure 4: Norepinephrine effects on the vasculature and blood pressure. Norepinephrine binds to α-1 adrenergic receptors in smooth muscle cells, which constricts blood vessels and increases peripheral resistance and increases blood pressure.



Neuronal nitric oxide synthase (nNOS) is found within VSMCs and plays a role in modulating calcium (Ca^{2+}) homeostasis (43). In eNOS knockout mise, nNOS has the ability to partially compensate for eNOS-dependent flow-induced dilation of coronary arteries (59). It is suggested this mechanism may also occur within the coronary vascular beds of humans, however the relative contributions of eNOS and nNOS have not been identified (60, 61). In cardiovascular cells, nNOS is expressed in cholinergic neurons and sympathetic neurons innervating the sinoatrial node and reduces heart rate. The role of nNOS dysfunction in pathogenesis of CVD is not well established, and eNOS therefore is thought to play the largest role in endothelial dysfunction and subsequent CVD (43). The third type of NOS is inducible

NOS (iNOS), transcription of which occurs in inflammatory conditions within cells such as endothelial cells, vascular smooth muscle cells, nerve cells, and cardiac myocytes (62, 63). Unlike nNOS and eNOS, iNOS is calcium independent (64), resulting in increased production of NO until all the substrate and cofactors are used up (65). However, iNOS is not considered to be expressed in healthy tissues and is suggested to be found only in states of pathological remodeling, such as HF (43, 66). The relationship between iNOS and CVDs is not known, but iNOS is thought to contribute less to NOS and vascular function than nNOS and eNOS (43, 67).

In CVD, dysfunction of the vascular and autonomic systems is often noted. When high amounts of NO are produced in response to inflammation caused by myocardial infarction, negative inotropic effects occur which compound dysfunction. Nitric oxide synthase (NOS) uncoupling results in increased production of ROS, increasing inflammation (46). NO-dependent endothelial dysfunction is recognized to be the first step in atherogenesis progression and in hypertension (43). Autonomic dysfunction may cause alterations to hemodynamic controls which will then disrupt homeostatic control mechanisms of the cardiovascular system. This, in turn, may promote cytokine storm which increases inflammation and increases cardiovascular disease risk (68, 69).

Vascular Assessment

The first human pulse recording was achieved in 1855 by Karl Vierordt who placed one end of a lever on top of the radial artery and dipped the other end in ink and placed it on a piece of paper. Étienne Jules Marey developed a simpler and more accurate sphymograph in the 1860s (70), which remained in use until the 1900s when Otto Frank began to work on a

manometer to measure pulsatile pressure. Today, manometers are still in use to measure pulse pressures, although modern manometers are more accurate and smaller than those of the 1900s due to revolutions in technology. However, these methods were unable to examine BF more directly via examining pulsatile flow because they relied on pulsatile pressure waveforms (39).

In 1997, continuous wave Doppler ultrasound was used to measure BF by detecting shifts in the frequency of reflected sound utilizing a piezoelectric crystal, and degree of ischemia. Blood flow velocity was calculated using the following equation:

$$\boldsymbol{f_d = \frac{f_t\ 2v\ cos\theta}{c}}$$

Where f_d is the Doppler frequency, f_t is transmitted frequency, v is velocity, θ is the angle between the direction of flow and the axis of the ultrasound beam looking toward the transducer, and c is the velocity of sound in blood (71, 72).

Concurrently, B-mode on the Doppler ultrasound is used to image blood vessel anatomy. Duplex instruments display a B-mode image and a spectral display; therefore, Doppler ultrasound duplex sonography is able to detect real-time changes in blood vessel anatomy as well as BF velocity (71, 72) Doppler images are taken 2-3 cm proximal to bifurcations to measure laminar flow as the disturbed flow found nearest to the bifurcations do not represent the true BF of the vessel (72, 73).

Peripheral Vascular Function Assessments

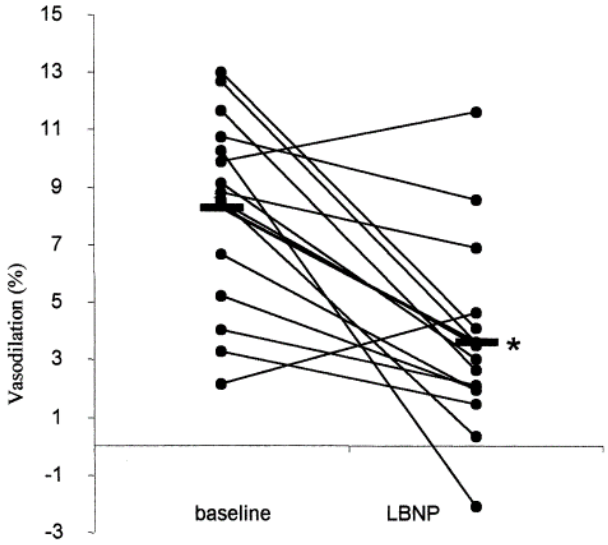
Brachial Artery FMD

The flow-mediated dilation (FMD) technique was developed in 1992 by Celermajer *et al*. (74) as a non-invasive technique used to measure vascular function. It is currently considered the 'gold-standard' non-invasive test of vascular function (23, 75), and it is

reflective of coronary vascular function (76). During the FMD, Doppler images are taken of the brachial artery at rest and after a 5 min cuff occlusion of the lower arm. The dilatory response of the brachial artery after cuff release is measured and is reported (77). Reactive hyperemia (RH) data collected from FMD is indicative of microvascular function. Assessing FMD requires a skilled sonographer as high-quality images must be captured, as analysis relies on detecting minute changes in artery diameter. The margin for error is small, as a ~0.1 mm difference in brachial artery diameter can result in significant differences in FMD between control and CVD groups (14). However, the literature is controversial in the degree to which FMD is NO-mediated. Previous literature has identified FMD to be primarily NO-dependent (78, 79), however this may be in part due to differences in FMD technique (80). Recent work by Wray *et al.* suggests the NO independent nature of FMD (80). The discussion of NO mediation within FMD is ongoing, therefore using FMD as a marker of NO bioavailability in young, healthy adults should be cautioned.

Many risk factors associated with impaired FMD function are also associated with increased sympathetic activity (81). Hijmering *et al.* examined the autonomic component of the FMD response by stimulating an increase in sympathetic activity using a lower body negative pressure box (LBNP) (82, 83). LBNP decreases venous return and increases baroreflex unloading, which results in increases in blood pressure and peripheral resistance as the central nervous system attempts to maintain cardiac output, despite lowered venous return (82, 84). FMD was reduced during LBNP (Figure 5), possibly due to increased vasoconstrictor tone, decreased shear stimulus, or decreased NO bioavailability. However, with the infusion of a local α-adrenergic blocker, FMD was not reduced with LBNP, suggesting sympathetic activity reduces FMD by an α-adrenergic mechanism (83).

Figure 5: MSNA reductions due to LBNP (83). When participants are placed in a LBNP chamber, sympathetic activity increases in order to restore blood pressure. However, this also results in systemic vasoconstriction, reducing the brachial artery vasodilatory response to FMD.

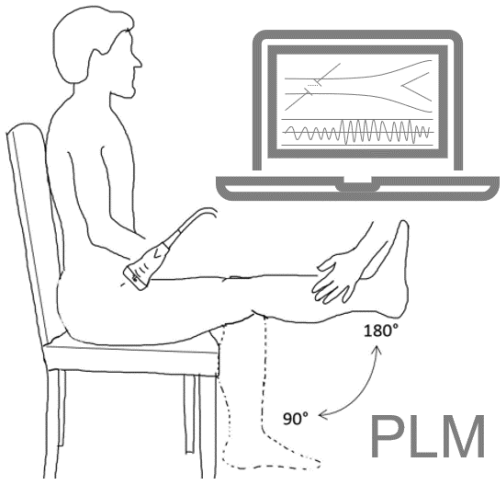


Further, brachial artery FMD strongly correlates with coronary vascular function (85), as well as predictions for cardiovascular event risk, as a 1% lower brachial artery FMD is associated with a 13% higher cardiovascular event risk (9, 86). In young, healthy adults, normal FMD is around 8.81% (87). In patients with HFrEF (2.63±1.57%; control: 5.62±2.60%), brachial artery FMD was significantly lower than age-matched healthy controls (88), as well as in those with chronic obstructive pulmonary disease (COPD: 5.40%; control: 10.10% (89)), coronary artery disease (CAD: 5.7±4.8%; control: 12.6±6.7% (90)), and peripheral artery disease (PAD: 6.0%; control: 8.0% (91)).

PLM

In 1999, Rådegran and Stalin used a passive movement of the leg to overcome the inertia of a flywheel prior to exercise on a knee extension ergometer. The authors observed an increase in femoral artery blood flow (BF), measured by Doppler ultrasound, after the passive limb movement (92). PLM is NO dependent, as there was a significant difference in femoral artery BF between the control and NO inhibition (by infusion of N^G-monomethyl-L-arginine; L-NMMA) trials (12). Modern use of the PLM technique consists of a member of the research team passively flexing and extending the participants leg in a full range of motion, without the participant helping, or resisting, the movement, as another research team member records femoral artery blood flow (Figure 6) (14).

