Sex Differences and Vascular Mechanisms of Elevated Cardiovascular Disease Risk in Non-Hispanic Black Individuals

by

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Dedication

To my mom and my sister. Your unyielding and unwavering support throughout my education and my life truly kept me moving forward, even when I did not think I had the courage or will to do so.

To Poppas and Grandma. Despite not seeing me through high school and college, I know that the two of you continue to watch over me every day, making sure I remember everything you taught me.

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Abstract

Sex Differences and Vascular Mechanisms of Elevated Cardiovascular Disease Risk in Non-Hispanic Black Individuals

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Cardiovascular diseases (CVD), including hypertension, disproportionately affect the non-Hispanic black (BL) population in the United States. Despite being an inherently multifactorial process, altered vascular function remains a critical hallmark in the development of CVD. Further, blunted vascular function is often observed in the BL population. Recent advancements have linked several observational and mechanistic findings to the reduced vascular function in BL individuals, though the breadth of functional disparities in this population, the mechanistic contributors to these differences across sexes, and possible interventions to improve vascular function remain unelucidated. Therefore, this dissertation aimed to expand our current understanding of the vascular contributors to CVD risk in the BL population. Study 1 provided observational evidence of divergent vascular function across the peripheral and cerebral circulations in young, BL men and women, highlighting a crucial role of sex in the presentation of blunted vascular function and, perhaps, the development of CVD. Study 2 utilized intradermal microdialysis to assess the mechanisms of blunted microvascular function in young, BL women. In this study, our data suggest that endothelin-1 (ET-1), acting primarily through ET-1 receptor

type A, partially restrained vasodilation during local heating-induced cutaneous hyperemia through a nitric oxide-dependent mechanism. Further, these data suggest that neither supplemental L-arginine nor ET-1 receptor type B antagonism improved cutaneous microvascular function. Finally, Study 3 aimed to compare the hemodynamic and cardiovascular responses to mental stress in young, BL and non-Hispanic white (WH) men and determine if acute nitrate supplementation could improve these hemodynamic and cardiovascular responses in the BL men. Indeed, the BL men possessed blunted hemodynamic and cardiovascular responses to mental stress relative to the WH men. Despite this blunting, a single dose of nitrate was not substantial enough to modify these responses. The research described herein represents essential steps in our understanding of disparate vascular function in the BL population. Perhaps more importantly, this research also clarifies the role of sex and potential treatment approaches related to vascular function and CVD risk within the BL population. Therefore, future research on these topics may leverage these findings to target additional candidate mechanisms and therapies to lessen the burden of CVD in the BL population.

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List of Abbreviations

ACA	Anterior Cerebral Artery
ACh	Acetylcholine
ADMA	Asymmetric Dimethylarginine
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
AU	Arbitrary Units
AUC	Area-Under-the-Curve
AUC_I	Incremental Area-Under-the-Curve
AUC_T	Total Area-Under-the-Curve
BH_4	Tetrahydrobiopterin
BL	Non-Hispanic Black
BM	Non-Hispanic Black Men
BMI	Body Mass Index
BR	Beetroot
BW	Non-Hispanic Black Women
cGMP	Cyclic Guanosine Monophosphate
CO_2	Carbon Dioxide
CVC	Cutaneous Vascular Conductance
CVCi	Cerebral Vascular Conductance Index
$\%CVC_{max}$	Cutaneous Vascular Conductance Percent of Maximum
CVD	Cardiovascular Diseases
CVLM	Caudal Ventrolateral Medulla
CVR	Cerebrovascular Reactivity *or* Cerebral Vasodilatory Reactivity
DAG	Diacylglycerol
DBP	Diastolic Blood Pressure
EDRF	Endothelium-Derived Relaxing Factors
eNOS	Endothelial Nitric Oxide Synthase
ET-1	Endothelin-1
ET_AR	Endothelin-1 Receptor Type A
ET_BR	Endothelin-1 Receptor Type B
FBF	Forearm Blood Flow
FMD	Flow-Mediated Dilation
FVC	Forearm Vascular Conductance
GC	Guanylate Cyclase
GPCR	G-Protein Coupled Receptor
GTP	Guanosine Triphosphate

HCO_3	Bicarbonate
HDL	High-Density Lipoprotein Cholesterol
HUVEC	Human Umbilical Vein Endothelial Cells
IP_3	Inositol 1,4,5-Triphosphate
K_D	Dissociation Constant
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NMMA	N^{G}-methyl-L-arginine
LDL	Low-Density Lipoprotein Cholesterol
MAP	Mean Arterial Blood Pressure
MCA	Middle Cerebral Artery
MRI	Magnetic Resonance Imaging
MSNA	Muscle Sympathetic Nerve Activity
NA	Nucleus Ambiguus
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOHA	N^{ω}-hydroxy arginine
NOS	Nitric Oxide Synthase
NOX	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NPY	Neuropeptide Y
NTS	Nucleus Tractus Solitarius
O_2	Oxygen
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
P2X	Purinergic 2X Purinoreceptors
P_aCO_2	Arterial Carbon Dioxide Partial Pressure
PAT	Pulse Amplitude Tonometry
PCA	Posterior Cerebral Artery
PCO_2	Partial Pressure of Carbon Dioxide
$P_{ET}CO_2$	End-Tidal Carbon Dioxide Partial Pressure
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKG	cGMP-Dependent Protein Kinase
PL	Placebo
PU	Perfusion Units
RBF	Red Blood Cell Flux
RH	Reactive Hyperemia

ROS	Reactive Oxygen Species
RVLM	Rostro-Ventrolateral Medulla
SBP	Systolic Blood Pressure
SD	Standard Deviation
SNA	Sympathetic Nerve Activity
SNP	Sodium Nitroprusside
S_pO_2	Peripheral Oxygen Saturation
SVT	Sympathetic Vascular Transduction
TCD	Transcranial Doppler Ultrasound
V_{BA}	Brachial Artery Blood Velocity
VLDL	Very Low-Density Lipoprotein Cholesterol
V_{MCA}	Middle Cerebral Artery Blood Velocity
WH	Non-Hispanic White
WW	Non-Hispanic White Women
XO	Xanthine Oxidase

CHAPTER 1: GENERAL INTRODUCTION

Cardiovascular diseases (CVD; including heart disease, heart failure, stroke, and hypertension) remain the leading cause of death in the United States and a tremendous societal burden (1). While mortality from CVD decreased substantially from 2000-2010, deaths in the subsequent decade have not fallen further (1). Indeed, after a brief downward trend starting in the 1990s, adult CVD prevalence increased from 35% to nearly 50% over the past 5-10 y (1, 2). Estimates expect the financial burden that accompanies the prevalence and mortality of CVD in the United States to rise from approximately $350 billion in 2015 to nearly $1.1 trillion in 2035 (3). Accordingly, a better understanding of the etiology of CVD and viable treatment options remains a necessity.

While CVD affects all groups indiscriminately, certain populations remain at greater risk than others. Indeed, non-Hispanic black (BL) Americans exhibit the greatest prevalence of CVD and hypertension relative to any other racial or ethnic group (e.g., non-Hispanic white Americans; WH) (1). Additionally, a stark sex difference emerges when comparing BL individuals to other groups. Typically, CVD prevalence in women is between 9-11% lower than in men. This comparison is lost, however, in BL individuals as black women (BW) have an ~1.5% lower total CVD prevalence compared to black men (BM; 58.8% versus 60.1%) (1). Several controlling factors may contribute to this disparity, though blunted vascular function through a variety of mechanisms is often implicated (4-8).

As a potent vasodilator that regulates vascular tone, nitric oxide (NO) is an important molecule often associated with attenuated vascular function (5, 6, 9-12). Any mechanism that reduces either the bioavailability of or the responsiveness to NO can impair vasodilation. Indeed, several pathways can influence NO, including oxidative stress (13-

15), endothelin-1 (ET-1) (16, 17), and the necessary precursors for NO production (15, 18-21), among other things. Accordingly, any imbalance in these factors limiting vasodilation can be detrimental to vascular health.

While NO and other vasodilators play a significant role in regulating vascular tone, vasoconstrictors also greatly impact tone. As a potent vasoconstrictor, ET-1 primarily serves to directly modulate vascular resistance, in addition to its functional impact on NO (16, 17, 22). In this regard, exaggerations in ET-1 concentrations or receptor sensitivity can significantly augment vasoconstriction and lead to altered vascular function. In lieu of an autocrine pathway, vascular tone is also heavily influenced by norepinephrine (NE) released from sympathetic nerve terminals secondary to increased sympathetic nerve activity in both health and disease (23, 24). While NE-mediated changes in vascular tone happen through altered release or receptor kinetics, any exaggeration in these pathways can also contribute to changes in vascular function.

Statement of the Problem

Vascular function is an incredibly complex balancing act with numerous contributing factors working in concert. Indeed, vascular function is not isolated to a single vascular bed, which needs to be accounted for when interpreting physiological and epidemiological evidence. Further, its manifestation in at-risk populations, particularly BL individuals, emphasizes the need to understand how it develops and how it can be ameliorated or prevented. As the rise in prevalence and mortality from all-cause CVD in the BL population remains a grave public health threat, understanding the etiology of CVD in the BL population requires a systematic approach to fully understand this population's health disparities. Therefore, this dissertation aims to accomplish several goals: 1) discuss several modulators of vascular function, identify methods to evaluate this function that are

relevant to the subsequent dissertation studies, and examine the underlying biological and socioeconomic contributors to disparate vascular function in BL individuals (Chapter 2); 2) clarify sex differences in peripheral and cerebral vascular function in the BL population (Chapter 3); 3) elucidate the underlying mechanisms related to blunted microvascular function in young, BL women (Chapter 4); and 4) determine if acute nitrate supplementation can augment vascular function in young, BL men (Chapter 5). Taken together, these studies aim to improve our understanding of the etiology of altered vascular function in the BL population and interventions that may alter this function.

CHAPTER 2: LITERATURE REVIEW AND BACKGROUND

Determinants of Vascular Health and Function

Altered vascular function remains inherently multifactorial and derives from several molecular and physiological pathways. While an incompletely understood condition, reduced vascular function can predict the onset of conditions such as hypertension (25), derived chiefly through the direct modulation of peripheral vascular resistance. Reduced vascular function can have additional indirect effects on peripheral vascular resistance by induction of vascular remodeling (26). Accordingly, understanding the key contributors to attenuated vascular function helps better target pathways for prevention and treatment. In the scope of this review, the focus will remain on NO, ET-1, and sympathetic neural activity and their contributions to vascular function.

KEY PATHWAYS MODULATING VASCULAR TONE

Nitric Oxide

CVD (including heart diseases, hypertension, and cerebrovascular diseases) genesis is an inherently multifactorial process, though blunted macro- and microvascular function remain invariable hallmarks of this progression. Indeed, impaired vascular function often triggers the development of conditions such as atherosclerosis and coronary disease (27), thereby catalyzing the progression to overt CVD. Interestingly, vascular function is a precarious balancing act between vasodilation and vasoconstriction, whereby any adverse modification can disrupt this balance (27). Of the pathways that have been studied in the past several decades, NO has been, perhaps, the most studied vasoactive molecule. Stemming from the seminal work of Furchgott & Zawadzki (28), scientific inquiry has

greatly reconsidered the role of NO in vascular health. Indeed, beyond its critical mediation of vasodilation (27), NO also inhibits leukocyte adhesion and smooth muscle proliferation, while possessing anti-inflammatory and anti-thrombotic properties essential to maintaining vascular health (29, 30).

The landmark work of Furchgott & Zawadski (28) helped identify the role of the endothelium in eliciting vasorelaxation to acetylcholine (ACh) by demonstrating disparate vasoreactivity in endothelium-intact and denuded tissue. These findings integrally improved our understanding of the modulation of vascular tone. Ultimately, the endothelium-derived relaxing factor (EDRF) released following ACh infusion was identified as NO (31, 32). This conclusion was drawn as the administration of ACh produced similar relaxation patterns as the perfusion of aqueous NO on *in vitro* isolated vessel preparations (31, 32). Importantly, these responses to ACh only occur in endothelium-intact samples (31), while the response to NO occurred in both intact and denuded samples (31, 32). Moreover, similar quantities of NO were recovered following the perfusion of aqueous NO and A23187, a calcium ionophore that elicits similar relaxation as NO and ACh (31). Together, these data emphasize the role of the endothelium in the production of NO and subsequent vasodilation.

The use of ACh and A23187, however, may not be of particular biological relevance as many, if not most, peripheral blood vessels do not have cholinergic innervation (27), while A23187 is a synthetic molecule. In this regard, other NO-producing pathways may provide greater biological relevance. Such stimuli include physical forces (e.g., shear stress) (33, 34), hormones (35-37), platelet products (38, 39), and autacoids (e.g., bradykinin, prostacyclin, histamine) (32, 40, 41). These studies, however, were completed *in vitro* using animal models. Therefore, *in vivo* human studies help deliver further insight into the progression of CVD. Indeed, techniques such as flow-mediated

dilation (FMD) (42-46), intra-arterial drug infusions (47-54), and cutaneous microdialysis (10, 15, 19, 55-64), alone or in combination with other techniques, allow for *in vivo* bioassays of NO-mediated vascular function in healthy, at-risk, and clinical populations.

Regardless of the means of induction, the pathway for NO production is similar following the initiation of the signaling cascade. Within the vasculature, endothelial nitric oxide synthase (eNOS) serves as the constitutive NO producing enzyme (65). Following the interaction of receptor-dependent or independent agonists with the endothelium, opening of endothelial ionic calcium (Ca^{2+}) channels, or an upregulation of shear stress along the endothelial glycocalyx, eNOS activity is augmented. This upregulation in activity occurs as a byproduct of both increased intracellular Ca^{2+} and phosphorylation of specific serine (e.g., Ser^{1177}, Ser^{114}, Ser^{633}), threonine (e.g., Thr^{495}), and tyrosine amino acids in the eNOS enzyme (66). As a Ca^{2+}-calmodulin (Ca^{2+}/CaM) dependent reaction, upregulations in intracellular Ca^{2+} yield increased Ca^{2+}/CaM formation (27, 65, 66), augmenting enzyme activity. Interestingly, CaM binding is only part of the response; phosphorylation of the Ser^{1177} and Thr^{495} residues greatly modulate the rate of NO production. Indeed, phosphorylation of Ser^{1177} by Akt, Protein Kinase A, AMP-activated protein kinase, or CaM-dependent protein kinase II can augment eNOS electron flux, and thus NO production, by two- to threefold above resting conditions (66). Contrarily, phosphorylation of Thr^{495} reduces enzyme activity by interfering with CaM binding, such that dephosphorylation can yield a 10- to 20-fold augmentation in eNOS activity (66).

Critically, in response to cell stimulation, several steps occur to increase eNOS activity and subsequent NO production. Upon stimulation, an immediate increase in intracellular Ca^{2+} occurs with simultaneous dephosphorylation of the CaM binding domain (65, 66). Depending on the stimulus, such as with shear stress, there may be an attenuated rise in intracellular Ca^{2+}, and thus Ca^{2+}/CaM; therefore, the increase in eNOS activity is

primarily driven by phosphorylation of Ser^{1177} and a subsequent increase in electron flux necessary for the eNOS redox reactions (66). Accordingly, the increase in eNOS activity catalyzes the conversion of L-arginine (the critical eNOS substrate) into NO and L-citrulline following a series of redox reactions that are reliant upon the presence of tetrahydrobiopterin (BH_4) and nicotinamide adenine dinucleotide phosphate (NADPH), among other key regulators (27, 65, 66).