Figure 6: Passive limb movement (14). One member of the research team moves the relaxed leg of the participant in a full range of motion from 90° to 180°.

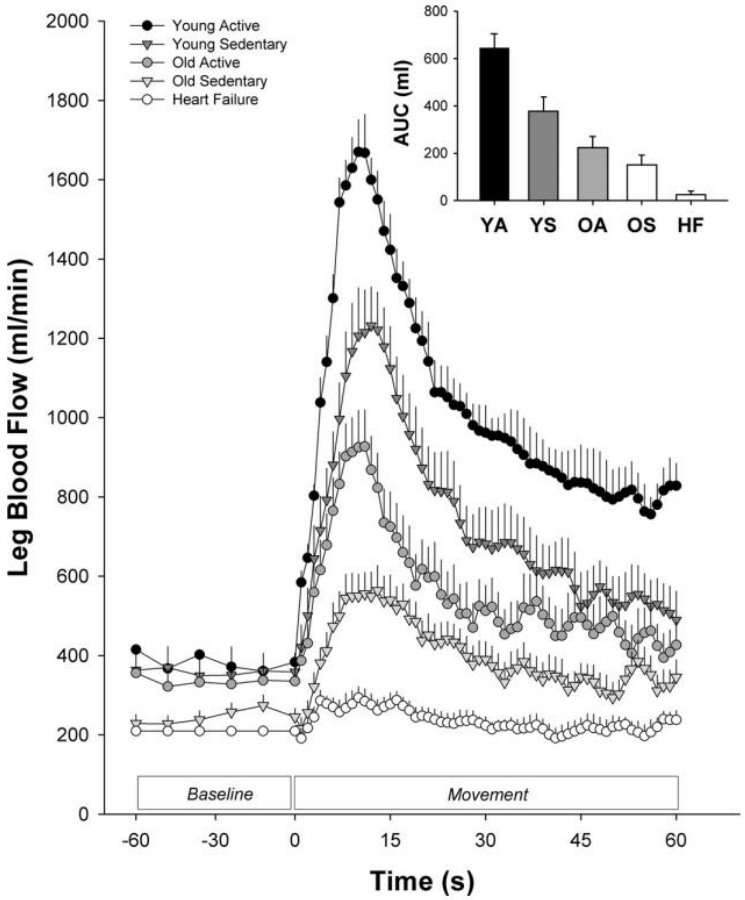


Assessment of PLM does not require as skilled of a sonographer as FMD, as a 0.1 mm error in PLM diameter assessment would minimally influence PLM response. Unlike FMD,

PLM assessment is predominantly NO-mediated and is not as highly influenced by shear stress. Both FMD and PLM assess RH and microvascular function, yet only FMD is able to assess macrovascular function (14).

During the PLM, heart rate (HR), stroke volume (SV), cardiac output (CO), and mean arterial pressure (MAP) tend to increase with movement, facilitating the increase in blood flow (BF) also observed (14). PLM response is influenced by both age and activity level, where older adults will have less of a BF response than young adults, and physically active individuals will have a larger BF response than their sedentary counterparts. The increasing slope of the BF response is indicative of NO synthesis, where a steeper slope means faster synthesis. The peak BF suggests the overall amount of NO synthesized and the descending slope is indicative of how quickly NO is washed out. Further, the area under the curve indicates the overall NO response across the entire movement and is thought to be indicative of overall NO bioavailability (12, 14, 15, 21). CVD can also reduce BF response to PLM, such as in those with HF (Figure 7).

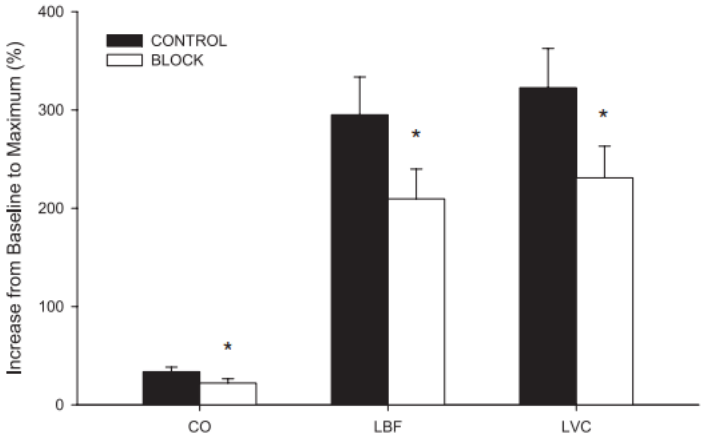
Figure 7: Passive limb movement blood flow response. YA, young physically active; YS, young sedentary; OA, old physically active; OS, old sedentary; HF, heart failure; AUC, area under the curve (14). Blood flow response to passive limb movement is reduced with age and increased with physical fitness.



Trinity *et al.* indirectly examined the central hemodynamic component of the hyperemic response to PLM (17). Authors performed PLM in two conditions, one normal and the other with a neural block (fentanyl) which would decrease feedback from group III and group IV afferent skeletal muscle fibers, as suggested by exercise in animal models (93, 94).

These fibers have been suggested to be the cause of the increased HR responses to PLM in humans (95). During the neural block condition, HR and SV did not significantly change with the passive movement. Interestingly, CO did increase in the neural block condition, although the increase was attenuated compared with the control condition. Further, MAP did not change during the neural block. The percent increase in BF was significantly reduced in the blocked condition as compared with control and the increase in vascular conductance (VC) was reduced in the block condition as compared with the control. VC is BF set relative to perfusion pressure (MAP) and is an indicator of vascular tone. Reductions in VC is associated with increased sympathetic activity (96). As blocking skeletal muscle afferent feedback altered hemodynamic responses (Figure 8), this suggests there is a central component to PLM response (17).

Figure 8: Percent increase of hemodynamic variables during a neural block compared with control (17). Under the block condition, hemodynamic responses are blunted.



Interestingly, body position seems to influence PLM responses, as noted by Trinity *et al.* (97). At baseline (BL), HR, SV, CO, MAP, and pulse pressure were numerically higher in the upright seated position compared with supine. This numerically higher values in the upright

position were also noted during the PLM. Compared with a supine position, the absolute change, the cumulative area under the curve (AUC) for HR, SV, and CO was larger in the upright position, indicating the increase in these variables was maintained for longer. Further, although BF and VC were not different between the two positions at BL, the PLM-induced increase in both was two times greater in the upright position compared with supine. The BFAUC was greater in the upright position compared with supine. AUC measurements are significant as they look at the entire response and not just the peak or the average values. The increased hydrostatic column of the upright position, as well as the possible contribution of the skeletal muscle pump, could be possible mechanisms behind these differences (97).

CPT

The cold pressor test (CPT) consists of the cutaneous application of ice water, such as by submerging a limb in ice water or placing an ice pack on the head, and assesses sympathetic and hemodynamic responses to the cold stimulus (98, 99). In response to CPT, the SNS is stimulated, releasing NE (100, 101) which binds to α-adrenergic receptors, increasing muscle sympathetic nerve activity (MSNA). In healthy, normotensive participants, the CPT causes vasoconstriction, and hence increases in blood pressure, and vascular resistance (22, 98). Heart rate (HR) response to CPT, in healthy, normotensive people, is variable. Some experience an increase in HR, which remains sustained throughout the entire CPT, whereas others experience an initial increase in HR which then slowly decreases towards pre-CPT levels over the course of the CPT (102-104). In those where their HR increases for the entirety of the CPT, it is suggested a decreased cardiac vagal outflow and increased sympathetic activity, whereas the opposite is found in those whose HR slowly declines over the course of the CPT (102).

Previous literature has noted interindividual variability in response to CPT, therefore this test is accepted to be reproducible. Those classified as 'responders' exhibit elevations in systolic blood pressure (SBP) by at least 16 mmHg or increases in diastolic blood pressure (DBP) by at least 12 mmHg in response to CPT. Those classified as 'non-responders' do not meet these criteria (105).

Given CPT is a pain stimulus, ratings of perceived pain are taken on a scale of 1-10, with 1 being no pain and 10 being the worst pain imaginable (106, 107). Males tend report lower pain ratings during the cold stimulus for longer durations of time when compared with females (108). However, males may rate perceptions of pain lower when a female researcher is present (109). Further, individuals who indicate a higher pain response typically have elevated MSNA and BP responses to CPT (110-112). In males, increases in MSNA due to CPT is more strongly coupled with increases in mean arterial pressure (MAP) and femoral vascular resistance, but these relations are not present in premenopausal females (26). Females also tend to paradoxically vasodilate in response to CPT, although the mechanism behind this response is unknown (113).

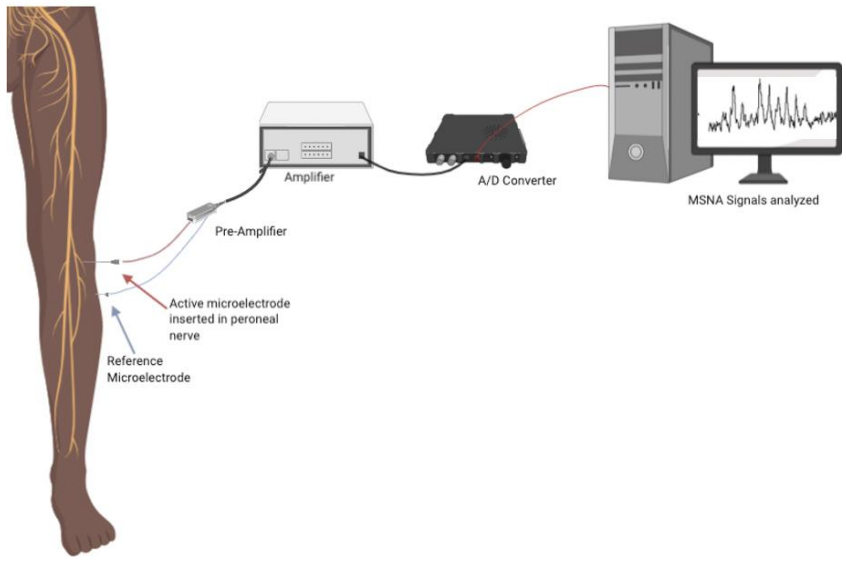
In relation to FMD, CPT has been utilized to examine the effect of acute increases in MSNA. Observations indicate that with increases in sympathetic activity, FMD is attenuated (114, 115). These alterations may be due to increases in blood pressure due to sympathetic vasoconstriction or possibly the binding of catecholamines to α and β adrenergic receptors (116). Only one source has linked PLM, and MSNA, however, this was done under the stimulus of an arm crank exercise. In this study, elevations in MSNA were induced by exercise. Results suggested the PLM-induced vasodilation was attenuated by exercise-induced MSNA,

suggesting sympathetic activity plays a large role in regulating skeletal muscle vascular conductance (20).

Microneurography

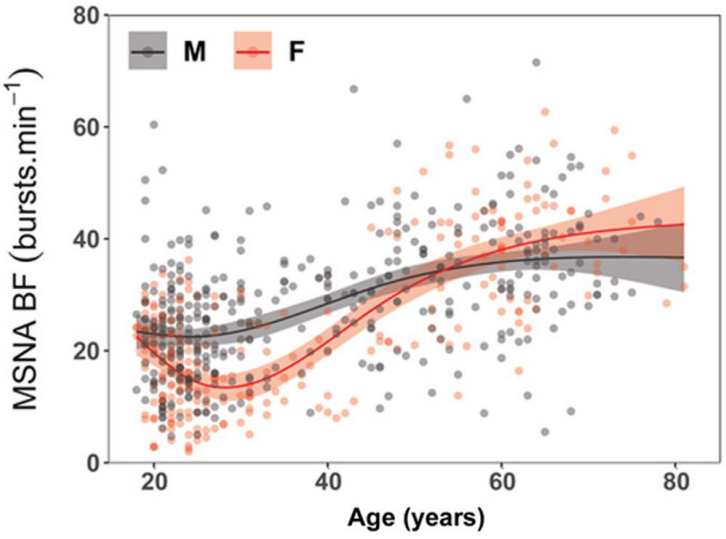
Pioneered by Karl-Eirk Hagbarth and Åke Valbo in 1965, microneurography is a technique used to record peripheral sympathetic nerve activity in humans, such as MSNA (117). Two tungsten electrodes are utilized during microneurography (Figure 9). One is a reference electrode which is placed subcutaneously and the second is an active electrode which is placed into the peripheral nerve, such as the peroneal nerve. The raw recordings of the nerve (via the active electrode) must be sent through a preamplifier, then an amplifier, before the raw signal is full-wave rectified, band pass filtered, and integrated. From there, the signal is run through an A/D converter, is analyzed, and processed (24).

Figure 9: Microneurography technique. After piercing the peroneal nerve with a tungsten microelectrode, the signal is amplified.



There are sex-differences in resting MSNA values between young women and men. Young women have lower MSNA than men and MSNA is not correlated with peripheral resistance in women (118). MSNA also increases with age (Figure 10), with the increase being larger in females than males (119). In disease, autonomic dysfunction is noted, such as elevations in MSNA in patients with systolic heart failure (120), hypertension (121), and COPD (122).

Figure 10: MSNA increases with age (123). MSNA is greater in males than females until females hit menopause.

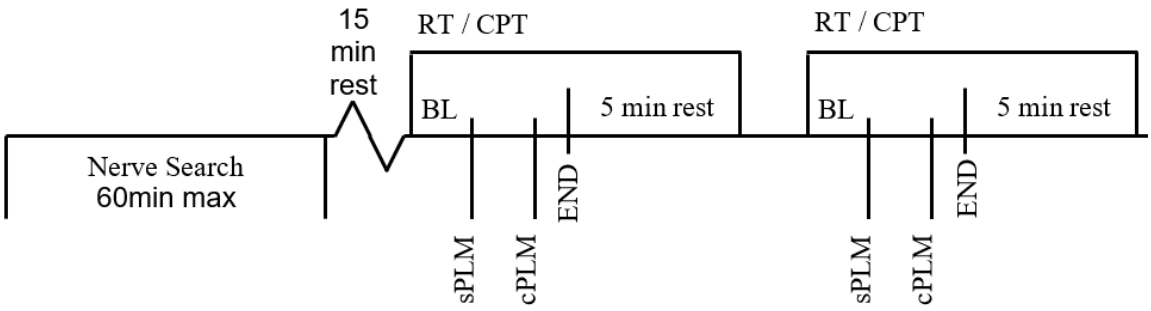


CHAPTER 3: METHODS

All procedures were approved by the Appalachian State University Institutional Review Board (IRB 20-0231). The participants provided informed consent, in accordance with the standards outlined by the Declaration of Helsinki prior to testing. Participants were relatively healthy and free from chronic metabolic and cardiovascular disease. Female participants were pre-menopausal, not pregnant, or trying to become pregnant. Participants were non-smokers and had no orthopedic limitations. Participants arrived for testing in a fasted state, having abstained from food and caffeine for 12 hr, and from exercise and alcohol for 24 hr before testing. Female subjects were tested during menstruation. All study procedures were performed in a quiet, thermoneutral environment after subjects had lain supine for 15 minutes.

Study Procedures. The study procedure is indicated in Figure 11.

Figure 11: Study Procedure. Room temperature and cold pressor test conditions were randomly assigned. Passive limb movement testing began after a successful nerve recording was found.



Participants reported to lab for one visit and were randomly assigned to the room temperature, non-water condition (RT) or the CPT condition. All study procedures were performed in a quiet, thermoneutral environment after 15 minutes of rest in the supine position,

following an acceptable nerve recording. Heart rate determined from lead II of the electrocardiogram (Biopac Systems, Goleta, CA, USA), beat-by-beat BP measured by finger photoplethysmography (NOVA, Finapres Medical Systems, Enschede, The Netherlands) and MSNA (662C-4, Department of Biomedical Engineering, University of Iowa, Iowa City, IA, USA) were continuously recorded during all tests.

Experimental Measures:

Vascular Function Assessments.

Femoral artery single passive leg movement. Femoral artery single PLM (sPLM) measurements were obtained from the right common femoral artery using current guidelines as a functional, lower-limb assessment of microvascular function (14). While in the supine position with the participant's left leg supported on a stool and right leg supported by a research team member at heart level, BL measurements of the common femoral artery diameter and blood velocity, at least 3 cm proximal the femoral artery bifurcation, were recorded for 1 min before PLM. Blood velocity was taken for 1min using a Doppler ultrasound system (GE Logiq eR7 and L4-12T-RS transducer, GE Medical Systems, Milwaukee, WI). Sample volume was optimized in relation to vessel diameter and centered within the vessel for each participant. Measurements of brachial artery diameter and velocity were obtained with the Doppler ultrasound in duplex mode with B-mode imaging frequency of ~12MHz and Doppler frequency of ~4MHz. An angle of intonation of ≤60° (124) was achieved for all measurements. Immediately following BL measurements, the research team member supporting the right thigh and ankle manually moved the knee joint one time through 90° range of motion, flexion-

extension, at 1Hz while common femoral artery diameter and blood velocity were recorded for 1 min after the movement. Common femoral artery diameter and blood velocity were analyzed offline for second-by-second measurements (Cardiovascular Suite v. 4.0, Quipu, Pisa, Italy). Cumulative values of the AUC for BF (total BF, BF; anterograde BF, aBF; and retrograde BF, rBF) were integrated via the trapezoid rule and calculated as: $\Sigma\{yi\ [x(I + 1) - xi\ ] + (½)[y(I + 1) - yi\ ][x(I + 1) - xi\ ]\}$; (x is time, y is shear rate, xi is initial time point, yi is initial blood velocity) (125). Blood flow (BF) was determined as: BF, $mL \bullet min^{-1}$ = [blood velocity • π • (arterial diameter • $2^{-1})^2$] • 60. Femoral artery BF was made relative to MAP (vascular conductance, VC) to account for daily changes in driving pressure. The average, BL, peak, change from BL to peak (ΔPeak), 30-s AUC (AUC30) and 60-s AUC (AUC60) were determined for all variables of interest.