NO has a very short half-life of ~3-5 seconds, given its gaseous and highly reactive nature (31). Accordingly, NO must travel quickly from the endothelium to the neighboring smooth muscle cells to have any vasoactive effect. As a gas, NO can freely diffuse apically from the endothelium into the lumen, whereby most is quickly scavenged by hemoglobin (67) or reactive oxygen/nitrogen species (68), or basolaterally into the smooth muscle cells. Once in the smooth muscle cell, NO binds to a heme group on the β-subunit of soluble guanylate cyclase (GC), subsequently activating the enzyme in a dose-dependent fashion (69, 70). Activation of GC catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), thus activating a cascade of enzymatically amplified phosphorylation reactions, particularly through cGMP-dependent protein kinase (PKG) (70). In vascular smooth muscle, some of the main targets of PKG include myosin light chain phosphatase (releases myosin light chain from a latch state), large-conductance Ca^{2+}-activated K^+ channels (hyperpolarizing the cell), and L-type Ca^{2+} channels (reduces Ca^{2+} influx), among others (70). The results of these phosphorylation reactions lead to smooth muscle cell relaxation and subsequent vasodilation.

Given the complex nature of the NO-cGMP-PKG pathway, any disruption of this pathway can lead to impaired relaxation. Reductions in NO bioavailability are among the predominant cause of attenuated vasodilation and are often seen in populations with impaired vascular function, including hypertension (6, 50, 51, 71, 72),

hypercholesterolemia (13, 18, 73-75), chronic kidney disease (15, 76, 77), type 2 diabetes mellitus (5, 10), and middle-to-older age (14, 19, 61, 78-80). Some of the critical downregulatory factors for NO bioavailability include lacking substrate (e.g., reduced L-arginine bioavailability), lacking cofactor (e.g., insufficient BH_4), and elevated oxidative stress (e.g., excess reactive oxygen species).

In populations with reduced vascular function or augmented CVD risk, the administration of L-arginine can favorably improve cardiovascular outcomes (15, 18, 19, 63, 81-83). Interestingly, even in the case of adequate intracellular L-arginine, administration of the amino acid can still augment the production of L-citrulline and NO, a phenomenon dubbed the L-arginine paradox (84). While insufficient intracellular L-arginine may drive reductions in NO production, the factors controlling this reduction are multifactorial. In this regard, L-arginine transport into the cell may be impaired such that intracellular L-arginine is reduced. Indeed, inhibition of the cationic amino acid transporter y^+, essential for L-arginine influx, reduces L-citrulline production in human umbilical vein endothelial cells (HUVECs) (85). Accordingly, reduced L-arginine influx reduces the production of NO. Once inside the cell, the L-arginine can be competitively scavenged by enzymes such as arginase (84), ultimately reducing the amount of L-arginine that eNOS can convert. In this regard, arginase inhibition can augment NO-mediated dilation in populations with heightened CVD risk or overt disease (19, 61, 71, 74, 86). Beyond arginase, competitive binding by the arginine analog asymmetric dimethylarginine (ADMA) can also reduce NO production through an L-arginine-related pathway (87). In any case, the inadequate oxidation of L-arginine to L-citrulline and NO through these pathways ultimately drives vascular dysfunction.

Insufficient BH_4 can also reduce the catalytic rate of eNOS. While mounting evidence suggests that BH_4 serves several functions in the synthesis of NO, the two key

roles that have been identified are the hydroxylation of L-arginine and oxidation of N^{ω}-hydroxy arginine (NOHA) (88). The former step converts L-arginine into NOHA, a key intermediary in the L-arginine to NO conversion pathway. Without BH_4, little to no NOHA formation occurs as BH_4 serves as a key electron donator to the heme-oxy complex found in each NOS subunit (88). On the other hand, the latter step uses a regenerated BH_4 molecule to oxidize a ferrous-dioxygen complex, which subsequently drives the formation of a ferrous nitroxyl complex and, consequently, NO (88). Studies have found that direct administration of BH_4 can augment vascular function in populations at-risk for the development of overt CVD (20, 21, 56). Therefore, BH_4 seems to serve as a permissive gatekeeper for the redox formation of NO and a potential source of blunted vascular function.

Oxidative stress is often a key mediator of blunted vascular function through its scavenging of NO. During metabolism, the production of adenosine triphosphate (ATP) during aerobic respiration yields intermediates referred to as reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radicals, and hydrogen peroxide (89). ROS typically play a role in maintaining normal cellular function, though they can lead to cellular damage and dysfunction when overproduced (89). Indeed, populations with elevated oxidative stress often have reductions in vascular function (15, 62, 90). ROS production, namely O_2^-, leads to a rapid reaction with NO, whereby NO bioavailability is reduced while peroxynitrite ($ONOO^-$) is formed (91). As a strong oxidant, $ONOO^-$ can lead to further cellular damage of vascular proteins, DNA, and lipids (92). Accordingly, excessive ROS may lead to attenuated vascular function through reduced NO bioavailability and the production of highly reactive molecules that damage cellular structures.

Endothelin-1

While the vascular endothelium is often revered for its production of NO, it is necessary to note that the endothelium generates other vasoactive molecules that work in an autocrine or paracrine fashion. Indeed, one of the most important regulators of vascular tone released from the endothelium is ET-1. First identified in 1988, ET-1 is a potent vasoconstrictive peptide (93) with two key receptor subtypes, ET-1 receptor type A (ET_AR) and type B (ET_BR), that yield disparate responses depending on the localization (94). The actions of ET-1 help modulate systemic hemodynamic and pressor responses at rest and during a variety of perturbations.

Coming from a family of 21 amino acid peptides, ET-1 is one of three endothelin isoforms, along with ET-2 and ET-3, and may be analogous to sarafotoxins (95). Endothelins begin their lifecycle as preproETs (~200 amino acids long), whereby proteolytic cleavage generates 38- to 39-residue long proETs (also referred to as big ETs) that are further broken into mature ET peptides via proteases such as cathepsin or endothelin converting enzyme (94, 96). ET-1 can be released through constitutive and regulatory pathways, though the predominant pathway for secretion derives from the former (94). Interestingly, the regulation of ET-1 gene transcription is controlled by other vasoactive substances, such that increases in gene transcription seem to be regulated by vasoconstrictive or inflammatory molecules (e.g., angiotensin II, IL-1), while vasodilatory molecules control decreases (e.g., NO, prostacyclins) (94). Much like NO, a predominant amount of ET-1 diffuses basolaterally (~80% of the total amount) (97) where it may then exert its vasoactive effects, which is vital as circulating ET-1 may not be in high enough concentrations to effectively bind to ET-1 receptors and exert a physiological effect (94).

ET-1 binding occurs at either ET_AR or ET_BR, though these receptors have different localizations, with sometimes opposite effects. ET_AR, in particular, are highly expressed

on vascular smooth muscle cells, but not on endothelial cells (98). Contrarily, ET_BR can be localized on both the vascular smooth muscle, where ET-1 exerts vasoconstriction, and on endothelial cells, where ET-1 elicits vasodilation (99). Both receptor subtypes operate through a transmembrane, G protein-coupled receptor (GPCR) signaling pathways (94, 96). On the vascular smooth muscle, ET-1 activation of both ET_AR and ET_BR elicit GPCR-mediated Ca^{2+} influx. Activation of the GPCR increases phospholipase C activity, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (100, 101). DAG elicits an increase in sarcolemmal receptor-operated Ca^{2+} channels, while IP_3 increases Ca^{2+} release from the sarcoplasmic reticulum, which activates sarcolemmal store-operated Ca^{2+} channels. In both cases, the end-product is an increase in intracellular free Ca^{2+}, which elicits contraction through increases and decreases in myosin light chain kinase and phosphatase activity, respectively (100, 101). ET_AR-mediated vasoconstriction can also be elicited through the Ca^{2+}-independent activation of Rho kinase, which leads to reductions in myosin phosphatase activity and potentiation of vascular smooth muscle cell contraction (100, 102). On the endothelium, ET_BR provoke vasodilation. Activation of an ET_BR-linked GPCR stimulates phosphoinositide 3-kinase that subsequently phosphorylates Akt and increases eNOS activity (100, 103). Additionally, endothelial ET_BR agonism leads to an increase in intracellular [Ca^{2+}], which activates both a Ca^{2+}/CaM dependent increase in NO production and a cyclooxygenase-mediated increase in prostacyclins (100), both of which elicit vascular smooth muscle relaxation. Remarkably, ET_BR also serve as physiological "sinks," whereby they facilitate the clearance of circulating ET-1 and serve an additional role to reduce the systemic activity of ET-1 (100). Despite these important functions in balancing the key vasoconstrictor properties of ET-1, ET_BR may serve a smaller role as the

K_D for ET_BR is substantially lesser than the K_D for ET_AR in various vessel preparations, suggesting preferential binding on ET_AR (104).

Given the important vasoactive properties of ET-1, any upregulation in ET-1 release or receptor activity can unfavorably increase vascular tone. Indeed, research indicates elevated ET-1 bioactivity in conditions such as hypertension (105-108) and aging (109, 110), whereby the risk for CVD amplifies. The increases in ET-1 bioactivity may therefore translate to augmented vascular tone or even altered vasodilatory properties. Indeed, the direct administration of ET-1 can reduce FMD and post-occlusive reactive hyperemia in young, otherwise healthy individuals (111, 112). The increased activity of ET-1 may reduce vascular function by offsetting endothelium-dependent vasodilation. In this regard, co-infusion of ET-1 and ACh blunts the rise in forearm blood flow in young, healthy individuals (112), while blockade of ET_AR alone or in combination with ET_BR can augment ACh-mediated vasodilation in hypertensive patients (112, 113). It is important to note that, independent of vasodilatory impairment, ET-1 can also reduce resting blood flow and, therefore, vascular shear stress (111, 114). Chronically, these reductions in blood flow and shear stress may leave the vasculature prone to injury (e.g., atherosclerosis), ultimately contributing to augmented CVD risk (115). Accordingly, any upregulation in ET-1 bioavailability or the modification to the properties of ET-1 receptors can contribute to the development and progression of overt CVD.

Sympathetic Nervous System

The sympathetic nervous system is a major controller of cardiovascular function and can have deleterious effects if overactive. This relation is particularly important because vascular function is a critical balancing act between vasodilation and

vasoconstriction. Often, the sympathetic nervous system picks up afferent reflex signals from a variety of receptors (e.g., baro-, chemo-, mechano-, metabo-), whereby they are integrated in the nucleus tractus solitarius (NTS) region of the brainstem (24). Following integration, efferent signals are sent through the caudal ventrolateral medulla (CVLM) and rostro-ventrolateral medulla (RVLM) or through the nucleus ambiguus (NA), whereby different outcomes occur. In the case of the former two pathways, efferent signals synapse onto interneurons or sympathetic preganglionic neurons followed by sympathetic postganglionic neurons and, eventually, the end organ such as the heart or blood vessels (24). In this manner, heightened efferent sympathetic nerve activity (SNA) or signal transduction at the receptor can augment vascular tone (i.e., sympathetic vascular transduction; SVT) and, subsequently, systemic vascular resistance.

To assess SNA and SVT, microneurography is often employed to gather direct recordings of sympathetic neural outflow (24, 116, 117), though indirect measures of sympathetic activity can be gathered using catecholamine measurements and organ-specific, norepinephrine spillover measurements (118, 119). In the former method, a thin tungsten needle is inserted percutaneously and directly into an underlying peripheral nerve, commonly the radial or peroneal nerves (24, 116). Once inside a nerve fascicle, the tip of the needle rests against one or more nerve fibers, all of which are destined for the same target organ or tissue (24). The impulses that travel through these fibers are often intermittent, spontaneous discharges that are separated by silent periods of varying duration. Accordingly, the bursts vary depending on the target organ (e.g., muscle versus skin); for instance, muscle SNA (MSNA) bursts seemingly contain only vasoconstrictor impulses that occur in rhythm with the cardiac cycle and in responses to reduced arterial blood pressure. These bursts can then be integrated (e.g., mean electrical activity) and quantified in terms of burst incidence (bursts per 100 heart beats), burst frequency (bursts

per minute), total activity (product of the number of bursts and average burst area), or total MSNA (burst area per heart beat) (24). While skin SNA provides insight into some mechanisms, MSNA is more commonly used due to the predominant vasoconstrictor activity it carries both at rest and during different physiological maneuvers. Additionally, MSNA is often coupled with blood pressure and direct vascular measures to assess sympathetic transduction in humans, providing important physiological insight into differences in the end-organ responses to SNA (120).

The assessment of sympathetic transduction helps to better understand the efficacy for conversion of SNA into an end-organ response. In particular, SVT illustrates the magnitude of vasoconstriction for a given MSNA input and the resulting changes in local vascular conductance/resistance (120). This evoked vasoconstriction occurs following the release of norepinephrine (NE) and the sympathetic cotransmitters adenosine triphosphate (ATP) and neuropeptide Y (NPY). Once released, these neurotransmitters bind to their respective receptors (NE: α- and β-adrenoreceptors; ATP: P2X purinoreceptors; NPY: NPY receptors) and elicit vasoconstriction or sensitization for vasoconstriction (120-122). In this manner, increased SNA (e.g., more neurotransmitter being released), binding (e.g., greater neurotransmitter release, reduced neurotransmitter reuptake or spillover, reduced K_D) or second-messenger signaling can augment the magnitude of vasoconstriction at rest and during physiological maneuvers.

Well-documented evidence points to augmentations in resting MSNA in both aging (123-125) and pathological conditions (126-129). Interestingly, differences in MSNA can also occur during different physiological stimuli in populations at risk for CVD development (130-140). Some of the differences in elevated MSNA, however, may not be detrimental and may be compensated in different ways. For instance, younger men with high MSNA also display lower cardiac output, which may offset the higher resistance

conferred by elevated MSNA (i.e., Ohm's law of fluid flow) and subsequently produces no relation to mean arterial blood pressure (MAP) (141). Further, in some populations (e.g., older men), SVT may be reduced, such that the observed elevations in MSNA may be requisite for the maintenance of MAP (120), though these findings remain incompletely understood. These cases of compensation, particularly at rest, may not completely uncover underlying cardiovascular risk, though. To illustrate this point, the cold pressor test, which elicits a rise in MSNA and, consequently, blood pressure, is predictive of the development of hypertension long term (142-144). Maneuvers like the cold pressor test may serve as a stimulus that disrupts homeostasis enough for physiological compensations to "fail." In cases where this balance is disrupted and the increase in MSNA or elevated SVT becomes incompensable, detrimental cardiovascular outcomes may occur. For instance, patients with chronic kidney disease exhibit graded MSNA with disease progression, which may exacerbate systemic injury in this population through heightened vascular resistance and modified blood flow (129). Further, augmented SNA may detrimentally blunt active vasodilation. In this regard, expected increases in middle cerebral artery blood velocity during a hypercapnic challenge are blunted by increased SNA during orthostasis (145). Accordingly, it is necessary to understand the contributors to elevated MSNA or SVT in different populations such that interventions may be utilized to ameliorate detrimental changes in these biological processes.

Several sources may contribute to the development of sympathetic overactivity and heightened SVT. Intriguingly, some of this phenomenon may be driven by impairments in the autonomic control provided by the arterial and cardiopulmonary baroreflexes or by exaggerations in sympathetic drive from somatic reflexes and chemoreflexes (128). Modulations in these control centers ultimately modify the afferent signaling pathways to the brainstem and yield augmented SNA. At the level of the brainstem, several functional

alterations are associated with pathological elevations in sympathetic outflow, including changes in NO (146, 147), angiotensin II (146, 148, 149), ROS (148-150), aldosterone (151, 152), and inflammatory cytokines (146). The influence of NO and ROS may also extend to the degree of SVT in different populations. Lembo *et al.* (153) previously demonstrated that forearm blood flow in response to brachial infusions of NE is significantly lower in hypertensive humans. This observation may be secondary to reduced NO bioavailability as demonstrated using concurrent infusion of N^G-methyl-L-arginine (L-NMMA), an NOS inhibitor. This coinfusion augmented constriction in normotensives, but not in hypertensives. Further, ROS scavenging using ascorbic acid ameliorated the augmented constriction in the hypertensive group, with no change in the normotensive group. Accordingly, several molecular pathways seem to contribute to changes in sympathetically mediated vascular function that may augment CVD risk, though more information is needed, particularly in human research.