Femoral artery continuous passive leg movement. Following the sPLM, a continuous PLM (cPLM) assessment was performed on the same leg. Measurements were repeated while the research team member manually moved the knee joint through 90° range of motion, flexion-extension, at 1Hz continuously for 1 min, while common femoral artery diameter and blood velocity were recorded for 1 min during this cPLM. Analysis was performed offline for continuous second-by-second measurements (Cardiovascular Suite v. 4.0, Quipu, Pisa, Italy). The BF and VC were determined as indices of microvascular function (14). Heart rate (HR), stroke volume (SV), cardiac output (CO), and MAP were determined noninvasively using photoplethysmography (Finometer, Finapres Medical Systems BV, Amsterdam, The Netherlands) throughout the cPLM test to determine central hemodynamic responses and are

reported as an average over each 60s stage. The average BL, peak, Δpeak, AUC30, and AUC60 were determined for all variables of interest.

Autonomic Function Testing:

Participants rested for 20 minutes prior to instrumentation. Utilizing microneurography, multiunit MSNA was assessed from the peroneal nerve at the fibular head. In brief, a recording electrode was inserted into the peroneal nerve using ultrasound guided microneurography, and a reference electrode was inserted subcutaneously 2-3 cm from the recording electrode. Nerve signals were amplified (70,000-160,000-fold), band-pass filtered (700-2000 Hz), full-wave rectified, and integrated with a resistance-capacitance circuit (time constant (0.1 s). The criteria for an adequate MSNA recording include pulse synchrony, increases in response to breath-holding, and sensitivity to a gentle skin touch or loud noise. Sympathetic and hemodynamic data were sampled at 625 Hz with a Biopac data acquisition system.

All participants performed a CPT where the hand was immersed in an ice water bath for 2 min. Participants were instructed to avoid breath holding and movement during the test. After the test, the participant's hand was immediately wrapped in a warmed towel for a 3 min recovery period. Immediately following recovery from CPT, participants were asked to rate their perception of pain on a scale of 1-10 with 1 being no pain and 10 being the worst pain imaginable (106, 107).

Sympathetic and hemodynamics data were sampled at 625 Hz with a commercial data acquisition system (BiopacSystems, Goleta, CA, USA). MSNA bursts were identified using computer software (LabView Software; National Instruments, Austin, TX, USA) with a 3:1

signal-to-noise ratio threshold within a 0.5 s search window and an expected burst reflex latency of 1.3 s from the preceding R-wave (126). All bursts were confirmed by an experienced microneurographer and re-running of the data with sound (to listen for sympathetic bursts from the raw signal) was performed using Spike2 8.08 software (Cambridge Electronic Design, Cambridge, UK) to confirm bursts. The number of bursts per minute (burst frequency; $MSNA_{BF}$) and per 100 heartbeats (burst incidence; $MSNA_{BI}$) were used as quantitative indices of MSNA.

Statistical Analysis

Statistical analyses were performed using commercially available software (IBM SPSS Statistics Version 26, IBM Corp., Armonk, NY, USA). A 2 x 3 repeated measures analysis of variance (ANOVA) (temperature, 2 levels: CPT and RT) (stage, 3 levels: BL, sPLM, cPLM) was performed to compare hemodynamic and autonomic responses during the CPT and RT conditions during passive movement. Bonferroni post hoc analyses were conducted when interactions were identified. Shapiro-Wilk tests were used to assess normality and Levene's test was used to assess the equality of variances. A Greenhouse–Geisser correction was used for variables where sphericity could not be assumed. Statistical significance was set at $p < 0.05$. Data are expressed as mean ± standard deviation (SD). Pearson correlations were conducted between the $\Delta MSNA_{BF}$ and $\Delta BF_{average}$ as well as between $\Delta MSNA_{BI}$ and $\Delta BF_{average}$ from BL to sPLM and from BL to cPLM for each condition (RT and CPT). Pearson correlations were also run for CPT condition minus the RT condition between $\Delta MSNA_{BF}$ and $\Delta BF_{average}$ and between $\Delta MSNA_{BI}$ and $\Delta BF_{average}$ from BL to sPLM and from BL to cPL.

CHAPTER 4: RESULTS

Participants. Participant characteristics are presented in **Table 1**. During the CPT, water temperature ranged from 0.3°C - 0.5°C across participants. Pain ratings during the CPT were 5±2 arbitrary units.

Table 1: Participant Characteristics

Characteristics	
Participants (n, M/F)	5 (1M/4F)
Age, yr	20 ± 2
Height, cm	165 ± 8
Weight, kg	59 ± 9
Body mass index, $kg \cdot m^2$	21.6 ± 1.4

M, male; F, female. Data are presented as mean ± SD.

Femoral Artery PLM Hemodynamic Response. Hemodynamic responses to the PLM can be found in **Table 2**. There was a main effect of condition (p=0.01) and stage (p=0.05) on MAP. A main effect of condition (p=0.02) on SBP was observed. Main effects of condition (p<0.01) and stage (p=0.03) were observed for DBP. A main effect of condition (p=0.02) and stage (p<0.01) on CO was observed. There was an interaction for SV during sPLM between conditions (p=0.04). A main effect of condition (p=0.03) on HR was observed.

Table 2: PLM Hemodynamic Response

Variable	RT	CPT
MAP, mmHg *, †		
BL	79 ± 10	100 ± 5
sPLM	77 ± 10	96 ± 9
cPLM	78 ± 10	95 ± 6
SBP, mmHg*		
BL	109 ± 12	131 ± 5
sPLM	107 ± 12	128 ± 5
cPLM	109 ± 12	127 ± 2
DBP, mmHg *, †		
BL	64 ± 10	85 ± 7
sPLM	63 ± 11	81 ± 12
cPLM	63 ± 11	79 ± 9
CO, $L \bullet min^{-1}$ *, †		
BL	4.3 ± 0.8	4.7 ± 0.7
sPLM	4.3 ± 0.8	4.8 ± 0.7
cPLM	4.6 ± 0.9	5.1 ± 0.7
SV, $mL \bullet min^{-1}$		
BL	65.2 ± 15.8	63.3 ± 16.4
sPLM ‡	64.8 ± 16.3	66.7 ± 16.1
cPLM	66.1 ± 16.5	67.8 ± 13.8
HR, bpm *		
BL	68 ± 10	76 ± 15
sPLM	68 ± 10	75 ± 13
cPLM	71 ± 8	76 ± 9

Data are mean ± SD. A 2 x 3 repeated measures ANOVA (condition 2 levels: RT and CPT; stage 3 levels: BL, sPLM, cPLM) was performed. BL, baseline; CO, cardiac output; cPLM, continuous passive limb movement; CPT, cold pressor test; DBP, diastolic blood pressure; HR, heart rate; MAP, mean arterial pressure; RT, room temperature; SBP, systolic blood pressure; sPLM, single passive limb movement; SV, stroke volume; * significantly different between conditions $p < 0.05$; † significantly different across stages $p < 0.05$; ‡, interaction between conditions $p < 0.05$.

Femoral Artery PLM Hyperemic Response. Raw data for hyperemic responses to the PLM can be found in Appendix A. Main findings are reported below.

There was no difference in BF_{BL} between conditions (p=0.94). There was a main effect of stage for $BF_{average}$ (p<0.01), with BL being less than cPLM (p=0.04). There was a main effect of stage for BF_{peak} (p<0.01), with BL being less than from sPLM (p=0.04) and from cPLM (p=0.02). There was a main effect of stage for $BF_{\Delta peak}$ (p<0.01), with BL being less than sPLM (p=0.04) and from cPLM (p=0.02). There was a main effect of stage for BF_{AUC30} (p<0.01), with BL being less than sPLM (p=0.01) and cPLM (p=0.01). There was a main effect of stage on BF_{AUC60} (p<0.01), with BL being less than from cPLM (p=0.04).

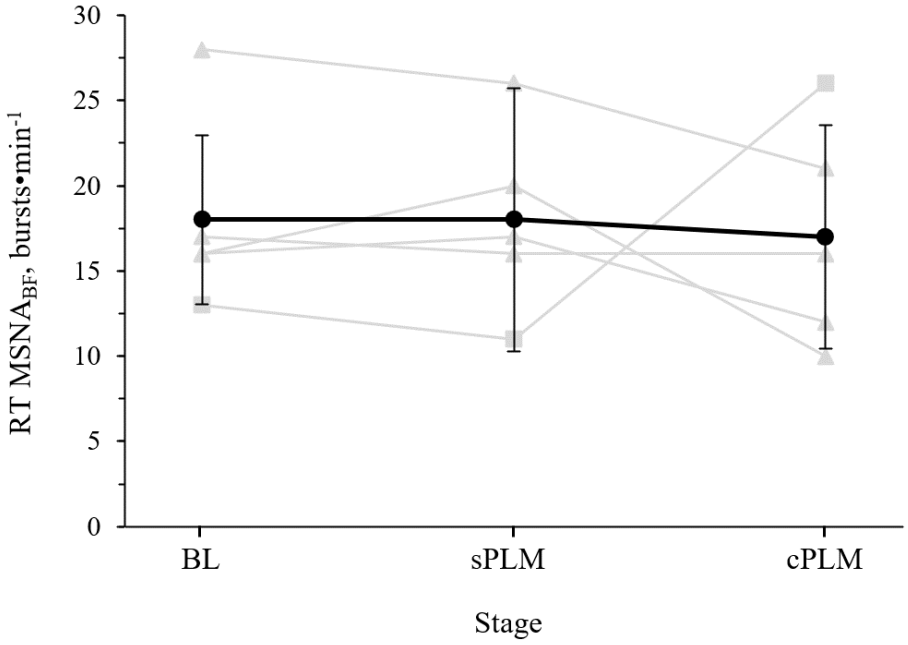
There was no difference in aBF_{BL} between conditions (p=0.92). A main effect of stage was observed on $aBF_{average}$ (p<0.01), with BL being less than cPLM (p=0.01). A main effect of stage on aBF_{peak} (p<0.01) was observed, with BL being less than cPLM (p=0.01). There was a main effect of stage on $aBF_{\Delta peak}$ (p<0.01), with BL being less than cPLM (p=0.01). A main effect of stage was observed on aBF_{AUC30} (p<0.01), with BL being less than sPLM (p=0.03) and cPLM (p<0.01). A main effect of stage on aBF_{AUC60} (p<0.01) was observed, with BL being less than cPLM (p=0.03).