METHODS TO ASSESS VASCULAR FUNCTION

Flow-Mediated Dilation

Brachial artery FMD began as a non-invasive, *in vivo* bioassay of macrovascular endothelial dysfunction in both children and adults (42). FMD has since become a widely adopted tool used by clinicians and researchers to better understand CVD development and predict the onset of clinical events (43, 154, 155). While non-invasive, administering an FMD test requires some technical equipment and proper training to ensure that any data collected is reliable. The most common method to assess brachial artery FMD is Doppler ultrasound before, during, and after a brief period of forearm circulatory arrest (42, 43, 154, 156). Doppler ultrasound, particularly modern Doppler ultrasound, is an effective tool for

this assessment as high-resolution, B-mode images are easily attained and can be maintained for the duration of the assessment. Often, the probes used for imaging emit sound waves at a frequency of 7-13 MHz and produce high-quality images of the insonated artery when perpendicular to the vessel, though most images are often taken at 60° due to technical considerations discussed below in *Post-Occlusive Reactive Hyperemia* (43). Once insonated, each image is optimized to produce a clear delineation between the lumen and vascular wall for acceptable post-processing of vessel diameter (43).

Before imaging, a pneumatic cuff (e.g., a standard sphygmomanometer or a rapidly inflating model) is typically wrapped around the forearm, just distal to the epicondyles, though it may be wrapped around the upper arm, just distal to the axilla (43). The former method is often the most employed as it produces a brachial artery dilation response with a greater dependence on NO (44). Once the artery is imaged, a period of 1-2 minutes is used for baseline analysis, followed by 5-minutes of suprasystolic cuff occlusion (e.g., ≥220 mmHg), and then, typically, a 3-minute recovery. During each phase, if using modern ultrasound with external video capture, brachial artery diameter is constantly recorded and then later analyzed by automated wall-tracking software to determine FMD without user bias (43). During the 5-minute ischemic period induced by cuff occlusion, the forearm microvasculature undergoes metabolic vasodilation, reducing vascular resistance (157). Accordingly, upon release of the cuff, a large, transient increase in blood flow occurs, augmenting the brachial artery shear stress and eliciting vasodilation (43), the degree of which often inversely relates to CVD risk (42, 43, 154-156, 158). Importantly, recent investigations have pushed for the allometric scaling of FMD to baseline brachial artery diameter, normalization of FMD to shear stress, or both (43, 159-165) to account for the inherent issues of different baseline diameters and to control for the stimulus for dilation.

While technically sound, the shift to these methods in the literature seems to be slow, though these techniques have become more commonplace.

Post-Occlusive Reactive Hyperemia

As mentioned above, the assessment of FMD requires a brief period of suprasystolic cuff occlusion of the forearm, which elicits a transient rise in blood flow as a result of the decreased forearm vascular resistance. This increase in blood flow is known as post-occlusive RH and is used to measure microvascular function (157). Post-occlusive RH requires the same setup as brachial artery FMD, though some technical considerations are different. While FMD requires B-mode imaging, post-occlusive RH requires the simultaneous measurement of brachial artery diameter and blood velocity using duplex mode (i.e., concurrent B-mode and pulse wave mode) (43, 157). As mentioned, brachial artery images are optimized when the probe is perpendicular to the blood vessel; the blood velocity signal, on the other hand, is optimized when the ultrasound is parallel (e.g., 0°) to the artery. Accordingly, to optimize both signals simultaneously, an insonation angle ≤60° is commonly used (43). Like FMD, post-occlusive RH is assessed during the 3-minutes following cuff release, though data are often only analyzed until peak diameter. The blood velocity is often analyzed concurrently with the brachial artery diameter using automated velocity profile tracing software (43). RH has been reported as peak blood velocity (166-168), peak blood flow (168, 169), velocity-time integral (i.e., velocity area-under-the-curve) (170-172), and flow-time integral (i.e., blood flow area-under-the-curve) (171, 173), though each may have its own utility and interpretation.

Mental Stress-Induced Brachial Artery Hyperemia

Temporary forearm ischemia is not the only method of inducing brachial artery hyperemia. Indeed, intra-arterial drug infusions are often used to manipulate brachial artery blood flow (48, 54, 174-176), though this methodology is highly invasive. In lieu of this, an additional technique that increases brachial artery blood flow and is sensitive to detect differences between groups with disparate endothelial function is the administration of mental stress. While mental stress can be elicited through several ways, including mirror tracing (177-179) and video game tasks (178, 179), rapid serial subtraction seems to be a popular modality (53, 140, 180-182). Many studies have previously used venous occlusion plethysmography to assess the hyperemic response to mental stress, particularly when coupled with intra-arterial infusions to assess mechanisms (53, 180-182). Duplex Doppler ultrasound can also be leveraged, however, to assess the hyperemic responses to mental stress (183), which can provide greater resolution for these measurements.

Interestingly, the mental stress task elicits an increase in sympathetic outflow (140, 183), which would typically yield vasoconstriction and a subsequent reduction in blood flow. This phenomenon does not appear consistently across vascular beds, though, as sympathetic outflow to the arm and to the leg is different (184). Accordingly, this dissociation leads to sympathetic withdrawal in the forearm and ultimate vasodilation. (185). The observed vasodilation in the forearm during mental stress has a few postulated mechanisms: 1) β-adrenoreceptor mediated dilation and 2) NO-mediated dilation. Collectively, it appears that both mechanisms contribute, and perhaps at different time points. Halliwill *et al*. (185) and Lindqvist *et al*. (186) both noted that the vasodilatory response to mental stress was attenuated during the administration of a β-adrenoreceptor antagonist. Despite this, evidence suggests that the involvement of β-adrenoreceptors is diminished during extended periods of mental stress (187), alluding to secondary

mechanisms involved in this vasodilation. Indeed, NO has been shown to be highly involved in the dilatory response to mental stress as the infusion of NOS inhibitors attenuates the hyperemic response (53, 181, 182, 188), though these results are equivocal (189). In the latter study by Lindqvist *et al*. (189), however, intravenous L-NMMA was used, which may obfuscate the comparison given the systemic influence of the NOS inhibitor (e.g., changes in cardiac and neural mechanisms). In this manner, early augmentations in forearm blood flow, perhaps driven by β-adrenoreceptor mediated dilation and increased cardiac output, lead to peripheral increases in vascular shear stress and NO production, whereby the combination of mechanisms contributes to the characteristic rise in forearm blood flow during mental stress.

Cerebral Vasomotor Reactivity

The cerebral circulation is highly sensitive to changes in arterial pH, such that lower pH elicits vasodilation. In this regard, elevated arterial CO_2 shifts the chemical balance in the carbonic anhydrase-bicarbonate reaction, favoring the formation of H^+ and HCO_3^- (190). Accordingly, the shift in CO_2 kinetics during hypercapnia and subsequent acid-base balance disruption stimulates a decrease in cerebrovascular resistance leading to augmented cerebral blood flow (191, 192). Experimentally, elevated arterial CO_2 can be provoked through several methods, including breath-holding (193, 194), acetazolamide administration (190, 193, 195, 196), steady-state CO_2 modulation (e.g., administration of a known PCO_2) (197-201), and CO_2 rebreathing (197, 202-204).

During experimentally induced changes in arterial CO_2 partial pressure (P_aCO_2), the subsequent changes in cerebral blood flow are often assessed via transcranial Doppler ultrasound (TCD) in the middle cerebral artery (MCA), anterior cerebral artery (ACA), or

posterior cerebral artery (PCA) (201-205). Working similarly as extracranial Doppler ultrasound, TCD emits high-frequency sound waves that are reflected off moving red blood cells in the cerebral vasculature. Unlike conventional ultrasound, current TCD systems are unable to project a "B-mode"-like image due to lack of resolution and the limitations of imaging through cranial bone, namely the temporal bones (205). Typically, the use of TCD during arterial CO_2 manipulation requires the assumption of constant arterial diameter, which prevents the user from measuring volumetric flow and necessitates the use of cerebral blood velocity as a surrogate. While this approach has been established and validated (206-209), questions regarding its reliability have been raised over the past decade (208, 210, 211). While still valid at small changes in P_aCO_2, larger changes may lead to substantial arterial vasodilation; using a cerebral blood velocity-only model may lead to underestimating cerebral blood flow (208, 210).

To mitigate some of the limitations with TCD, several approaches have been adopted in the recent literature: 1) utilizing magnetic resonance imaging (MRI) to target regional and global cerebral blood flow (210-212); 2) new approaches to TCD (e.g., imaging TCD), such that vessel diameter may be assessed (213); and 3) simultaneous use of TCD for intracranial hemodynamics and conventional Doppler ultrasound to measure extracranial hemodynamics in the internal carotid and vertebral arteries (198, 214). This last technique is particularly compelling as changes in extracranial blood flow directly reflect changes in intracranial flow. Further extracranial Doppler assessment may also be fundamentally less technical and less expensive than MRI studies (214), while concurrently being a more validated approach than imaging TCD (215). Accordingly, while TCD may remain valid for certain assessments, the addition of extracranial Doppler ultrasound in future studies will help elucidate cerebrovascular disparities in health and disease.

Cutaneous Thermal Hyperemia and Microdialysis

Much of the previously discussed techniques are either 1) too invasive (e.g., intra-arterial drug infusions), 2) too expensive for large scale use (e.g., MRI), or 3) not mechanistic enough (e.g., FMD, RH, CO_2 rebreathing) to fully elucidated any underlying physiology. In this regard, the cutaneous microvasculature has emerged as an easily accessible vascular bed that may represent global microvascular function (216). Using the same Doppler shift phenomenon that is utilized for B-mode or pulse wave imaging, cutaneous blood flow, indexed as red blood cell flux in perfusion units, can be assessed using laser Doppler flowmetry. This technique uses low-grade laser emitted into the skin, whereby the wavelength is altered upon hitting the underlying red blood cells, and changes proportionally to the number of red blood cells and their velocity (217). In this regard, any perturbation that modifies cutaneous blood flow (e.g., heat, pharmaceuticals) can be detected and recorded.

Cutaneous blood flow is highly sensitive to both whole body (71, 78, 133, 218, 219) and local tissue thermal load (15, 58, 62, 220-222). The use of local heating as a non-invasive external stimulus to increase cutaneous blood flow has been a popular experimental procedure for several decades. This popularity stems, in part, from the technique's ease of administration and relative applicability to vascular function, as certain thermal loads (e.g., 39°C local temperature) induce vasodilation that is predominantly NO-dependent (58, 222). During a typical local heating protocol, participants undergo several phases: 1) a baseline period during which skin temperature is clamped to ~33°C (roughly basal skin temperature); 2) a period of heating during which local temperature is often raised to between 39°C and 42°C; and 3) a period of heating during which local temperature

is further raised to either 43°C or 44°C (58, 222). During the second period of heating, three phases emerge: 1) an initial dilation, often termed an initial "peak," that is mediated by axon reflexes; 2) a brief nadir in cutaneous blood flow; and 3) a sustained dilation that is predominantly mediated by NO, the percentage of which is determined by the temperature used (58, 223). Following this phase, NOS inhibitors (e.g., L-NAME) can be infused using intradermal microdialysis (discussed later) to assess the contribution of NO to the 39°C plateau phase (58, 223). The third phase of heating serves as the maximal blood flow response and is site-specific. Given the sizeable intersite variability in cutaneous blood flow responses to heating, a maximal blood flow response (i.e., site-specific maximum) is typically gathered for normalization of the previous periods and often co-administered with intradermal sodium nitroprusside (SNP), an NO doner and endothelium-independent dilator (217).

While heating alone provides important insight into cardiovascular responsiveness in different populations, pairing local heating with intradermal microdialysis provides a pathway to understanding the etiology of CVD better. A vast majority of mechanistic *in vivo* studies utilize intra-arterial or intravenous drug administration, which can have larger or more systemic effects than necessary. Additionally, intra-arterial and intravenous drug delivery limits researchers to testing one condition at a time, leading to tedious data collection with long washout periods. Intradermal microdialysis, contrarily, allows for multiple drugs and conditions to be tested simultaneously without systemic effect (224, 225). Though intradermal microdialysis originated as a technique to assess the rate and mode of penetration of different drugs and toxic compounds (225, 226), it has evolved to elucidate the mechanisms of different pathways that modulate vascular tone and the changes that occur from health to disease (15, 19, 56, 58, 62, 219, 220, 222, 227).

With intradermal microdialysis, a microdialysis fiber consisting of a thin, hollow tube (typically 0.2-0.5 mm) connected to a semipermeable membrane (pore size of 6-3000 kDa, though smaller pores are more common) is inserted into the intradermal space using a guide canula. This guide canula (e.g., a 23-25 gauge needle) is inserted into the intradermal space, exiting approximately 2-2.5 cm away (224). Guide canula insertion may occur with or without local anesthesia to alleviate some of the pain associated with this technique (228, 229). Following guide canula insertion, the microdialysis fiber is threaded through the canula, which is subsequently removed, leaving the fiber in the intradermal space. Once the membrane is in the intradermal space, it is connected to a syringe placed on an infusion pump to control infusion rates (typically 0.1-5 $\mu L \cdot min^{-1}$) of different physiological solutions (224). These physiological solutions can be used to elucidate the mechanisms underpinning vasodilation and vasoconstriction in different conditions and populations while measured using the previously discussed laser Doppler flowmetry. Further physiological information can be gathered from the collection of the dialysate, which provides insight into the molecular dynamics of the interstitial space (224, 225). Utilizing the microdialysis approach, in-depth mechanistic understanding of vascular function in both health and disease can be uncovered.

Racial Disparities in Vascular Function

While CVD affects all sub-populations, some populations are at greater risk for developing CVD and its sequelae. In particular, the BL population suffers from the highest prevalences of CVD and hypertension, among other conditions, and one of the highest mortality rates from CVD (1). BL individuals also develop prehypertension and hypertension far earlier than other racial/ethnic groups (230) and experience greater detrimental side effects from hypertension (231). Accordingly, it is imperative that the mechanisms and factors contributing to the heightened risk in BL individuals. As previously discussed, blunted vascular function stemming from various sources contributes to CVD and hypertension pathogenesis. In this way, reduced vascular function most certainly precipitates the development of CVD in BL individuals.

PERIPHERAL VASCULAR FUNCTION IN NON-HISPANIC BLACK INDIVIDUALS

Given the relation between attenuated macrovascular function (e.g., FMD) and CVD risk (42, 158), this extension is easily made to the BL population. Specifically, previous reports note reduced FMD in the BL population (45, 46, 232), though these results are equivocal (233). Despite the similarities in FMD in the studies by Perregaux *et al.* (45) and Campia *et al.* (46), the former study found no difference in the brachial artery nitroglycerin response, while the latter study found an attenuation in the BL participants. Moreover, data from Gokce *et al.* (233) demonstrate no impairments in the nitroglycerin response, underscoring the variable brachial artery responsiveness in this population. Indeed, some of the differences may be derivative of structural rather than functional differences, representing a different matter, although still detrimental. Of note, the lack of normalization for shear stress (163) or baseline brachial artery diameter (160) in these

studies may account for some of the observed differences or non-differences. Equally important is the lack of direct sex comparisons in these studies. While studies present data from women independently, only Perregaux *et al.* (45) analyzed racial/ethnic differences in the women directly, further limiting conclusions made on the important race-by-sex interaction.

While FMD is an instrumental research measurement, intra-arterial drug infusions help determine other differences in vascular reactivity, particularly in the microvasculature, between BL versus WH individuals. In this regard, BL individuals exhibit blunted forearm blood flow responses during intra-arterial infusions of the endothelial-dependent vasodilators ACh, methacholine, and bradykinin (54, 176, 234, 235), though these results are equivocal (48). These findings of blunted vasodilator function in BL individuals extend to other pathways, including attenuated isoproterenol-mediated dilation (e.g., β-adrenoreceptor agonism) (54, 175, 176) and sodium nitroprusside (SNP) (54, 176), though the findings with SNP are not consistent across studies (48, 235). Accordingly, these data suggest that endothelial-dependent dilation is severely attenuated in BL individuals, though this may or may not occur in the presence of vascular smooth muscle function alterations.

As discussed in *Methods to Assess Vascular Function*, hyperemic responses can also occur following ischemic occlusion and during mental stress. Using these methods, several studies have demonstrated differences in the hyperemic patterns between BL and WH individuals. Heffernan *et al.* (236), Duck and Hoffman (237), and Ranadive *et al.* (238) each demonstrated reduced post-occlusive RH in BL versus WH individuals using five minutes of suprasystolic cuff occlusion and venous occlusion plethysmography. Interestingly, these data were observed in adolescents and young adults (i.e., < 30 years), which suggests the presence of reduced endothelial function at this young age, potentially contributing to the augmented prevalence of prehypertension and hypertension in young,

BL individuals (230). Further data from Morris *et al.* (9) highlight these racial disparities in RH using digital pulse amplitude tonometry (PAT) in the fingertip. This report helps to shed further light on possible race-by-sex differences in RH, seemingly demonstrating the BL women have similar PAT responses to WH women, while BL men have impaired RH relative to WH men. Of importance, though, are two key points: 1) this study did not specifically test the race-by-sex interaction, and thus the conclusions regarding this finding are limited; and 2) the age range used (20-70 years) produced a mean age of ~48 years, whereby these findings may be obscured by the onset of overt cardiovascular disease, particularly in the BL population. Accordingly, more research is needed to better understand the possible race-by-sex differences in reactive hyperemia.

Beyond post-occlusive RH, the BL population also experiences blunted cardiovascular responses to mental stress. Cardillo *et al.* (182) demonstrated that middle-aged BL men demonstrate measurably lower forearm hyperemia during mental arithmetic relative to age-matched WH men. Remarkably, the increase in forearm blood flow from baseline during the stressor was almost halved in the BL men. Unfortunately, this is one of the few reports that looks at racial differences in mental stress-induced hyperemia. Of the other studies that did assess this perturbation between BL and WH individuals, one did not find any differences in the hyperemic response between these two groups (180), one found differences in the response but utilized the calf rather than the forearm (239), and one did not measure blood flow (140).

Interestingly, the differences in the findings between this first study and Cardillo *et al.* (182) may derive from the age differences in the populations (~20 years vs. ~45 years) and subtle differences in the way the venous occlusion plethysmography was used in these studies. While in the calf, the data presented by Fredrikson *et al.* (239) make a natural extension of previous findings in the arm (182). Though a dissociation exists between the

arm and leg in response to mental stress (184), the augmented calf vasoconstriction and greater blood flow reduction in the BL versus WH participants studied by Fredrikson *et al.* (239) further establishes the presence of disparate vascular function in the BL population. Unfortunately, no studies seem to have investigated sex differences in the BL versus WH populations in response to mental stress, which opens a new avenue for future research.

CEREBRAL VASCULAR FUNCTION IN NON-HISPANIC BLACK INDIVIDUALS

With the elevated risk of cerebrovascular diseases (e.g., stroke, Alzheimer's disease and related dementias) in the BL population (1, 240), elucidating differences in cerebrovascular function is critical. Unfortunately, few studies have investigated racial/ethnic differences in cerebrovascular reactivity (CVR) in BL individuals. Moreover, while some studies have looked at sex differences in CVR (199, 200, 241), none of them have further divided their groups by race. In the studies investigating cerebrovascular function in BL individuals, the data demonstrated a significantly lower CVR during rebreathing-induced hypercapnia in young, BL versus WH individuals (203, 242). Interestingly, the differences in reactivity (e.g., MCA blood velocity, cerebral vascular conductance index) predominantly derive from an attenuated maximal vasodilatory responsiveness, without a difference in the slope of the relation between partial end-tidal carbon dioxide tension ($P_{ET}CO_2$) and the reactivity measure (203). These data suggest that reactivity, per se, is not altered, but that maximal cerebral vasodilation is blunted in a young, BL population. Interestingly, while women were included in this study, there were no direct sex comparisons made, limiting the applicability of these findings. A follow-up study demonstrated a similarly blunted maximal vasodilatory capacity, without impairment in the reactivity slope, in a young, BL versus WH population (242). While providing some

supporting evidence for these racial differences, this study also established that most, if not all, of this reduced vasodilation could be improved using flavanol supplementation, which speaks to some of the underlying mechanisms that will be covered in *Possible Contributing Biological Factors* later.

CUTANEOUS MICROVASCULAR FUNCTION IN NON-HISPANIC BLACK INDIVIDUALS

As mentioned previously in *Cutaneous Thermal Hyperemia and Microdialysis*, the cutaneous circulation presents an easily accessible vascular bed that is proposed to represent global microvascular function (216). In this regard, cutaneous microvascular function has been studied extensively in BL individuals, particularly over the past decade (62-64, 220, 243-246). In a few studies utilizing iontophoresis, a technique that delivers drugs transdermally using changes in applied electrical current, cutaneous vasodilation to ACh was substantially lower in the BL individuals compared to the WH individuals (243, 244), though these results were not consistent across skin sites (e.g., glabrous versus non-glabrous skin, finger versus forearm) (243). Additional data from Pienaar *et al.* (245) further equivocate the differences in cutaneous microvascular function in BL individuals as no differences in endothelium-dependent dilation were found, particularly after controlling for skin resistance. Interestingly, though, was their finding of differences in endothelium-independent dilation using iontophoretic application of SNP. Some of the findings from Pienaar *et al.* (245), though, obfuscate the conclusions as they utilized the forearm, which was shown to produce different responses than other cutaneous circulations (243).

Much of the remaining work studying the cutaneous circulation utilizes intradermal microdialysis and local heating to assess vascular function. Indeed, one of the first studies

to assess cutaneous thermal hyperemia in BL individuals found that the vasodilation in response to 39°C local heating, and the corresponding contribution of NO to that dilation, was blunted in BL relative to WH participants (64). Interestingly, these data also demonstrate that tempol, a superoxide dismutase mimetic, was able to sufficiently augment cutaneous vasodilation through NO-mediated pathways such that racial differences were abolished. A subsequent series of studies found similar blunting in cutaneous thermal hyperemia in BL individuals during the same 39°C stimulus (62) and a warmer 42°C stimulus (63), which produces a response that is proportionately less NO-mediated (222). While both studies further investigated mechanisms of blunted cutaneous microvascular function in the BL population, of note are the sex differences in the mechanisms leading to reduced function that Patik *et al.* (62) identified. These mechanisms will be discussed further in *Possible Contributing Biological Factors*. Last, a recent publication from Wolf *et al.* (246) corroborated earlier findings that suggest blunted NO-mediated dilation to 39°C heating in BL individuals, which was restored following tempol administration.

Beyond sustained dilation, a few studies have also noted alterations in the initial dilation to local heating in the BL population. As discussed previously, the initial dilation to local heating is axon-reflex mediated (58). In this manner, Wong *et al.* (220) noted that axon-reflex mediated dilation in normotensive BL individuals is blunted relative to normotensive WH individuals. Moreover, this dilation is blunted in prehypertensive WH individuals, but not further blunted in prehypertensive BL individuals, suggesting that even young, normotensive, BL people exhibit substantial alterations in cutaneous vascular function. Additional data from Wolf *et al.* (246) and Patik *et al.* (62) support these findings and further reveal that this attenuated vasodilation can be ameliorated through different vasoactive compounds (e.g., tempol, apocynin, allopurinol).

POSSIBLE CONTRIBUTING BIOLOGICAL FACTORS

While several mechanisms may contribute to the reduced vascular function observed in the BL population, key contributors include reduced NO bioavailability, augmented ET-1 bioactivity, and greater SNA influences. In this regard, detrimental alterations in each of these contributing factors have been observed in the BL population, potentially contributing to the heightened CVD risk.

Attenuated NO bioavailability and NO-mediated dilation, frequent hallmarks of reduced vascular function, are often observed in BL individuals (247). Indeed, numerous studies have observed blunted vasodilation to both endothelium-dependent dilators (e.g., ACh) (54, 176, 234, 235) and local heating (62-64, 246). Interestingly, this phenomenon has also been observed during non-traditional (e.g., intra-arterial drug infusions, heating) maneuvers such as mental stress (182), whereby BL men exhibit blunted increases in blood flow that are predominantly NO-mediated. In sum, these data would suggest that blunted NO bioactivity, and thus altered endothelial function, precipitates attenuated vascular function in BL individuals. Some data from intra-brachial drug infusions of SNP, though, would suggest that there may be a degree of reduction in vascular smooth muscle function beyond any changes in the endothelium. In fact, a few studies have demonstrated markedly larger blood flow responses in WH individuals during the same dose of SNP as in BL individuals (54, 176, 234). Within these data, though, are two important distinctions to be made. First, most of these participants were middle-aged (54, 234), whereas younger (< 25 y) BL individuals do not demonstrate this reduced endothelium-independent dilation (235), alluding to a potentially important racial difference in aging. Second, the studies by Cardillo *et al.* (54) and Stein *et al.* (176) primarily present the hemodynamic responses to drug infusion, but fail to report changes in vascular conductance or resistance. In light of this shortcoming, while Ozkor *et al.* (234) demonstrated racial differences in blood flow

during ACh and SNP, these differences only persisted for the ACh infusion when evaluated as resistance. Accordingly, endothelium-independent vasodilation may not be different in the BL population and the predominance of vascular function differences may stem from reduced NO bioavailability.

While NO bioavailability may be blunted in the BL population, the causes of this reduction are variable. One of the prime candidates for this physiological process is elevated oxidative stress, namely the overproduction of O_2^-. In this regard, *in vitro* experiments of both endothelial cells (247, 248) and peripheral blood mononuclear cells (249) demonstrate elevated O_2^- production in BL relative to WH participants. This rise in O_2^- is concomitantly met with a reduction in NO bioavailability as the two rapidly react to form $ONOO^-$, which is also elevated in BL individuals (247, 248). The elevated O_2^- production has been linked to increased NADPH oxidase (NOX) activity and protein expression in BL individuals (247, 248, 250). Indeed, *in vitro* treatment of endothelial cells from BL and WH donors with apocynin, an NOX antagonist, reduces the production of O_2^- and augments the ratio of NO to $ONOO^-$ (248). More importantly, *in vivo* mechanistic studies using microdialysis demonstrate that reducing O_2^- through tempol, apocynin, or allopurinol (xanthine oxidase antagonist) and augment NO-mediated vasodilation in BL individuals (62, 64, 246). Strikingly, the work by Patik *et al.* (62) suggests that this may not apply uniformly in the BL population. Indeed, this research demonstrates that apocynin and allopurinol improve NO-mediated vasodilation in BL men, but these findings did not extend to BL women. These data, therefore, suggest that unique sex differences in vascular function exist within the BL population that are not wholly explained by oxidative stress.

One possible NO-related pathway leading to reduced vascular function independent of oxidative stress is reduced L-arginine bioavailability. An essential precursor of NO-production, reductions in L-arginine from increased activity of enzymes such as arginase

(19, 61, 251) or creatine kinase (252) can subsequently reduce vasodilatory function. Regarding the effects of L-arginine in BL individuals, previous studies have noted that supplemental L-arginine can improve vasodilation in this population. A study by Houghton *et al.* (253) demonstrated that supplemental L-arginine augments ACh-induced increases in coronary artery blood flow in BL patients but not WH patients. Extending these findings, Kim *et al.* (63) determined that L-arginine administered via microdialysis can moderately improve vasodilation in response to 42°C local heating. Interestingly, the effect of the L-arginine may have been more significant at a lower temperature (e.g., 39°C) given the additional contribution of non-NO sources at this higher temperature (222).

Though reduced NO bioavailability can lead to greater basal vascular resistance, other factors may contribute to blunted vascular function in BL individuals. As a potent vasoconstrictor, ET-1 has been implicated in prehypertension and hypertension (108, 254-256), which may be different across races/ethnicities as hypertensive BL individuals possess greater circulating ET-1 (105, 257, 258) and modified receptor expression (257, 259). This physiological process may be primarily mediated through ET_AR (260) and may exert a synergistic effect with reduced NO bioavailability, further increasing vascular tone (16). Interestingly, this may also extend to BL individuals who are not patients as plasma ET-1 concentrations are elevated at rest (261, 262) and during acute stress responses (262). While Treiber *et al.* (262) demonstrated that BL male adolescents had greater circulating ET-1 concentrations than their female counterparts, this may not illuminate the entire vascular interaction with ET-1. Though BL men and women may have similar ET-1 receptor densities (259) and ET-1 concentrations when hypertensive (105), the end interaction may be of greater severity in BL women. Indeed, work by du Plooy *et al.* (263) in South Africans demonstrated similarly high plasma ET-1 in BL men and women, but only demonstrated relations between ET-1, blood pressure, and arterial stiffness in BL

women, suggesting that the detrimental effects of ET-1 are somehow exacerbated in BL women. However, considerations should be made to account for the fact that these participants were South African and may not fully relate to the United States BL population. Regardless, given this race-by-sex disparity, differences in ET_AR and ET_BR localization and activity may serve as key regulators of reduced vascular function, particularly in BL women.

While NO and ET-1 are potent regulators of vascular tone and subsequent vascular function, the sympathetic nervous system can also produce deleterious outcomes in the BL population. Typically, a rise in SNA yields vasoconstriction. However, during different maneuvers, the rise in SNA has been observed to be greater (139), equal (8, 131, 137, 140), or less (8, 131, 137, 138, 140) in BL versus WH participants. In many of these studies, though, the blood pressure reactivity was noted to be similar or greater in the BL participants, suggesting that SNA is not the only factor determining sympathetically mediated hemodynamic changes in the BL population. Indeed, sympathetic transduction may be a more significant contributor to blood flow and pressure regulation in BL individuals, which may leave this population at greater risk for the development of blunted vascular function and CVD.

In both younger and older BL adults, augmented transduction has been previously noted. Indeed, Ray and Monahan (8) noted that young, BL men and women exhibit larger changes in vascular resistance for a given change in total MSNA during both -5 and -40 mmHg lower body negative pressure. Okada *et al.* corroborated these findings in elderly individuals during both orthostasis (138) and a cold pressor test (137). During orthostasis, older, WH individuals were shown to have a reduction in transduction from the supine position, while older, BL individuals maintained transduction across postures, suggestive of alterations in transduction during stressors (138). While using the change in diastolic

blood pressure per unit change in total MSNA, older BL, individuals were also shown to exhibit nearly two-fold greater sympathetic transduction relative to age-matched WH individuals (138). Though many studies note augmented transduction in the BL population, these data remain equivocal. Interestingly, Jarvis *et al.* did not find the same augmented transduction in young, BL women (131), though a tendency for an increase was noted.