There was no difference in rBF_{BL} between conditions (p=0.89). A main effect of stage on $rBF_{average}$ (p=0.01) was observed, with sPLM being different than cPLM (p=0.02). There was a main effect of stage on rBF_{peak} (p=0.03). A main effect of stage on $rBF_{\Delta peak}$ (p=0.03) was observed. No differene was observed in rBF_{AUC30} between conditions (p=0.40) or stages (p=0.18). A main effect of stage on rBF_{AUC60} (p=0.01) was observed, with sPLM being different than cPLM (p=0.02).

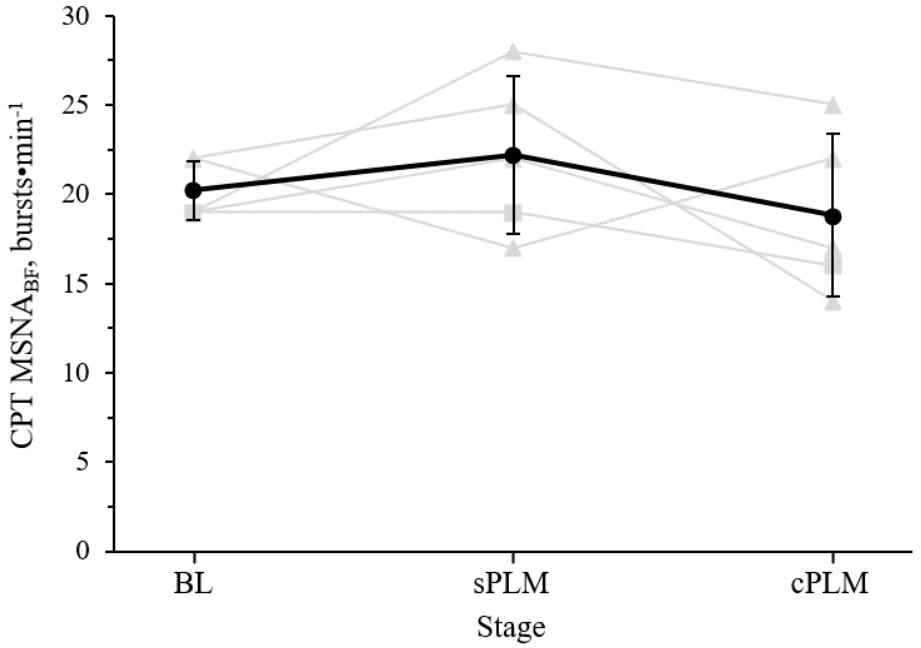
No difference was observed in VC_{BL} between conditions (p=0.24). There was a main effect of stage on $VC_{average}$ (p<0.01), with sPLM being less than cPLM (p-=0.02). A main effect of stage on VC_{peak} (p=0.01) was observed, with BL being less than sPLM (p<0.01) and cPLM (p=0.02). There was a main effect of stage on $VC_{\Delta peak}$ (p=0.01), with BL being less than sPLM (p<0.01) and cPLM (p=0.02). A main effect of stage on VC_{AUC30} (p<0.01), with BL being less than sPLM (p=0.02) and cPLM (p=0.01). A main effect of stage on VC_{AUC60} (p<0.01) was observed, with BL being less than cPLM (p=0.02).

Sympathetic Response to PLM. Indices of sympathetic activity are presented in Figure 12**.** No significant difference was observed in $MSNA_{BF}$ across condition (p=0.31) or stage (p=0.62). There was no significant difference in $MSNA_{BI}$ across condition (p=0.57) or stage (p=0.57).

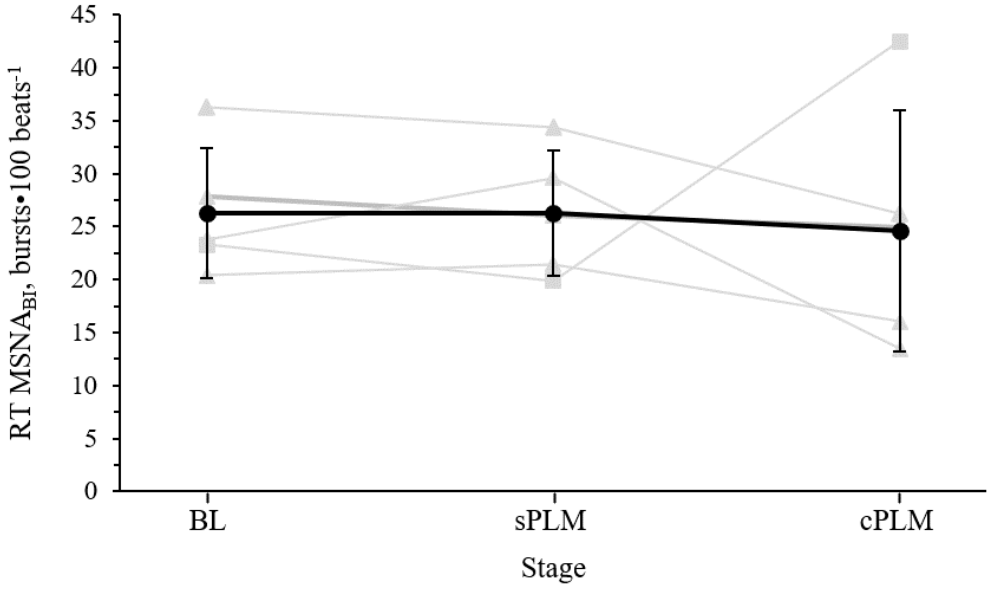
Figure 12: Sympathetic Response to PLM.
A.



B.


30
25
20
15
10
5
0
CPT MSNA$_{BF}$, bursts•min^{-1}
BL
sPLM
cPLM
Stage

C.


45
40
35
30
25
20
15
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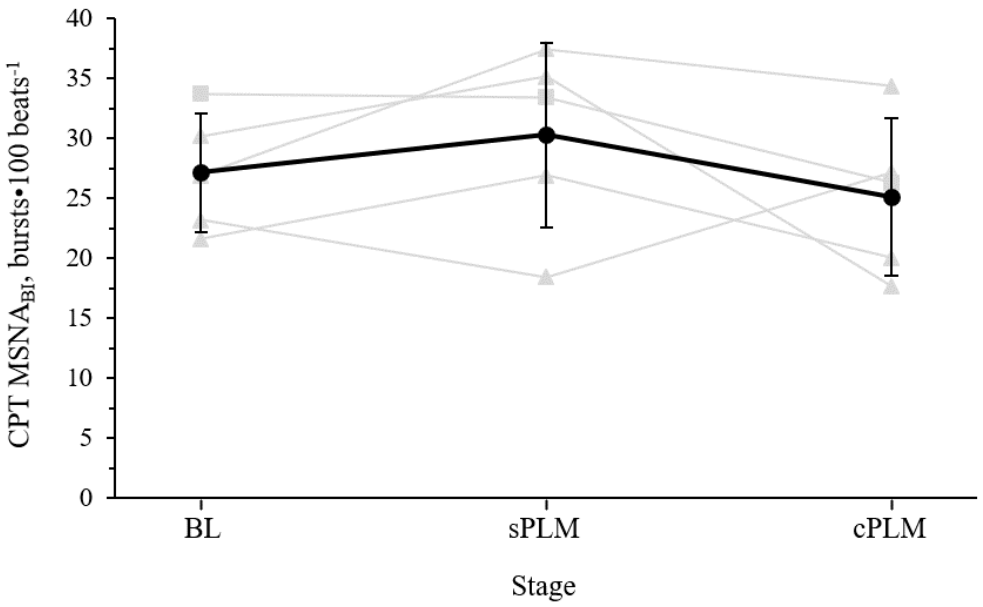
0
RT MSNA$_{BI}$, bursts•100 beats^{-1}
BL
sPLM
cPLM
Stage

D.



Muscle sympathetic nerve activity burst frequency ($MSNA_{BF}$) during the room temperature (RT) condition (A), cold pressor test (CPT; B;) MSNA burst incidence ($MSNA_{BI}$) during the RT (C) and CPT (D) conditions. BL, baseline; sPLM, single passive limb movement; cPLM, continuous passive limb movement. Individual data are presented as closed triangles (female participants, n=4) and closed squares (male subject, n=1). Data are mean ± SD.