Beyond physiological maneuvers, augmented transduction in young, BL individuals may occur at rest as well. Indeed, in a cohort of young, BL and WH men, Vranish *et al.* (7) demonstrated that, for a given burst of spontaneous SNA, young, BL men produced a greater reduction in vascular conductance and a more considerable increase in mean arterial pressure, which may be further controlled by burst pattern. The augmented sympathetic transduction in these studies may directly be related to alterations in adrenoreceptor sensitivity. Indeed, BL individuals exhibit augmented vasoconstriction to graded intra-arterial infusions of phenylephrine, an α_1-adrenoreceptor agonist, such that the effective dose to elicit half-maximal constriction is nearly half that of WH individuals (47, 264). This may not be the case in BL women (264), though, further highlighting potential sex differences in this population. Conversely, β-adrenoreceptor sensitivity may be reduced in the BL population as demonstrated by attenuated responsiveness to isoproterenol (47, 54, 175, 176). Additionally, under behavioral stressors that generally produce less vasodilation or physical stressors that produce greater vasoconstriction in BL versus WH men, the administration of propranolol (β-adrenoreceptor antagonist) elicits little change in the responses in BL men, but completely reverses the response in WH men. Remarkably, this same response does not seem to occur in BL women, highlighting the important race-by-sex interaction in the BL population (265). To summarize, BL men exhibit increased α-adrenoreceptor and blunted β-adrenoreceptor sensitivity that may

contribute to the heightened vascular transduction in this population, though BL women may not experience the same physiological responses.

CONTRIBUTIONS OF SOCIAL DETERMINANTS OF HEALTH

While this dissertation primarily focuses on observations of reduced vascular function in BL men and women focusing on the biological underpinnings, it is important to identify and discuss some of the social determinants of health that play a vital role in the observed vascular responses. The worsening health status in the United States BL population may be linked within the broader context of social determinants of health, including socioeconomic status, education, racial bias, and perceived discrimination (266). Indeed, BL individuals are reportedly less likely to have completed more than 12 years of school and graduate college, are much more likely to have a yearly household income <$30,000, are much more likely to experience multiple events of discrimination, particularly on a day-to-day basis, and are more likely to experience general life and financial stress (266). While this study was conducted over two decades ago, these findings maintain their relevance. Indeed, following the COVID-19 pandemic caused by SARS-CoV-2 and the social justice protests of 2020, these disparities were only amplified (267). Accordingly, it is essential to understand how such disparities can contribute to worsened health status.

Viewed through a more biological lens, the worsened health outcomes from social factors in the BL population may derive from several sources. Of these factors, the most succinct description comes from the phenomenon termed *John Henryism*. Sherman James (268) described *John Henryism* as a form of high effort, prolonged, active coping with stressful environments. These stressors often include chronic financial strain, job

insecurity, and subtle or overt racial or social discrimination. Most often, the deleterious effects of these stressors (e.g., hypertension) are observed in individuals that persist and "successfully" cope (268). Interestingly, this coping process may be producing negative biological responses, such as augmented oxidative stress and inflammation (269, 270), that may be impairing vascular function (62, 64, 90). Accordingly, it is important to put the biological findings of future research into a broader context that also considers social determinants of health. Further, most of the previous research into social stressors takes the simple approach of racial/ethnic differences and fails to capture the additional influence of sex. In this regard, Brownlow *et al.* (271) note that BL men and women experience stress differently, which may further contribute to some of the sex differences observed in the physiological responses in this population.

Conclusions

Reduced vascular function is a highly complex and multifactorial condition that serves as a key contributor to CVD development. While CVD and blunted vascular function occur in all populations, the United States BL population is disproportionately affected (1, 45, 46, 53, 54, 272, 273). These disparities also extend to a unique race-by-sex interaction, whereby BL men and women often exhibit disease prevalence unlike sex differences in any other racial/ethnic population (1, 62, 272, 273). Though numerous factors likely contribute to the development of these conditions, controlling mechanisms such as NO bioavailability (54, 62-64, 176, 182, 234), ET-1 bioactivity (105, 113, 258, 260), and SNA/sympathetic transduction (8, 47, 137, 138, 145) can all impact peripheral, cerebral, and cutaneous vascular function, particularly in BL individuals, and may be further modulated by social determinants of health.

Much of this research, however, is lacking in determining whether sex differences in vascular function exist in the BL population. Further, of the sex differences in vascular function that have been observed in the BL population, the controlling mechanisms remain predominantly unelucidated. Last, understanding the mechanisms of altered vascular function in the BL population is not enough; finding ways to intervene and improve function is needed. Accordingly, the remainder of this dissertation will be used to explore: 1) peripheral and cerebral vascular function in BL men and women, 2) additional mechanisms of reduced cutaneous vascular function in BL women, and 3) interventions to improve vascular function in BL men.

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CHAPTER 3: YOUNG, NON-HISPANIC BLACK MEN AND WOMEN EXHIBIT DIVERGENT PERIPHERAL AND CEREBRAL VASCULAR REACTIVITY

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Abstract

In the U.S., cardiovascular and cerebrovascular diseases remain more prominent in the non-Hispanic black (BL) population relative to other racial/ethnic groups. Typically, sex differences emerge in the manifestation of these diseases, though unique patterns appear in the BL population. Although numerous mechanisms are implicated, differences in vascular function likely contribute. Research has demonstrated blunted vasodilation in several vascular regions in BL versus non-Hispanic white individuals, though much of this work did not assess sex differences. Therefore, this study aimed to ascertain if several indices of vascular function are different between young, BL women (BW) and men (BM). Eleven BW and 15 BM (22 (4) vs. 23 (3) y) participated in this study. Each participant underwent testing for brachial artery flow-mediated dilation (FMD), post-occlusive reactive hyperemia (RH), and cerebral vasodilatory reactivity (CVR) during rebreathing-induced hypercapnia. BW exhibited greater FMD than BM ($P = 0.02$), but similar or lower RH when assessed as blood velocity ($P > 0.39$ for all) or blood flow reactivity ($P < 0.05$ for all), respectively, relative to BM. Across a range of hypercapnia, BW had greater middle cerebral artery blood velocity and cerebrovascular conductance index than BM ($P < 0.001$ for both). These data suggest that young, BW have greater vascular function relative to young, BM, though this was inconsistent across the different indices. Our vascular reactivity findings may shed insight on the divergent epidemiological findings between BL men and women. Further research is needed to elucidate possible mechanistic underpinnings and relate these physiological responses to epidemiological observations.

Introduction

All-cause cardiovascular disease (CVD) prevalence and mortality remain prominent issues in the United States (1) despite continued medical advancements and interventions. Indeed, after a brief downward trend starting in the 1990s, adult CVD prevalence increased from 35% to nearly 50% over the past 5-10 y (1, 2). Both men (~36% to ~54%, respectively) and women (~33% to ~44%, respectively) experienced this rise in disease prevalence, which ultimately manifests as an additional 70,000 CVD-related deaths per year (1, 2).

While CVD prevalence and mortality continue an upward trajectory, not all population groups are affected equally. For instance, non-Hispanic black (BL) individuals exhibit the highest prevalence of CVD and hypertension and one of the highest CVD mortality rates compared to other populations (1). Additionally, BL individuals develop prehypertension and hypertension at a much younger age (3) and experience greater morbidity and mortality from hypertension (4). These observations extend to cerebrovascular diseases and mortality, as the BL population has a greater cerebrovascular disease incidence, prevalence, and age-adjusted mortality than other racial/ethnic groups (1). Later in life, BL individuals also suffer from greater cognitive impairment (5) and a higher prevalence of Alzheimer's disease and related dementias (6), relative to other racial/ethnic groups. Interestingly, sex differences emerge such that BL women (BW) have distinct responses depending on the epidemiological category. For instance, while women of other racial/ethnic groups have a lower prevalence of all-cause CVD relative to their respective male counterparts, BW suffer from a nearly identical CVD prevalence as BL men (BM) (1). Contrarily, BW experience reduced age-adjusted mortality from stroke compared to BM, which is not seen in other groups (1). Accordingly, the CVD disparities between BM and BW seem to be unique and remain incompletely understood.

CVD and cerebrovascular disease genesis remain inherently multifactorial processes. Differences in macro- and microvascular function, however, prevail as invariable hallmarks of various CVD and cerebrovascular disease. Reduced vascular function often precipitates the development of conditions such as atherosclerosis and coronary disease (7). Accordingly, these reductions may catalyze the development of overt disease. In this regard, the BL population exhibits blunted vasodilation (8-12) and exaggerated vasoconstriction (9, 13-15) at rest and to varying stimuli compared to matched non-Hispanic, white (WH) individuals. Mechanistically, the blunted vasodilation in BL individuals often derives from reduced nitric oxide (NO) bioavailability (12, 16), while the exaggerated vasoconstriction may stem from several sources, as detailed by Brothers et al. in a recent review (17).

The extent of differences in the control of vascular tone often changes responsiveness to different physiological maneuvers used to assess vascular function. In this regard, brachial artery flow-mediated dilation (FMD; an index of macrovascular function) is blunted in BL versus WH men and women following a period of brief cuff occlusion (8, 11), although these results are equivocal (18). Further, cerebrovascular responsiveness to rebreathing-induced hypercapnia is reduced in BL versus WH participants (10). In these studies, however, comprehensive measures of reactive hyperemia (RH), an index of microvascular function (8, 11), or direct sex comparisons (8, 10, 11, 18) were not elucidated. Therefore, the purpose of this study was to better understand the peripheral and cerebral vasodilatory responses in young, BM and BW. We hypothesized that BM and BW would exhibit divergent 1) FMD and RH responses to a period of brief cuff occlusion and 2) cerebral vasomotor reactivity to rebreathing-induced hypercapnia.

Methods

Ethical Approval

The Institutional Review Board at the University of Texas at Arlington approved all procedures for this study (UTA IRB #2016-0847, #2017-0856, and #2019-0318). Participants were given a verbal description of all procedures, purposes, and risks involved before providing their informed, written consent. This study conformed to the standards set by the Declaration of Helsinki (apart from registration in a database).

Participant Characteristics

Eleven young, BW and 15 young, BM participated in this study. Subject characteristics are presented in Table 1. To determine eligibility, each participant self-reported their racial identity. Smokers and competitive athletes were excluded. Additionally, all participants were free from overt cardiovascular, metabolic, and neurological disease and were not taking vasoactive prescription medications or supplements. Participants fasted a minimum of 4-h, abstained from caffeine a minimum of 12-h, and abstained from alcohol and strenuous activity for 24-h before each experimental visit. Women were studied in the low hormone phase of their menstrual cycles as assessed by venous blood sample analysis (LabCorp; Dallas, TX; Table 1). Following arrival to the lab, measures of height and mass were collected for each participant using a digital scale and stadiometer (Seca 769, Seca North America; Chino, CA). Then, each participant laid supine on a patient bed for instrumentation, measures of baseline cardiovascular parameters, and the assessment of cerebral vascular function.

INSTRUMENTATION

Each participant was instrumented for the continuous measurement of heart rate, via electrocardiography (CardioCard, Nasiff Associates; Central Square, NY), and intermittent blood pressure, via electrosphygmomanometry (Tango+, SunTech; Raleigh, NC). On the arm contralateral to the intermittent blood pressure cuff, non-invasive, beat-to-beat mean arterial blood pressure was measured via finger photoplethysmography during the rebreathing protocol (MAP; Finometer Pro, Finapres Medical Systems; Netherlands).

Peripheral vascular function was assessed using FMD and RH, as previously described (19). Briefly, a pneumatic cuff connected to a rapid inflation device (Hokanson Model E20 Rapid Cuff Inflator; Bellvue, WA) was placed just distal to the antecubital fossa and the brachial artery was imaged using high-resolution, duplex Doppler ultrasound. An adjustable frequency (10-13 MHz) linear array transducer (LOGIQ P5, GE Healthcare; Chicago, IL) was selected for optimal B-mode signals of the brachial artery and held in a stereotactic clamp 5-10 cm proximal to the antecubital fossa. Once a suitable image was obtained and optimized for clear delineation between the lumen and vessel walls, duplex mode (at a pulsed frequency of 5 MHz) was utilized for the continuous measurement of brachial artery diameter and blood velocity. The sample volume was set to encompass the entire lumen, without extending into the surrounding tissue, at an insonation angle of 60°. All images were recorded using a commercially available screen capture software (Elgato Video Capture, Corsair; Fremont, CA). Each image was analyzed offline using continuous edge-detection software (Cardiovascular Suite, Quipu; Pisa, ITA) along a section of the artery with clearly defined vessel walls, while second-by-second velocity was taken as the entire envelope.

Cerebral blood flow was indexed as middle cerebral artery (MCA) mean velocity (V_{MCA}) using transcranial Doppler ultrasound (TCD) following standard procedures (20).

Briefly, a 2-MHz TCD probe (Neurovision TC, Multigon Industries Inc.; Yonkers, NY) was placed on the left temple, superior to the zygomatic arch, and attached using an adjustable headband to maintain probe placement. Following insonation of the MCA through the transtemporal window, the TCD signal was optimized by adjusting the probe angle and insonation depth, gain, and amplitude. Each participant was then fitted with a mouthpiece attached to a three-way stopcock (Hans Rudolph; Shawnee, KS) that permitted rapid switching between ambient air and a 5-L rubber rebreathing bag (GPC Medical Ltd.; New Delhi, India) pre-filled with the participant's expired air. Partial end-tidal CO_2 tension ($P_{ET}CO_2$), a surrogate for the partial pressure of arterial CO_2 (P_aCO_2), was measured continuously through a sampling line connecting the mouthpiece to a capnograph (Capnocheck Plus, Smiths Medical; Dublin, OH). Peripheral oxygen saturation (S_pO_2) was monitored throughout the protocol with a digital pulse oximeter (Capnocheck Plus, Smiths Medical; Dublin, OH) placed on a finger. Respiratory excursions were measured with a piezo-electric respiration transducer (Pneumotrace II, UFI; Morro Bay, CA) placed around the abdomen.

PROTOCOL

Following instrumentation and a 15 min stabilization period, FMD and RH were assessed. After a 2 min baseline, during which brachial artery diameter and velocity were continuously measured, the pneumatic cuff was inflated to ~220 mmHg for 5 min to elicit ischemia. Upon cuff deflation, brachial artery diameter and blood velocity were recorded for an additional 3 min.

Immediately after the FMD/RH assessment, each participant was instrumented as outlined above for the hypercapnic challenge. Each participant breathed ambient air for 3

min for baseline data collection of V_{MCA}, MAP, HR, $P_{ET}CO_2$, S_pO_2, and respiratory rate. Immediately after this baseline period, each participant performed the rebreathing protocol as previously described (10). Briefly, the three-way stopcock Y-valve was switched from ambient air to the rebreathing bag such that the participant expired into and inspired from the 5-L bag, slowly raising P_aCO_2 and therefore $P_{ET}CO_2$. Rebreathing was continued until the participant reached discomfort, a discernable plateau in $P_{ET}CO_2$ was established, or 3-min elapsed, whichever came first. Following rebreathing, the Y-valve was switched back to ambient air for a 3-min recovery period. Throughout the rebreathe protocol, 100% oxygen was continuously administered into the 5-L bag to maintain arterial normoxia (S_pO_2 ~97%) (10).