Correlations. A summary of the correlations are presented in Table 3. Plots can be found in Appendix B. There were no significant correlations except during the CPT condition during the cPLM, $\Delta BF_{average}$ was inversely correlated to $\Delta MSNA_{BI}$ (*r*: -0.94).

Table 3: Correlations Summary

Correlations	
Variable	***p*-value**
CPT minus RT	
cPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.50
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.50
sPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.85
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.98
CPT	
cPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.02*
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.13
sPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.84
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.98
RT	
cPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.12
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.17
sPLM	
$\Delta BF_{average}$ and $\Delta MSNA_{BI}$	p=0.70
$\Delta BF_{average}$ and $\Delta MSNA_{BF}$	p=0.57

$\Delta BF_{average}$, change in average blood flow across 60s; cPLM, continuous passive limb movement; CPT, cold pressor test; $\Delta MSNA_{BF}$, change in muscle sympathetic nerve activity burst frequency; $\Delta MSNA_{BI}$, change in muscle sympathetic nerve activity burst incidence; RT, room temperature; sPLM, single passive limb movement. *, $p<0.05$.

CHAPTER 5: DISCUSSION

The purpose of this investigation was to elucidate the contribution of the sympathetic nervous system on vascular function measures during the PLM. We were unable to elucidate the role of MSNA on vascular responses during PLM as we did not have an adequate sample size due to the absence of increase in sympathetic activity during CPT determined via MSNA.

Lack of an increase in MSNA during the CPT is unexpected, and contrary to existing literature as the CPT is a well-established test known to increase MSNA in both young and old healthy individuals (19, 26, 98), as well as in those with HF with coronary artery disease (127). Increases in MSNA in response to CPT is independent of biological sex in young adults (19). However, change in MSNA during the CPT is inversely related to cardiorespiratory fitness in young, normotensive females (103). This difference may be due to differences in baroreflex sensitivity, where females are more efficient in controlling changes in blood pressures induced by CPT (128). During the CPT, sympathetic outflow increases occur when nociceptors in the hand, specifically C fibers, sense the reduction in skin temperature and produce the sensation of pain (129). As MSNA did not change in the current study, it is possible these pain receptors may not have responded to the noxious cold stimulus. However, this is not supported by the participant's perceived pain responses. Even in pharmaceutical conditions hypothesized to decrease MSNA due to a reduction in pain perception, such as in low dose ketamine (110) or low dose morphine (130) conditions, an increase in MSNA is still observed during a CPT. Medication usage was not controlled for, and therefore may have impacted the response to CPT. Further, with the strict inclusion criteria of no history of cardiovascular, autonomic, metabolic, or pulmonary disease, it is possible, but unlikely, that one or more of the participants had an unknown underlying condition.

Our current findings of no change in MSNA during the CPT as compared with the RT condition could be due to several factors, such as the study design. Victor *et al*. performed the CPT test for two minutes, during which the hemodynamic and MSNA response were noted; they observed MSNA peaked during the second minute of the CPT, returning towards the BL values at around minute 2 of recovery (98). Although the CPT test has been performed for up to 5 min, a limit set by the research group (108), most common practice is to leave the hand submerged for 2 min or 3 min, eliciting an increase in MSNA (26, 131, 132). However, MSNA response has not been examined in CPT lasting longer than 3min. To the author's knowledge, no study has utilized the current method to examine the relationship between PLM and the autonomic nervous system, specifically MSNA. As recording of BL occurred at CPT minute 2, when MSNA is likely to peak, it is likely that no difference was observed because MSNA was already elevated during the BL measurements and remained elevated throughout the PLM movements. Previous studies have observed no difference in MSNA during the CPT at the end of minute 3 as compared with the end of minute 1 when peak MSNA occurs (131) at water temperatures near 0°C (129).

Further, there was substantial inter-individual variability in MSNA responses within this sample, which is common as microneurography recordings are generally highly reproducible within participants yet basal MSNA differs between individuals (133, 134). In response to the CPT one female participant's $MSNA_{BF}$ and $MSNA_{BI}$ decreased at each stage as compared with RT. One male participant's $MSNA_{BF}$ and $MSNA_{BI}$ decreased during the CPT condition as compared with RT during cPLM. One female participant's $MSNA_{BF}$ and $MSNA_{BI}$ decreased during the CPT condition as compared with RT during sPLM.

However, these divergent sympathetic outflow findings are likely a byproduct of having an underpowered sample and measurement error. Even with a within-subjects design, a sample size of n=5 is underpowered to statistically identify differences between conditions. Based on these similar studies, an effect size of 1.31 was determined and a priori power analyses using 1-tailed and alpha error of probability set to 0.05 determined we would have needed 10 participants to complete the study. Indeed, a larger sample size may make the current trends more apparent or may elucidate other trends more in line with the current literature.

The possibility of measurement error also needs to be considered. Typically, sympathetic neural recordings are recorded in a stationary limb, as any movement of the musculature near the electrode may cause it to shift, altering the MSNA recording and increasing noise artifact (24). Ideally, the limb that is being assessed utilizing microneurography is immobilized and remains relaxed. However, the aim of this study was to investigate the role of MSNA, the autonomic nervous system, in the microvascular BF response to PLM. Even when performed by a highly trained research team, PLM causes movement throughout the body, including the active leg and the leg immobilized for microneurographic recordings.

This study was the first to attempt microneurographic recordings in the contralateral leg during PLM. Increased artifact noise made the processing of the neurograms challenging. Once PLM began, increased afferent feedback (seen and heard via neurogram) combined with the augmented noise artifact that comes with moving the contralateral limb added to occasional shifts in the neurogram BL. Additionally, maintaining a good sympathetic recording was difficult as adjustments of the microelectrode took place in several of the subjects, sometimes

between conditions. This limited our interpretation of these findings as we could not calculate burst amplitude.

Further evidence for error in our MSNA recording is that hemodynamic responses were intact. Increases in MAP, SBP, DBP, CO, and HR observed during the CPT all indicate that vascular regulation is responding as expected to the pain stimulus (19, 22). Although basal MSNA is not coupled to blood pressure in young adults (135), there is an expected reactivity to the CPT, which was indicated by perceived pain ratings but not indicated by changes in MSNA, results seen possibly due to these factors. Based on the previously noted pain perception responses, and hemodynamic changes to CPT, it is possible that we were unable to statistically show increases in MSNA due to being underpowered and due to interindividual differences. Further research should examine the contribution of MSNA and sympathetic activity on the hemodynamic changes associated with PLM with a larger sample size.

In young healthy male adults, during the cPLM, central hemodynamics (MAP, CO, SV, HR) increased transiently before returning to BL within 30s (18, 85). During the sPLM, however, there was a transient reduction in MAP (18, 85). Agreeing with the existing literature, within the current study central hemodynamic responses of MAP, SBP, DBP, and CO differed by stage, however we were underpowered and unable to see exactly where those differences were. Contrary to the literature, average HR did not change between stages, nor did SV. In young healthy male adults, BF_{peak} and $BF_{\Delta peak}$ did not change across stages. BF_{AUC60} was greater during the cPLM than the sPLM, where the change in BF during the cPLM was due to significant increases in both rBF and aBF, and the change during sPLM was due to increases in aBF (18). The results of the current study partially agree with the existing literature as significant changes were observed in BF_{AUC60} and aBF_{AUC60} between BL and cPLM,

suggesting total NO response to the cold stimulus, and therefore a greater NO bioavailability. However, no significant changes were noted in rBF_{AUC60}, suggesting NO bioavailability has less of an impact on rBF. VC_{AUC60} was elevated between BL and cPLM but not between BL and sPLM, which is supported by existing literature as it is thought the sPLM is not a large enough stimulus to induce significant changes in VC during sPLM (14, 18, 85). Changes in VC_{AUC60} is thought to be a consequence of NO, therefore the change observed suggests NO mediation of the vasculature, which is supported by the BF_{AUC60} and aBF_{AUC60} findings. It is important to note these differences may be influenced by sex, as the current study sample is predominantly females and existing literature is of males only.

To the author's knowledge, there is no literature on MSNA being measured at the same time as PLM during a CPT. Coovadia *et al.* examined sympathetic and hemodynamic responses to CPT in young, healthy adults. They observed significant increases in $MSNA_{BI}$, MAP, SBP, DBP, and HR during the CPT, but no change in CO. Average femoral BF also tended to decrease during the CPT (26). The results of the current study partially agree with the existing literature; however, we observed significant changes in CO between temperatures and no difference in $MSNA_{BI}$.

Interestingly, Coovadia *et al.* identified sex differences in responses to CPT. In males, the magnitude of CPT-induced changes in MSNA was related to increases in MAP and femoral vascular resistance. In females, this relationship was not present; data which are supported by previous literature (118, 136). These data suggest males rely more in sympathetic activation to alter blood pressure during the CPT (26). The absence of a relationship between MSNA and vascular response in females has been hypothesized to be due to greater density or sensitivity of β2-adrenergic receptors (136), however further research is needed to elucidate if this

relationship exists. It is possible there is no relationship between MSNA and vascular responses to PLM, as the current study suggests. In order to elucidate this, further research with a larger sample size powered for sex differences is needed.