DATA ACQUISITION AND ANALYSIS

Brachial artery responses following a brief period of forearm ischemia were continuously measured on a second-by-second basis. Brachial artery diameter (*D*; cm) and mean blood velocity (V_{BA}; mean of summed anterograde and retrograde; cm • s^{-1}) were used to subsequently calculate shear rate ($4 \bullet V_{BA} \bullet D^{-1}$; s^{-1}) and blood flow ($\pi \bullet [D \bullet 0.5]^2 \bullet V_{BA} \bullet 60$; mL • min^{-1}). Peak V_{BA}, shear rate, and blood flow were identified as the highest single second recorded values. V_{BA} and shear area-under-the-curve (AUC) were calculated as the sum of second-by-second velocity and shear rate, respectively, from the end of occlusion until peak brachial artery diameter (19). Both total (Flow AUC_T) and incremental (i.e., above baseline; Flow AUC_I) blood flow AUC were calculated using second-by-second flow data from end occlusion to peak dilation. The latter was calculated to account for possible differences in baseline blood flow between groups.

All data during the hypercapnic challenge were collected using a data acquisition system and software (PowerLab and LabChart 8, ADInstruments; Colorado Springs, CO) and stored on a laboratory computer for offline analysis. All variables were taken as a 1 min average during baseline and on a breath-by-breath basis during the rebreathing protocol. Cerebrovascular conductance index (CVCi) was calculated as $V_{MCA} \bullet MAP^{-1}$ and expressed in both absolute and relative (i.e., percent change from baseline; ΔCVCi) terms. The absolute change in $P_{ET}CO_2$ ($\Delta P_{ET}CO_2$) was assessed over the entire rebreathe and MAP and CVCi were recorded as three breath averages at predetermined stages (i.e., $\Delta P_{ET}CO_2$ of 3, 6, 9, and 12 mmHg). The slope of each individual's absolute and relative response was calculated and averaged to determine mean cerebrovascular reactivity (CVR) during hypercapnic rebreathing.

STATISTICAL ANALYSIS

All comparisons for FMD and RH were completed using unpaired, two-tailed, Welch's t-tests. Hemodynamic responses at baseline and during the rebreathe were analyzed via two-way, mixed model ANOVA with the repeated factor of $\Delta P_{ET}CO_2$ stage (baseline, Δ3 mmHg, Δ6 mmHg, Δ9 mmHg, and Δ12 mmHg) and the non-repeated factor of sex (BM and BW). In the case of significant interactions, post-hoc Holm-Sidak corrections were performed. Unpaired, two-tailed, Welch's t-tests were performed for the slopes of the $P_{ET}CO_2$ versus CVCi (absolute and relative) relation. All data were analyzed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA) and SPSS 24 (IBM Corp.; Armonk, NY) and presented as mean (SD). The level of statistical significance was set *a priori* at $\alpha = 0.05$.

Results

FLOW-MEDIATED DILATION AND REACTIVE HYPEREMIA

At baseline, BW had a smaller brachial artery diameter than BM (2.98 (0.38) vs. 3.69 (0.65) mm, respectively; P = 0.002; Table 2). While no difference was observed between BW and BM for the absolute FMD (Δ0.27 (0.10) vs. Δ0.21 (0.12) mm; P = 0.214; Table 2), BW exhibited significantly greater %FMD than BM (9.10 (3.49) vs. 5.84 (2.78)%; P = 0.020; Figure 1A). Given the differences in baseline diameter between the BW and BM, we also compared the %FMD response, adjusted for baseline diameter, using allometric scaling as previously described (21). Following allometric scaling, the %FMD response remained greater in the BW compared to the BM (9.00 (3.19) vs. 5.95 (3.09)%; P = 0.044). To account for the influence of shear rate, the primary stimulus for vasodilation, %FMD was analyzed using shear AUC as a covariate. Accordingly, shear-adjusted %FMD remained significantly greater in the BW versus BM (9.15 (3.32) vs. 5.79 (3.87)%; P = 0.009).

Following cuff occlusion, neither peak V_{BA} (77.1 (19.9) vs. 79.4 (19.6) cm • s^{-1}, respectively; P = 0.778; Table 2) nor V_{BA} AUC (42.6 (12.6) vs. 47.5 (15.8) cm, respectively; P = 0.387; Table 2) were different between the BW and BM. Additionally, no differences were noted between BW and BM for peak shear (1,040.1 (208.4) vs. 917.4 (315.4) s^{-1}, respectively; P = 0.245; Table 2) or shear AUC (28,988 (7,121) vs. 30,276 (13,368) A.U., respectively; P = 0.754; Figure 1B) Peak blood flow, however, was different between BW and BM (343.6 (169.8) vs. 495.4 (197.9) mL • min^{-1}, respectively; P = 0.046; Table 2). This difference was abolished after normalizing peak to baseline blood flow in BW and BM (654 (211) vs. 865 (764) %baseline; P = 0.322; Table 2). Compared to BM, BW had a reduced $FlowAUC_I$ (133.6 (61.1) vs. 220.3 (130.7) mL, respectively; P = 0.035;

Figure 1C) and FlowAUC_T (175.9 (90.1) vs. 303.4 (206.9) mL, respectively; P = 0.046; Figure 1D).

REBREATHING-INDUCED HYPERCAPNIA

No differences in MAP (Table 3) were observed between the BW and BM at baseline (91 (9) vs. 89 (7) mmHg) nor during any stage of hypercapnia ($\Delta P_{ET}CO_2$; Δ3: 88 (11) vs. 88 (9) mmHg; Δ6: 93 (8) vs. 89 (8) mmHg; Δ9: 94 (8) vs. 90 (9) mmHg; Δ12: 95 (10) vs. 91 (9) mmHg; interaction P = 0.114). Further, no differences between groups were noted for either respiratory rate or S_pO_2 during hypercapnia (P > 0.519 for both; Table 3). At baseline, the BW had a greater V_{MCA} than the BM (72.8 (11.3) vs. 59.5 (9.4) cm • s^{-1}), which extended to each degree of hypercapnia (Δ3: 80.0 (14.7) vs. 59.3 (11.2) cm • s^{-1}; Δ6: 91.6 (14.7) vs. 62.9 (11.4) cm • s^{-1}; Δ9: 98.1 (15.8) vs. 70.0 (12.4) cm • s^{-1}; Δ12: 105.8 (18.2) vs. 72.5 (14.7) cm • s^{-1}; all P < 0.001; Table 2).

Given the lack of difference in MAP, the difference in V_{MCA} manifested in our measures of cerebral vascular tone as BW exhibited an augmented absolute CVCi at baseline relative to the BM (0.80 (0.10) vs. 0.68 (0.13) cm • s^{-1} • $mmHg^{-1;}$ Figure 2A). This pattern was also observed at each $\Delta P_{ET}CO_2$ stage (Δ3: 0.91 (0.11) vs. 0.67 (0.13) cm • s^{-1} • $mmHg^{-1}$; Δ6: 0.98 (0.10) vs. 0.71 (0.12) cm • s^{-1} • $mmHg^{-1}$; Δ9: 1.04 (0.09) vs. 0.78 (0.13) cm • s^{-1} • $mmHg^{-1}$; Δ12: 1.11 (0.11) vs. 0.80 (0.16) cm • s^{-1} • $mmHg^{-1}$; all P < 0.001; Figure 2A). These absolute CVCi differences also manifested in the slope of the $P_{ET}CO_2$ and CVCi relation (i.e., cerebrovascular reactivity; CVR), whereby BW had a greater slope than BM (0.024 (0.010) vs. 0.012 (0.006) cm • s^{-1} • $mmHg_{MAP}^{-1}$ • $mmHg_{PETCO2}^{-1}$; P = 0.004; Figure 2B)

While baseline absolute CVCi differences existed between the BW and BM, they did not affect the relative CVCi response. At each stage of $\Delta P_{ET}CO_2$, the BW exhibited an augmented ΔCVCi relative to the BM (Δ3: 12.9 (5.3) vs. 0.1 (9.4)%; Δ6: 23.0 (6.8) vs. 5.5 (10.2)%; Δ9: 29.9 (12.9) vs. 16.0 (11.2)%; Δ12: 40.7 (16.8) vs. 19.0 (12.3)%; all $P < 0.001$; Figure 3A). Further, these baseline differences did not affect CVR, such that BW maintained a greater slope than BM (3.07 (1.50) vs. 1.77 (0.94) %Δ • $mmHg^{-1}$; $P = 0.022$; Figure 3B).

Discussion

The purpose of this study was to determine whether young, BM and BW exhibit disparate vascular function. Accordingly, we tested brachial artery FMD and RH following a brief period of forearm ischemia for peripheral conduit and resistance artery function, respectively, while changes in V_{MCA} in CVCi during hypercapnia represented cerebral vascular function. The primary findings were that young, BW had 1) greater FMD, 2) similar shear rate, but lower blood flow reactivity following cuff occlusion, and 3) greater V_{MCA} and CVCi responses to a hypercapnic challenge relative to young, BM. Taken together, these data suggest that young, BW exhibit greater peripheral conduit artery function, similar peripheral resistance vessel function, and greater cerebral blood vessel function relative to young, BM.

The finding of a greater FMD in the young, BW departs from previous literature including race-by-sex differences in brachial artery reactivity (8, 11, 22). Indeed, these studies noted almost no difference between BM and BW. Several explanations may contribute to the different results in these previous studies. The participants in these studies 1) had a larger D_{base} (e.g., at least 0.4 mm greater than the current study), 2) had variable absolute diameter changes (e.g., men were not different, but women had smaller absolute dilation than the present study), 3) were older (>7 y), and 4) had a greater BMI (>3 kg • m^{-2}). Noting these differences, we can at least partially determine the interplay of D_{base} and shear rate on FMD. Despite allometrically scaling %FMD for D_{base} (21) or covarying for the shear stimulus (23), %FMD remained significantly greater in the young, BW in this study. Accordingly, it appears that these women have preserved, if not greater, conduit artery endothelial function relative to the young, BM. Based on the findings in middle-aged and overweight/obese individuals, however, this function may not be immune to the typical effects of aging (24, 25) or increases in BMI (26, 27), which may explain some of

the discrepancy between the current study and previous studies (8, 11, 22). Further, the protected function in young, BW may be lost at an earlier age given the augmented prevalence of CVD in BW (1).

Interestingly, despite our findings for %FMD, the RH response was variable, depending on the measurement. Our data suggest that reactive hyperemia-related sex differences exist in young, BL individuals as assessed by blood flow. Peak flow, flow AUC_T, and flow AUC_I were all greater in the BM, although the difference in peak flow was abolished after normalizing to baseline flow. Remarkably, previous data from Morris *et al*. (28) would suggest the opposite of our reactive hyperemia findings. Using digital pulse amplitude tonometry (PAT) in the fingertip, they found that BM exhibited lower RH index measures than BW. As PAT has been demonstrated to correlate well with RH assessed through Doppler ultrasound in both health and disease (29), other explanations may contribute to the disparate RH findings. Indeed, the population studied by Morris *et al.* (28) was ~25 y older than the BW and BM in this study, which may obfuscate comparisons with the present data as microvascular function degrades with age, particularly in the presence of CVD risk factors (30, 31). Perhaps another reason for these disparate RH findings is that PAT measures augmentations in finger pressure, while Doppler ultrasound uses direct measures of blood flow.

Simple calculations of brachial artery blood flow involve both V_{BA} and D (e.g., $D^2 \cdot 4^{-1}$). In this regard, blood flow can be modified by either variable, either individually or in combination. Immediately following cuff occlusion, the rapid surge in blood flow is primarily driven by a large augmentation of V_{BA}, rather than changes in D. In the current study, no differences between BW and BM were noted for either peak or AUC V_{BA}. Accordingly, the gradient for blood flow may not have differed between the BW and BM. While peak and AUC V_{BA} are commonly used measures of RH (32) that relate well to CVD

event free survival (33), they do not fully account for the differences in blood flow. Indeed, in the present study, the primary influence for the flow differences between the BW and BM is the sizable difference in *D* (~0.7 mm). While our observed difference in *D* helps mathematically explain the dissimilarity in blood flow, and thus RH, it does not explain why.

As the increase in blood flow corrects for the ischemic stimulus (30, 32, 34), it is reasonable that the greater the stimulus, the greater the RH. Indeed, previous data suggest that after correcting for tissue desaturation (i.e., ischemia), differences in RH are abrogated (34). In the present study, while BMI was similar, the BM weighed nearly 14 kg more than the BW. While direct measures of adiposity or forearm tissue mass were not made, it is highly likely that the BM in this study had a greater amount of lean forearm tissue. Indeed, previously reported sex differences for post-occlusive RH were ameliorated after normalizing for muscle mass (35). Therefore, the observed sex difference in RH as determined by blood flow may be directly related to the greater amount of ischemic tissue rather than an inherent disparity in peripheral microvascular function. Therefore, interpretations of RH in the present study warrant caution.

The present data extend previous findings from our group (10) demonstrating reduced CVR in young, BL individuals by establishing sex differences within this group. Previous work by Favre *et al.* (36) and Tallon *et al.* (37) included measures of cerebral vasomotor tone (e.g., cerebral vascular resistance) during hypercapnia in young men and women. While not the primary outcome of these studies, neither group noted differences in the change in vascular resistance across a change in $P_{ET}CO_2$ of ~6-10 mmHg while the participants were supine (36, 37). These differences accounted for an absolute reduction in resistance of ~0.12-0.19 mmHg $\cdot$ cm^{-1} $\cdot$ s^{-1} at Δ6 mmHg $P_{ET}CO_2$ (36) and ~0.41-0.48 mmHg $\cdot$ cm^{-1} $\cdot$ s^{-1} at Δ10 mmHg $P_{ET}CO_2$ (37), which equate to reductions in vascular

resistance of ~13-17% and ~32-40%, respectively. By comparison, the BW in the present study would fall at the low end of these ranges for similar $P_{ET}CO_2$ values, while the BM barely achieve half of this indexed vasodilation. Interestingly, despite our apparent differences in cerebral vasomotor tone with these previous studies, our observed differences for baseline and hypercapnic V_{MCA} were also noted by Tallon *et al.* (37). These findings are corroborated by a recent cross-sectional examination looking at sex differences in V_{MCA} across the lifespan. For the same age bracket (18-30 y), similar baseline differences in mean V_{MCA} were noted for the men and women in the present study, highlighting basal sex differences in cerebral blood velocity at this young age (38). None of these studies, however, reported the racial/ethnic composition of their participants. In the event of a mixed cohort, racial/ethnic differences in cerebral hemodynamics may not be readily apparent. Accordingly, while our differences in V_{MCA} mirror data from other research groups, the additional sex difference in cerebral vasodilation may be a characteristic unique to young, BL individuals.

As each of these vasoreactivity tests elicit dilation to varying degrees, it is important to understand their underlying mechanisms and how they relate back to the BL population. As perhaps one of the most studied vasoactive molecules of the past few decades, NO is important for understanding the variability within these measures of vascular reactivity between young, BM and BW. Previous literature demonstrates blunted NO bioavailability in BL individuals (12, 16), manifesting as blunted vasodilation. In this regard, NO has been implicated in the FMD responses in different populations. For instance, distal forearm occlusion, such as the protocol in the present study, has been demonstrated to elicit FMD that is ~60-70% NO-mediated (39). Ultimately the differences in FMD between the BM and BW may be the byproduct of altered NO status. Additional pathways include the production of prostacyclins and endothelial derived hyperpolarizing factors (39),

particularly as compensating factors, though their role in the FMD response between BW and BM have, to our knowledge, not been elucidated. An interesting intersection of the current race-by-sex difference in FMD and epidemiological data now emerges. While FMD can be used as a tool for the non-invasive determination of vascular health and prediction of CVD progression (19, 40), the greater FMD in the BW in this study does not parallel the development of CVD in this population. Perhaps this discrepancy stems from the age of the participants in the present study (e.g., ~23 y) and the onset age of overt CVD (e.g., midlife). As FMD declines with age (24), the greater FMD in these young, BM versus BW and equivalent epidemiological CVD prevalence between groups may indicate an accelerated vascular aging phenotype in BW.