Venturelli *et al* performed PLM in an upright seated position in 9 participants (6M/3F) after an arm crank exercise, which was utilized to increase MSNA. Performing the PLM in an upright position may decrease the contribution of NO and blunt the BF response (14, 15). Despite the differences in body position during the PLM, Venturelli *et al.* provide valuable insight to the influence on the role of the autonomic nervous system on vascular responses to PLM. They observed an inverse relationship between VC and MSNA, suggesting NO-induced vasoconstriction is blunted by the exercise induced MSNA (12, 20). They also observed increased BF response to the PLM movement after progressively higher arm crank intensities, which resulted in increased MSNA (20). In response to the passive movement of the leg, other molecules that regulate vascular tone may also be released, such as calcium, ROS, and RNS (137). However, their contribution to the PLM response is not known. One major limitation of this study, however, is the presence of exercising metabolites from the arm-crank exercise, which may confound the results of the PLM. In the current study, the change in BF_{AUC60} was correlated to the change in $MSNA_{BI}$ during the cPLM during the CPT, however BF_{AUC60} was not correlated to $MSNA_{BF}$ or $MSNA_{BI}$ under any other condition.

The strong correlation observed during the CPT when cPLM was performed, between the change in $BF_{average}$ and the change in $MSNA_{BI}$, suggests there is a relationship between sympathetic outflow and limb BF. However, this relationship is likely not seen within the other correlations performed during this study due to the small sample size.

Limitations. There are several limitations to this study. First, the sample size was underpowered as indicated by our initial power analyses. Secondly, it is possible there are sex differences in responses to PLM, however we were unable to run analyses to examine sex differences. As both males and females within the sample, the possibility of underlying sex differences is important to note. Physical activity level was not controlled for, nor was medication, or contraceptive usage. However, female participants reported to the lab during menstruation, therefore all females should have been tested during the early follicular phase. Finally, readjustment of the microneurographic electrode between stages and conditions made determination of burst amplitude impossible. Burst amplitude would have allowed for identification of burst size, quantifying the degree of sympathetic response, and would have allowed for standardization of burst sizes between participants. Future studies can learn from these initial findings and should learn from the current study design as well as increase their sample size.

In conclusion, the results of the study suggests MSNA has no impact on vascular responses to PLM. However, the lack of MSNA response during the CPT indicates the possibility of measurement error, as previous literature indicates the CPT test is sympathoexcitatory, perhaps due to the movement of the contralateral limb during PLM. Further studies are needed to elucidate the role of the autonomic nervous system in vascular responses to the PLM, without the impact of exercising metabolites.

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APPENDICES

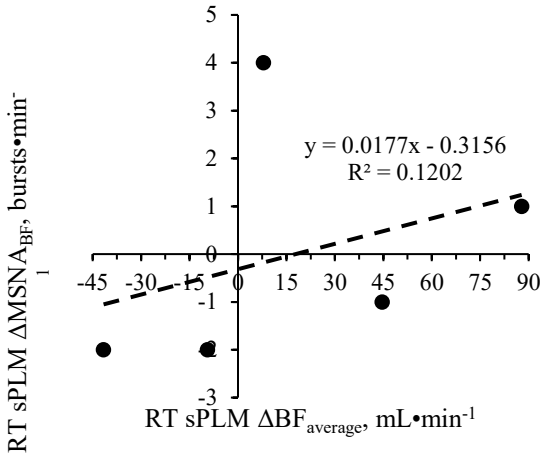
Appendix A- Hyperemic response to PLM

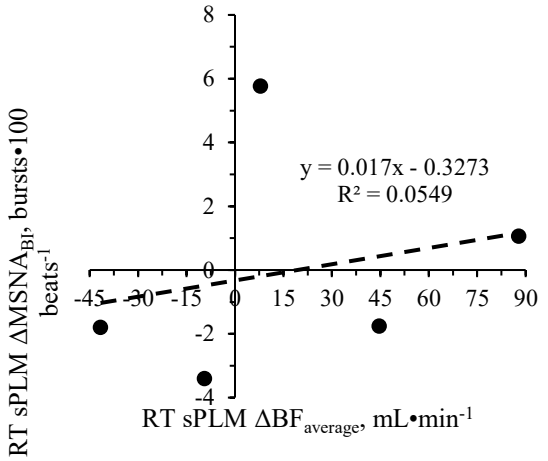
Variable	RT	CPT
Blood Flow		
Baseline		
Average, mL•min^{-1}	269 ± 93	273 ± 99
Peak, mL•min^{-1}	315 ± 108	371 ± 197
ΔPeak, mL•min^{-1}	46 ± 27	98 ± 103
AUC30, mL•min^{-1}	8083 ± 2023	8188 ± 3046
AUC60, mL•min^{-1}	16147 ± 5566	16351 ± 5954
sPLM		
Average, mL•min^{-1}	287 ± 88	313 ± 93
Peak, mL•min^{-1}	484 ± 118	487 ± 157
ΔPeak, mL•min^{-1}	214 ± 56	215 ± 118
AUC30, mL•min^{-1}	10174 ± 2882	10060 ± 2742
AUC60, mL•min^{-1}	17218 ± 5200	18787 ± 5577
cPLM		
Average, mL•min^{-1}	395 ± 89	388 ± 133
Peak, mL•min^{-1}	605 ± 133	631 ± 198
ΔPeak, mL•min^{-1}	336 ± 119	359 ± 133
AUC30, mL•min^{-1}	13138 ± 2771	13062 ± 4096
AUC60, mL•min^{-1}	23700 ± 5352	23281 ± 8001
Anterograde Blood Flow		
Baseline		
Average, mL•min^{-1}	331 ± 96	336 ± 126
Peak, mL•min^{-1}	372 ± 109	422 ± 207
ΔPeak, mL•min^{-1}	41 ± 23	86 ± 85
AUC30, mL•min^{-1}	9839 ± 3010	10228 ± 3753
AUC60, mL•min^{-1}	19830 ± 5752	20145 ± 7534
sPLM		
Average, mL•min^{-1}	351 ± 87	370 ± 123
Peak, mL•min^{-1}	522 ± 112	529 ± 173
ΔPeak, mL•min^{-1}	192 ± 58	194 ± 128
AUC30, mL•min^{-1}	11865 ± 2767	11737 ± 3514
AUC60, mL•min^{-1}	21044 ± 5215	22226 ± 7381
cPLM		
Average, mL•min^{-1}	453 ± 112	471 ± 153
Peak, mL•min^{-1}	629 ± 144	665 ± 210
ΔPeak, mL•min^{-1}	299 ± 95	330 ± 121
AUC30, mL•min^{-1}	14670 ± 3480	15195 ± 4664

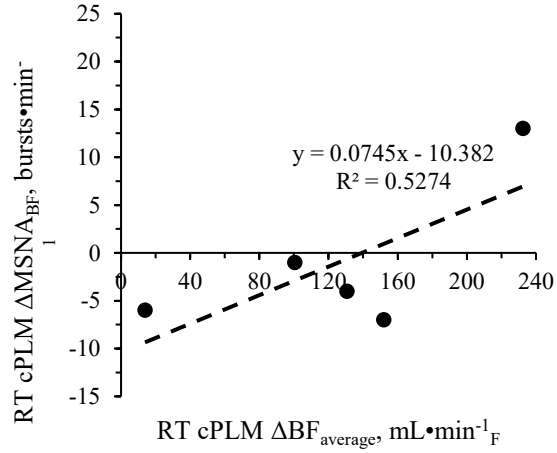
AUC60, mL•min^{-1}	27170 ± 6707	28286 ± 9160
Retrograde Blood Flow		
Baseline		
Average, mL•min^{-1}	-61 ± 43	-63 ± 40
Peak, mL•min^{-1}	-44 ± 38	-39 ± 30
ΔPeak, mL•min^{-1}	17 ± 6	24 ± 14
AUC30, mL•min^{-1}	-1757 ± 1178	-2040 ± 1312
AUC60, mL•min^{-1}	-3684 ± 2558	-3794 ± 2380
sPLM		
Average, mL•min^{-1}	-64 ± 45	-57 ± 40
Peak, mL•min^{-1}	-38 ± 37	-38 ± 33
ΔPeak, mL•min^{-1}	23 ± 8	25 ± 10
AUC30, mL•min^{-1}	-1691 ± 1361	-1676 ± 1235
AUC60, mL•min^{-1}	-3827 ±2702	-3439 ± 2391
cPLM		
Average, mL•min^{-1}	-58 ± 30	-83 ± 58
Peak, mL•min^{-1}	-17 ± 16	-29 ± 26
ΔPeak, mL•min^{-1}	45 ± 31	34 ± 19
AUC30, mL•min^{-1}	-1532 ± 1038	-2133 ± 1546
AUC60, mL•min^{-1}	-3471 ± 1770	-5005 ± 3470
Vascular Conductance		
Baseline		
Average, mL•mmHg^{-1}•min^{-1}	3.5 ± 1.2	2.8 ± 1.1
Peak, mL•mmHg^{-1}•min^{-1}	4.1 ± 1.4	3.8 ± 2.1
ΔPeak, mL•mmHg^{-1}•min^{-1}	0.7 ± 0.5	1.0 ± 1.1
AUC30, mL•mmHg^{-1}•min^{-1}	103 ± 40	82 ± 34
AUC60, mL•mmHg^{-1}•min^{-1}	208 ± 75	165 ± 67
sPLM		
Average, mL•mmHg^{-1}•min^{-1}	3.8 ± 1.2	3.3 ± 1.3
Peak, mL•mmHg^{-1}•min^{-1}	6.5 ± 1.7	5.3 ± 2.3
ΔPeak, mL•mmHg^{-1}•min^{-1}	3.0 ± 0.9	2.6 ± 1.6
AUC30, mL•mmHg^{-1}•min^{-1}	134 ± 41	106 ± 36
AUC60, mL•mmHg^{-1}•min^{-1}	227 ± 74	201 ± 77
cPLM		
Average, mL•mmHg^{-1}•min^{-1}	5.2 ± 1.5	4.2 ± 1.6
Peak, mL•mmHg^{-1}•min^{-1}	8.1 ± 2.9	6.8 ± 2.4
ΔPeak, mL•mmHg^{-1}•min^{-1}	4.7 ± 2.3	4.0 ± 1.5
AUC30, mL•mmHg^{-1}•min^{-1}	174 ± 55	139 ± 49
AUC60, mL•mmHg^{-1}•min^{-1}	311 ± 88	250 ± 94