Unlike FMD, the constitutive role of NO during post-occlusive RH is uncertain. While some studies have found small to large contributions of NO during RH (41-44), these findings are equivocal (45). Further, in the studies that found an influence of NO on RH, the specific temporal contribution has been variable. Some data suggest that NO contributes only to peak RH (42), only to the RH AUC (41, 43), or to both RH phases (44). Accordingly, while NO may contribute to RH, it may predominate during the prolonged phase (i.e., AUC) rather than the peak response. Other mechanisms may include prostaglandins (42), inward-rectifying K^+ channels (46), and Na^+-K^+-ATPase (46). In the scope of the present study, it appears that BL sex differences in RH depend on the measure (i.e., velocity versus flow), rather than the temporal response (i.e., peak versus AUC). Though the BL population typically exhibits reduced NO bioavailability (12, 16), the potentially low dependence of RH on NO complicates interpretation of the present data. These inconsistencies, however, may suggest several things: 1) RH has a negligible NO component; 2) sex differences in microvascular function may not exist in young, BL individuals; 3) RH in BL individuals, specifically, is primarily influenced by non-NO

mechanisms. Regarding the last point, there is no literature to our knowledge specifically investigating racial/ethnic or race-by-sex differences for these other controlling mechanisms in RH. Therefore, further research is needed to better understand the RH responses in the present study, the mechanisms behind them, and how they may contribute to the observed epidemiological data.

Increased arterial CO_2 reduces blood pH, leading to cerebral vasodilation and robust increases in cerebral blood flow (47, 48). This vasodilation is, at least partially, mediated by NO (49). During hypercapnia, however, NO may act indirectly and serve as a permissive molecule for other cerebral vasodilators. Indeed, NO-inhibition reduces cerebral blood flow at rest and during hypercapnia, the latter of which may be side-effect of reductions in the efficacy for other molecules to elicit vasodilation (50). This may be of particular importance in the BL population, as reductions in NO bioavailability may reduce overall cerebral vasodilation through this synergistic mechanism. Other factors that may control hypercapnia-induced cerebral vasodilation include prostaglandins, cyclic nucleotides, and inward-rectifying K^+ channels (48, 51), though the effect of these mechanisms is unknown in young, BL individuals, let alone between young, BM and BW. Beyond active vasodilation, augmented sympathetic nerve activity (SNA) may attenuate hypercapnia-induced vasodilation, ultimately dampening the typical increases in cerebral blood flow. Previous data from Jordan *et al.* (52) suggest that increases in SNA blunt the relation between $\Delta P_{ET}CO_2$ and ΔV_{MCA}. As young, BL individuals exhibit augmented sympathetic reactivity relative to young, WH individuals (13, 14), any hypercapnic vasodilation may be attenuated by this SNA. Remarkably, augmented sympathetic reactivity may not be consistent between BW and BM, as BW seemingly have similar reactivity as WH women (53).

Ultimately, any reduction in cerebral blood flow is detrimental to cerebral health, particularly when the effects are cumulative from early life. The observed reductions in CVR in young, BL individuals (10) may explain the greater risk for stroke (1), cognitive impairment (5, 54), and Alzheimer's disease and related dementias (6). When accounting for metrics such as age-adjusted stroke mortality, further differences emerge such that BW are seemingly protected relative to BM (1), which may be a result of the greater CVR in BW. These early life differences in CVR may provide some degree of cerebrovascular protection that delays stroke-related mortality to later life. More research, however, is needed to understand the mechanisms leading to altered cerebral blood flow and the disparate cerebrovascular disease risk in this unique race-by-sex manner.

While this study presents unique sex differences in vascular function in the young, BL population, there are considerations that should be made when interpreting these data. First, sex hormones can exert strong influences on vascular function (55, 56), which may be causing the observed sex differences. The women in this study were tested during a low hormone phase (see Table 1). Therefore, given the typical augmentation in vascular function during the high hormone phase of the menstrual cycle, we may be underestimating differences in vasomotor reactivity. Second, the use of TCD to estimate changes in cerebral blood flow is limited by its inability to measure vessel diameter. Given the hypercapnic challenge in the present study, TCD may still be a valid approach for assessing cerebral hemodynamics (48). Interpretation of these data, however, may become more challenging at greater $\Delta P_{ET}CO_2$, whereby changes in vessel diameter might lead to underreporting of cerebral hemodynamic changes (57). In this regard, it may be that the differences in CVR between BM and BW are underestimated at high $\Delta P_{ET}CO_2$, though the results at lower $\Delta P_{ET}CO_2$ (i.e., $< \Delta 9$ mmHg) remain valid. Third, we did not include a reference group (e.g., WH) in this study as the scope of this research was focused solely on sex differences within

the BL population. Given the extensive body of research demonstrating independent racial/ethnic or sex differences in vascular function (8, 10-12, 18, 24, 35), often including WH individuals, we aimed to assess this unique interaction between BL men and women across several vascular beds. Investigation across several vascular beds is of particular importance because differences in one vessel may not directly relate to another, particularly between the peripheral and cerebral circulations (58). Last, we did not explore the potential influence of various social determinants of health (e.g., socioeconomic status, perceived discrimination) on our observed sex differences in vascular function. Chronic elevations in social stressors are associated with augmented oxidative stress and inflammation (59, 60), among other detrimental biological mechanisms, which lead to reductions in NO-mediated vascular function (12, 61). While much of the previous research regarding these social stressors has focused on racial/ethnic differences, the additional influence of sex is an important consideration. Indeed, BL men and women seem to experience social stressors differently (62). Accordingly, future research should incorporate measures of social stress to better understand the impact it may have on sex differences in black individuals' vascular function.

In conclusion, we found that young, BW exhibit disparate peripheral and cerebral vascular reactivity relative to young, BM, including augmented FMD and CVR, yet similar post-occlusive RH. These sex differences allude to mechanistic differences that may be blunting or augmenting certain indices of vascular function in young, BM and BW. Accordingly, these differences may be driving such things as the reduced age-adjusted stroke mortality, but elevated CVD prevalence in BW. Further research is needed to better understand 1) the mechanisms underlying vascular function differences between young, BW and BM, 2) how any augmented function in BW, relative to BM, deteriorates across

the lifespan, and 3) how these functional disparities at a young age contribute to CVD development in later life.

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Tables and Figures

Table 3.1: Subject characteristics for the black men and women. BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure

	Black Men	*Black Women*
Age (y)	23 (3)	22 (4)
Height (cm)	177.7 (6.0)	160.6 (5.0)
Mass (kg)	74.9 (11.3)	61.0 (9.0)
BMI ($kg \cdot m^{-2}$)	23.7 (3.2)	23.8 (4.2)
SBP (mmHg)	119 (8)	120 (8)
DBP (mmHg)	71 (6)	73 (8)
Serum Estradiol ($pg \bullet mL^{-1}$)	-	39.1 (12.9)
Serum Progesterone ($ng \bullet mL^{-1}$)	-	0.5 (0.4)

Table 3.2: Additional brachial artery flow-mediated dilation (FMD) and hemodynamic parameters following a brief period of forearm ischemia. Black Men: n = 15; Black Women: n = 11. *D*: Diameter; V_{BA}: Brachial Artery Blood Velocity; AU: Arbitrary Units

	Black Men	*Black Women*	*P-value*
D_{base}	3.91 (0.68)	2.98 (0.38)	0.002
Absolute ΔD (mm)	0.21 (0.12)	0.27 (0.10)	0.214
V_{BA} Peak ($cm \cdot s^{-1}$)	79.4 (19.6)	77.1 (19.9)	0.778
V_{BA} AUC (cm)	47.5 (15.8)	42.6 (12.6)	0.387
Peak Shear (s^{-1})	917.4 (315.4)	1040.1 (208.4)	0.245
Shear AUC (AU)	30,276 (13,368)	28,988 (7,121)	0.754
Baseline Flow	84.3 (67.6)	52.8 (43.3)	0.162
Peak Flow ($mL \bullet min^{-1}$)	495.4 (196.9)	343.6 (169.8)	0.047
ΔFlow (%baseline-peak)	865.9 (764.4)	654.4 (211.2)	0.322

Table 3.3: Respiratory and cardiovascular parameters at baseline and during the hypercapnic challenge. Black Men: n = 15; Black Women: n = 11, except for Δ12 mmHg, where n = 8. S_pO_2: Peripheral Oxygen Saturation; MAP: Mean Arterial Blood Pressure; V_{MCA}: Middle Cerebral Artery Blood Velocity; $P_{ET}CO_2$: Partial End-Tidal Carbon Dioxide Pressure. (*): $P < 0.05$; (#): $P < 0.01$; (§): $P < 0.001$ vs. black men at the same $\Delta P_{ET}CO_2$ stage.

	Hypercapnia Stage ($\Delta P_{ET}CO_2$)					*P* - values		
	Baseline	Δ3 mmHg	Δ6 mmHg	Δ9 mmHg	Δ12 mmHg	Group	$P_{ET}CO_2$	Interaction
Respiratory Rate (breaths · min^{-1})								
Black Men	15 (4)	13 (5)	13 (5)	13 (5)	14 (5)	0.519	0.018	0.071
Black Women	12 (4)	11 (2)	12 (3)	13 (3)	15 (4)			
S_pO_2 (%)								
Black Men	97 (1)	97 (1)	98 (1)	98 (1)	98 (1)	0.842	0.010	0.212
Black Women	97 (1)	96 (3)	98 (2)	98 (1)	98 (1)			
MAP (mmHg)								
Black Men	89 (7)	88 (9)	89 (8)	90 (9)	91 (9)	0.415	<0.001	0.114
Black Women	91 (9)	88 (11)	93 (8)	94 (8)	95 (10)			
V_{MCA} (cm · s^{-1})								
Black Men	59.5 (9.4)	59.3 (11.2)	62.9 (11.4)	70.0 (12.4)	72.5 (14.7)	<0.001	<0.001	<0.001
Black Women	72.8 (11.3)*	80.0 (14.7)#	91.6 (14.7)§	98.1 (15.8)§	105.8 (18.2)#			

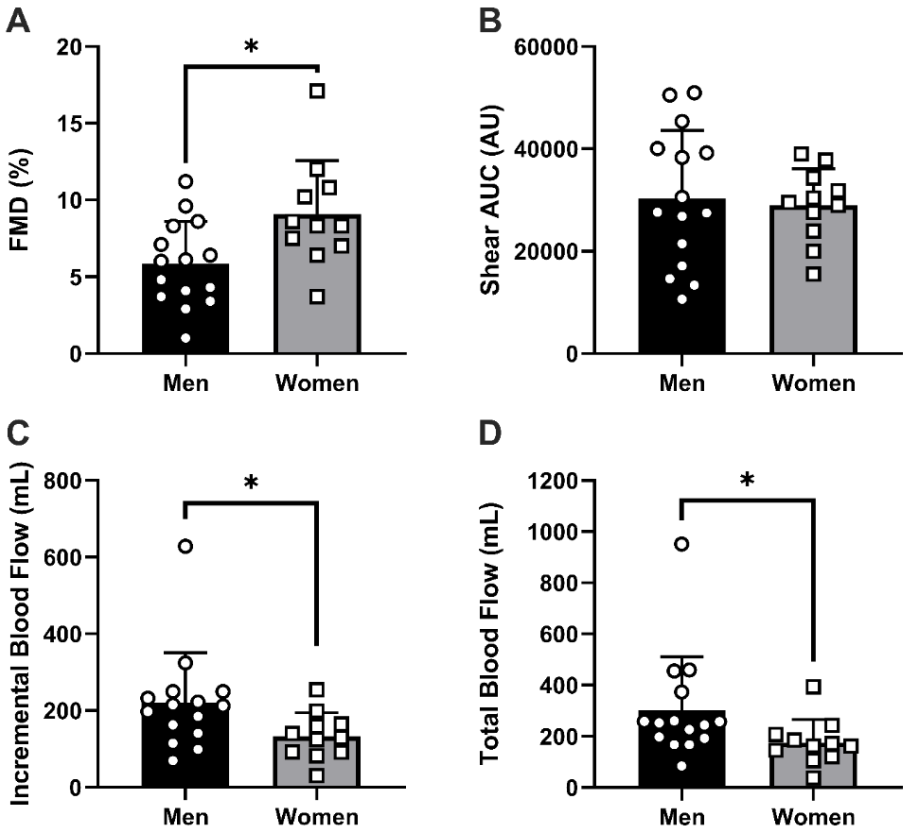

Figure 3.1: Brachial artery flow-mediated dilation (FMD; A), shear area-under-the-curve (AUC; B), incremental flow AUC (C), and total flow AUC (D) responses to a brief period of forearm ischemia. Shear AUC and flow AUC are summed from end-occlusion to peak brachial artery dilation. Data are from 11 BW and 15 BM. (*): $P < 0.05$

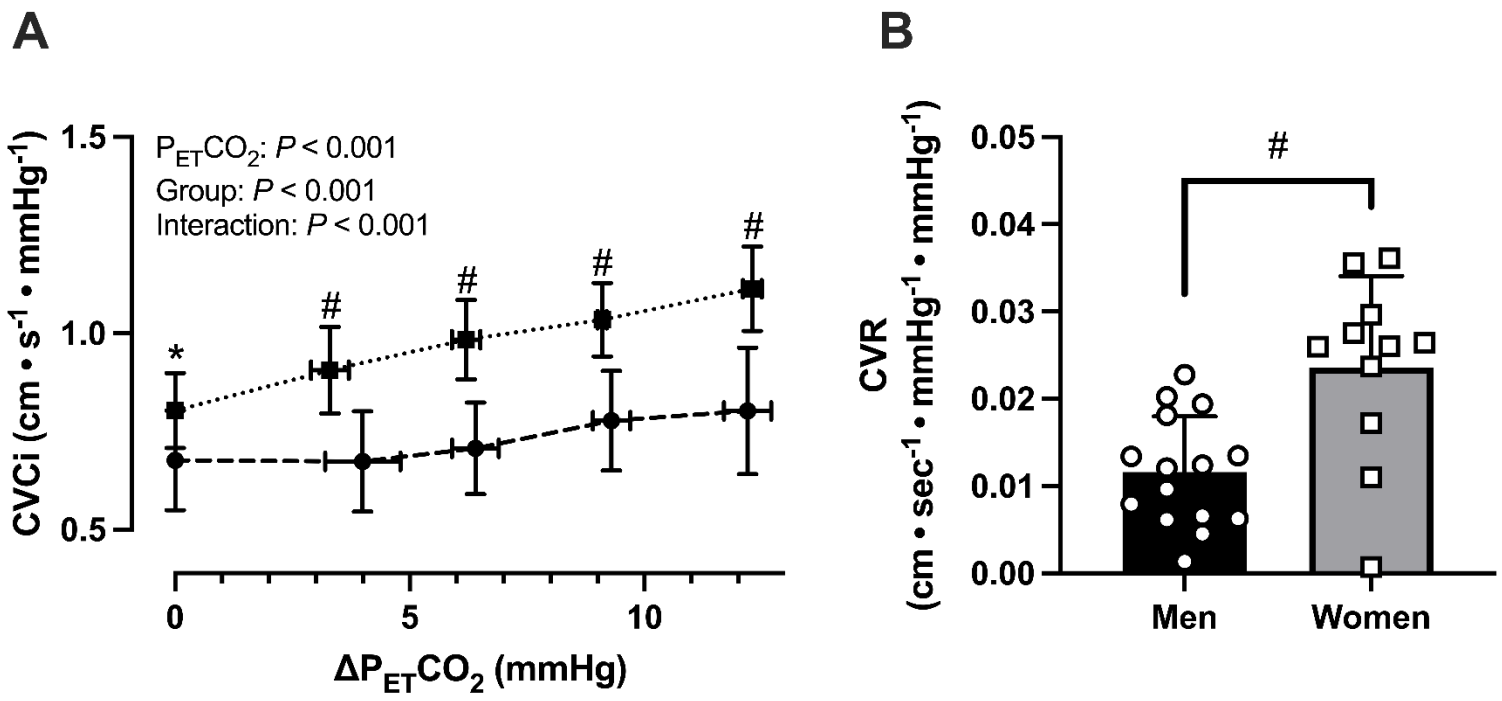

Figure 3.2: Absolute cerebral vascular conductance index (CVCi; A) and the slope of the partial end-tidal CO_2 tension ($P_{ET}CO_2$) and CVCi relation (i.e., cerebrovascular reactivity; CVR; B) at baseline and during rebreathing-induced hypercapnia. BW are represented by squares and BM are represented by circles. Data are from 11 BW and 15 BM, except for a $P_{ET}CO_2$ of Δ12 mmHg, whereby only 8 BW reached this stage of hypercapnia. CVR is measured as the change in blood velocity per unit change in blood pressure, per unit change in $P_{ET}CO_2$. (*): $P < 0.05$; (#): $P < 0.01$