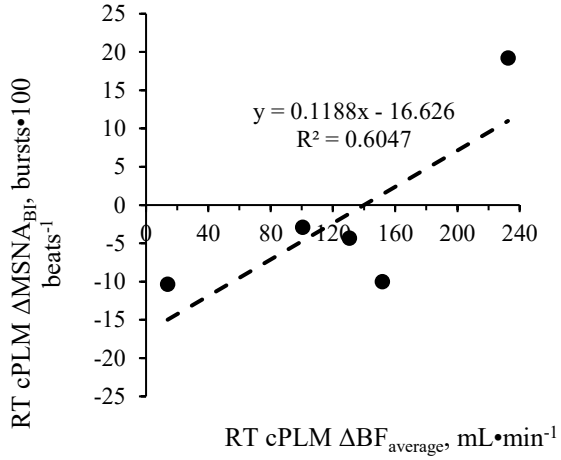
Data are mean ± SD. A 2 x 3 repeated measures ANOVA (condition 2 levels: RT and CPT; stage 3 levels: BL, sPLM, cPLM) was performed. cPLM, continuous passive limb movement; CPT, cold pressor test; RT, room temperature; sPLM, single passive limb movement

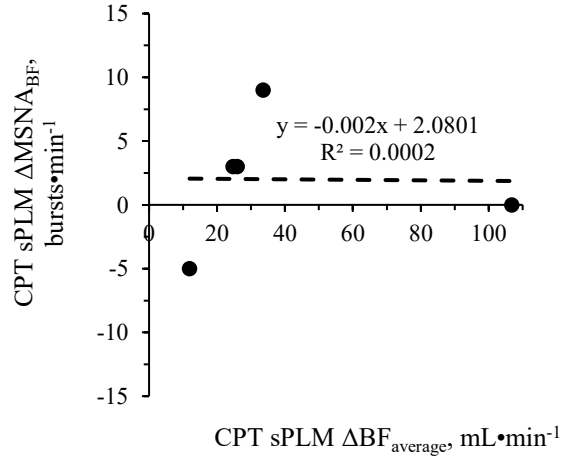
Appendix B- Correlation Plots

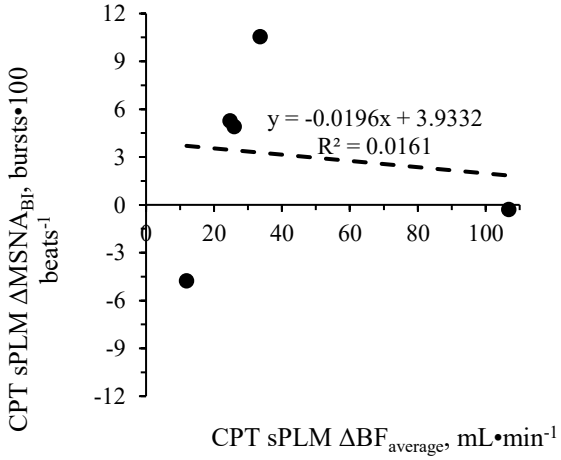


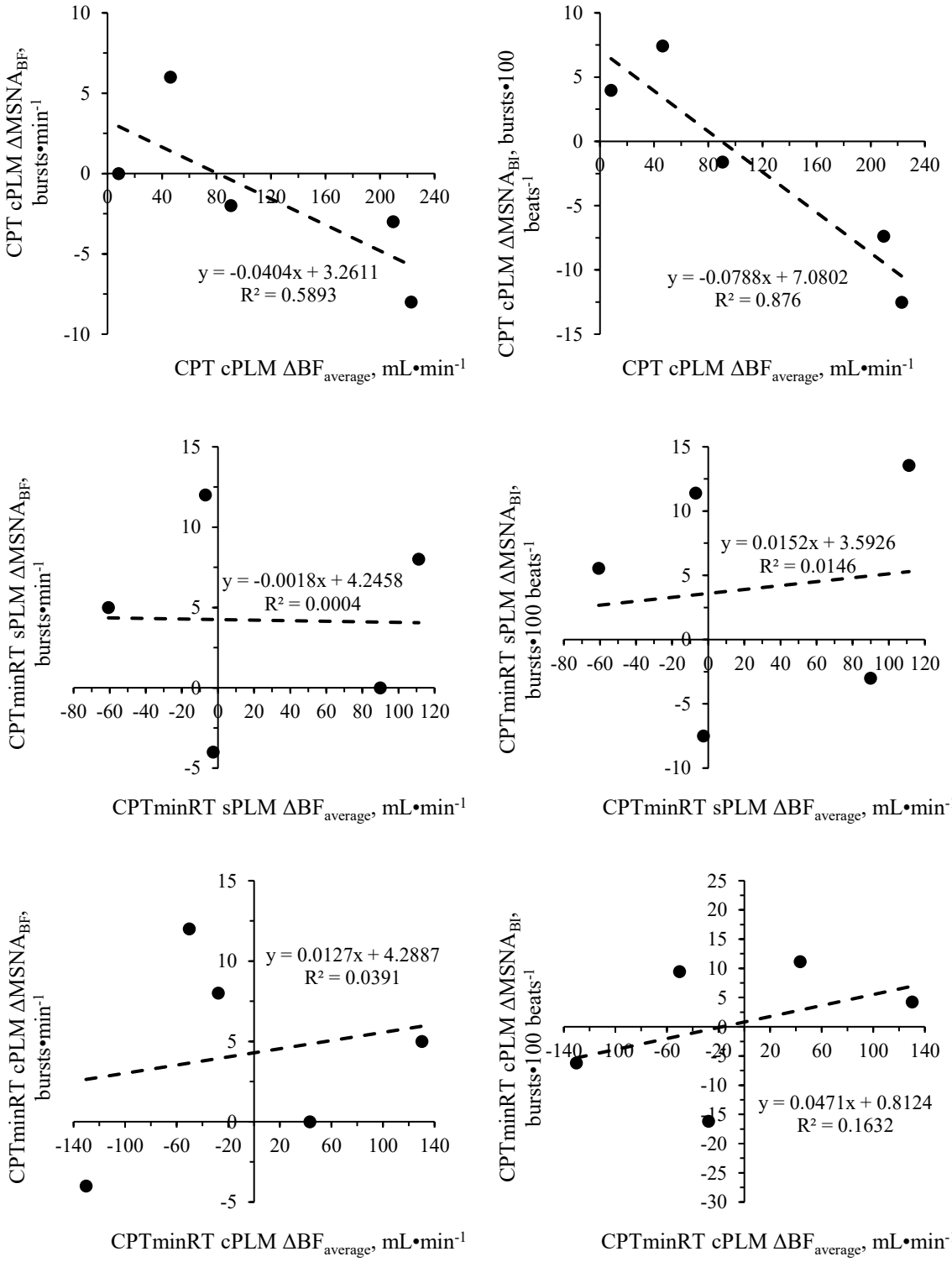












$\Delta BF_{average}$, change in average blood flow across 60s; cPLM, continuous passive limb movement; CPT, cold pressor test; $\Delta MSNA_{BF}$, change in muscle sympathetic nerve activity

burst frequency; $\Delta MSNA_{BI}$, change in muscle sympathetic nerve activity burst incidence; RT, room temperature; sPLM, single passive limb movement.

VITA

Rachel Elizabeth Szeghy was born in Manassas, Virginia to Cindy and Mark Szeghy, She has a younger sibling named Renee Szeghy. Rachel graduated from Appalachian State University in 2021 with her B.S. in Exercise Science. Immediately afterwards she began her M.S. Exercise Science degree at Appalachian State University, which was awarded in May 2023.

Rachel has no idea where she is going next, but is excited for wherever life will take her. She aspires to work within medical device clinical trials to channel her desire to keep learning and to help others.