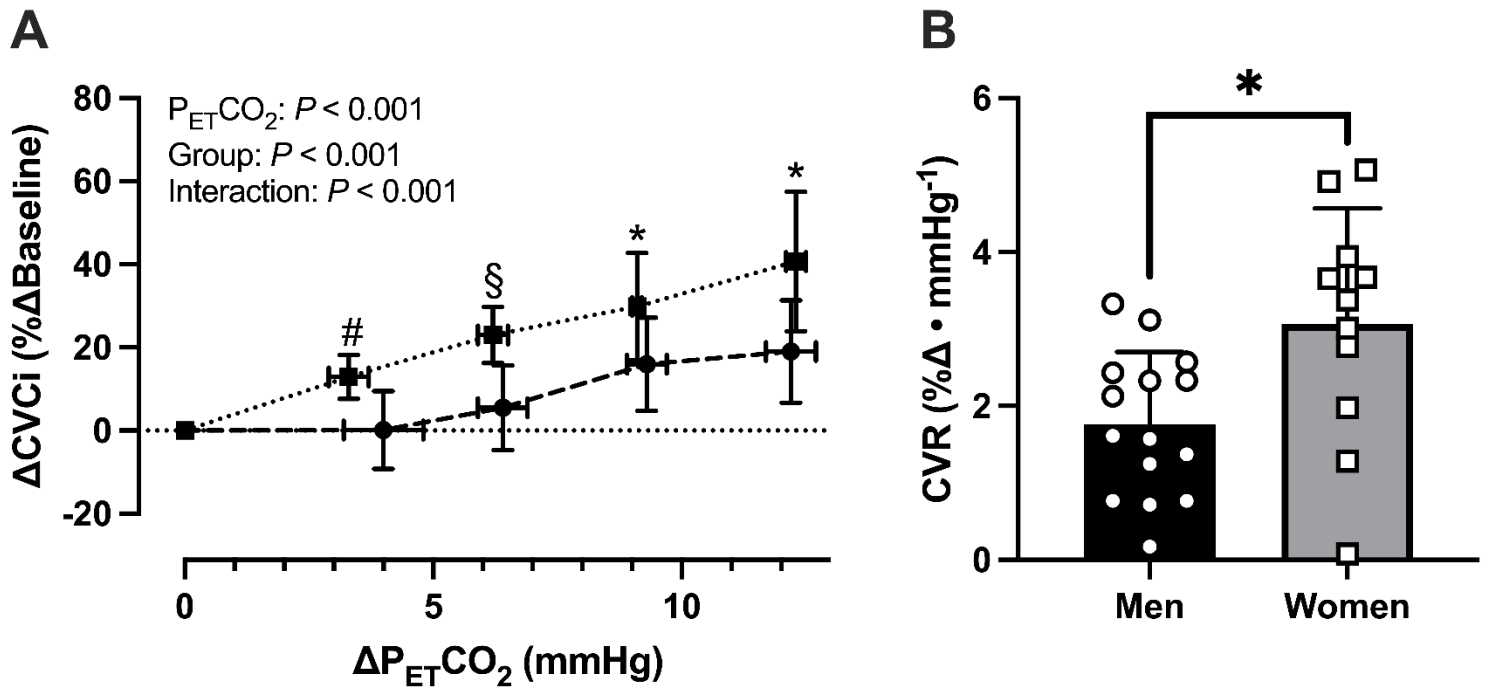

Figure 3.3: Relative cerebral vascular conductance index (ΔCVCi; A) and the slope of the partial end-tidal CO_2 tension ($P_{ET}CO_2$) and ΔCVCi relation (i.e., cerebrovascular reactivity; CVR; B) at baseline and during rebreathing-induced hypercapnia. BW are represented by squares and BM are represented by circles. Data are from 11 BW and 15 BM, except for a $P_{ET}CO_2$ of Δ12 mmHg, whereby only 8 BW reached this stage of hypercapnia. (*): $P < 0.05$; (#): $P < 0.01$; (§): $P < 0.001$

CHAPTER 4: CONTRIBUTIONS OF ENDOTHELIN-1 AND L-ARGININE TO BLUNTED CUTANEOUS MICROVASCULAR FUNCTION IN YOUNG, BLACK WOMEN

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Abstract

Non-Hispanic black (BL) individuals in the United States suffer from the greatest prevalence of cardiovascular disease (CVC) and hypertension, relative to other racial/ethnic groups (e.g., non-Hispanic white population; WH). This increased prevalence may be secondary to blunted vascular function. Interestingly, while women typically present with reduced CVD prevalence relative to men of the same racial/ethnic group, BL women (BW) experience similar CVD prevalence and reduced vascular function as BL men, though the mechanisms are different. Accordingly, this study tested the hypothesis that reduced microvascular function in young, BW is associated with endothelin-1 (ET-1) overactivity or insufficient L-arginine bioavailability. Nine BW and 9 WH women (WW) participated in this study (age: 20 ± 2 vs. 22 ± 2 y, respectively). Each participant underwent testing for cutaneous microvascular function during 39°C local heating, while Lactated Ringer's (control), BQ-123 (ET-1 receptor type A antagonist), BQ-788 (ET-1 receptor type B antagonist), or L-arginine was infused via intradermal microdialysis to modify cutaneous vascular conductance (CVC; red blood cell flux/mean arterial blood pressure). Subsequent infusion of N^{ω}-nitro-L-arginine methyl ester allowed for quantification of the nitric oxide (NO) contribution to vasodilation, while combined sodium nitroprusside and 43°C heating allowed for normalization to maximal CVC (%CVC_{max}). BW relative to WW had blunted %CVC_{max} and NO contribution to dilation during the 39°C plateau ($P < 0.027$ for both). BQ-123 improved the 39°C plateau response through augmented NO-mediated dilation ($P < 0.048$ for both). Interestingly, neither L-arginine nor BQ-788 modified the CVC response to heating ($P > 0.835$ for both) or the NO contribution to dilation ($P > 0.371$ for both). These findings suggest that cutaneous microvascular function is reduced in BW, and ET-1 receptor type A may contribute to this

dysfunction. Further research is needed to better characterize these mechanisms and continue to elucidate additional pathways by which this dysfunction manifests in young, BW.

Introduction

Cardiovascular diseases (CVD) represent a persistent societal burden in the United States as the prevalence of CVD approaches 50% in those older than 20 y and the annual cost of CVD is projected to reach $1.1 trillion by 2035 (1). Accordingly, identifying means to combat any substantiated rise in, and even reduce, CVD burden are warranted. While substantial progress has been made over the past two decades, as evidenced by the plateau in mortality related to CVD (1), significant problems remain. In particular, the non-Hispanic black (BL) population exhibits a >10% greater prevalence of CVD, predominantly via the prevalence of hypertension, compared to any other racial or ethnic population (1, 2). Additional sex differences are also apparent within each racial and ethnic group; BL women (BW) are more susceptible to manifest CVD relative to women from other racial/ethnic groups, leading to CVD risk comparable to BL men (BM) (1).

While the genesis of CVD is multifactorial, blunted vascular function is a major contributing factor. Reduced vascular function occurs through numerous mechanistic pathways, with attenuated nitric oxide (NO) mediated vasodilation, either via reduced NO bioavailability or responsiveness, often implicated as one of these primary pathways (3-5). Indeed, reductions in NO bioavailability following reactive oxygen species-mediated uncoupling of nitric oxide synthase (NOS) have been previously noted (6-8). These findings may be of particular importance as BL individuals, a group demonstrating blunted microvascular function (9-12), exhibit elevated oxidative stress relative to non-Hispanic white (WH) individuals (13, 14). A previous study by our group demonstrated blunted cutaneous microvascular reactivity to a local heating stimulus in BM compared to WH men, which was mediated by NADPH oxidase (NOX) and xanthine oxidase (XO) derived oxidative stress (11). Despite a similarly blunted cutaneous

microvascular reactivity, however, BW did not respond to NOX or XO antagonism (11). These data suggest sex differences in the mechanisms contributing to the previously observed race-by-sex differences in CVD prevalence (1). Accordingly, other factors that influence vascular tone may contribute to the observed functional differences in BW. Two possible candidate mechanisms that may blunt vasodilator function are increased endothelin-1 (ET-1) bioactivity and reduced L-arginine bioavailability.

As a potent vasoconstrictor released by the endothelium (15), ET-1 plays an essential role in the maintenance of basal vascular tone (16). Accordingly, any change in the interaction between ET-1 and its receptors can further modulate vascular tone. Indeed, the expression of ET-1 is augmented in prehypertension and hypertension (17, 18), which may be different across racial/ethnic groups, as hypertensive BL individuals exhibit elevated plasma ET-1 concentrations (19, 20) and modified receptor expression (20). These findings may extend to young, apparently healthy, BL individuals as plasma ET-1 concentrations are elevated at rest (21, 22) and during acute stress responses (22). Interestingly, modulations in ET-1 may also alter vascular structure (e.g., changes in arterial stiffness) in BW (23), ultimately leading to the genesis of CVD. Accordingly, ET-1 may play a role in the previously observed vascular dysfunction in BW (11).

While we did not observe oxidative stress-mediated reductions in dilation and NO in our previous study (11), it may be that the blunted vasodilation in BW is a result of endothelial NOS (eNOS) uncoupling. One pathway that may lead to eNOS uncoupling is a reduction in the key substrate L-arginine. In this regard, L-arginine performs a critical role in vascular function by serving as the precursor for the production of NO through eNOS (24, 25). Therefore, alterations in L-arginine metabolism or bioavailability can affect NO production and thus vasodilation. Indeed, endothelial-dependent vasodilation has been

augmented with the acute administration of L-arginine in multiple vascular beds in populations with blunted vasodilatory function (26, 27). These findings extend to young, BL individuals as research using intradermal L-arginine infusion during local cutaneous heating demonstrated improved vasodilation in BL participants (10). Though this study included women, it was not designed to elucidate sex differences. Therefore, the role of L-arginine in the blunted cutaneous microvascular reactivity in BW warrants further investigation.

Our objective was to test the hypothesis that exaggerated ET-1 activity diminishes cutaneous vasodilation in young, BW such that ET-1 receptor antagonism would restore this blunted function. Further, we hypothesized that reduced L-arginine bioavailability blunts cutaneous NO production in young, BW, and thus vasodilation, in a manner that can be improved by acute intradermal administration of L-arginine.

Methods

Ethical Approval

All procedures for this study were approved by the Institutional Review Board at the University of Texas at Arlington (UTA IRB: 2018-0648). Participants were given a verbal description of all procedures, purpose, and risks involved before providing their informed, written consent. This study conformed to the Declaration of Helsinki and was a registered clinical trial under the identifier NCT03679780 at www.clinicaltrials.gov.

Participant Characteristics

Nine young, BL and 9 young, WH women were studied. To determine eligibility, participants self-reported their racial and ethnic identity and that of their parents. Individuals of mixed-race were excluded from the study to prevent a mixed racial background confounding the interpretation of the results. All participants were normotensive during screening and free of overt cardiovascular, metabolic, and neurological disease and not currently taking vitamin and mineral-based supplements. Smokers and competitive athletes were also excluded.

Participants were asked to fast overnight and instructed to abstain from caffeine for 12 h and alcohol, strenuous exercise, and medications for 24 h before the study. All participants were studied during the early follicular phase of the menstrual cycle or during withdrawal bleeding if on hormonal contraception (mean ± SD; 4 ± 2 days from the start of menstruation). Height and weight were measured using a digital scale and a standard stadiometer (Seca 769, Seca North America; Chino, CA). Waist and hip circumferences were assessed with a flexible tape measure at the level of the umbilicus and the largest

circumference around the glutes, respectively. Blood was drawn via venipuncture from an antecubital vein into serum separator vacuum tubes, allowed to clot for 30-min, and centrifuged at 3,000 *g* for 10-min. Serum samples were then sent to a local laboratory for metabolic biomarker analysis (LabCorp; Dallas, TX; Table 1).

INSTRUMENTATION

After lying semi-recumbent on a laboratory bed, each participant was instrumented for heart rate, via electrocardiography (CardioCard, Nasiff Associates; Central Square, NY) and intermittent blood pressure (Tango+, SunTech; Raleigh, NC). Following at least 10-min of quiet rest, blood pressure was measured, and each subject was then instrumented for microdialysis and the assessment of cutaneous blood flow via laser-Doppler flowmetry (see remainder of *Instrumentation* and *Microdialysis Protocol*).

The left arm was supported at approximately heart level and, using clean technique, a 23-gauge needle was inserted intradermally in the dorsal forearm at four sites such that ~2.5 cm of the needle remained under the skin. Each site was located at least 2.5 cm apart and care was taken to avoid superficial veins. A microdialysis fiber (CMA 31, Harvard Apparatus; Holliston, MA) was then inserted through each needle, with subsequent needle removal such that the 10 mm semipermeable membrane (55 kDa) remained under the skin. Each fiber was then perfused with lactated Ringer's solution at 2 $\mu L \cdot min^{-1}$ via a syringe pump (Model 11, Harvard Apparatus; Holliston, MA). An integrated, multifiber laser-Doppler flowmetry probe (VP7a/T, Moor Instruments; Wilmington, DE) housed inside of a local heating unit (PF-450, Perimed AB; Stockholm, SWE) was placed over each site for the assessment of cutaneous blood flow as red blood cell flux (RBF) and the manipulation of local temperature, respectively. Continuous measurements of RBF and local temperature

were recorded at 400 Hz via a data-acquisition system and saved locally for offline analysis (PowerLab and LabChart 8, ADInstruments; Colorado Springs, CO).

PHARMACOLOGICAL AGENTS

L-arginine (10 mM, Millipore Sigma; St. Louis, MO) was used as a supplement, while BQ-123 (500 nM, Millipore Sigma), and BQ-788 (300 nM, Millipore Sigma) were used to inhibit ET-1 Type A (ET_AR) and Type B (ET_BR) receptors, respectively. The concentrations of these substances were chosen based on previous literature (10, 28-30). Each site was randomly assigned either one of the three agents or a continued infusion of lactated Ringer's to serve as a control site. The NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20 mM; Millipore Sigma) and the NO donor sodium nitroprusside dihydrate (SNP; 28mM; Millipore Sigma) were later infused through all sites to assess the NO contribution to dilation and maximal RBF, respectively (31, 32). Each drug was prepared via dilution with lactated Ringer's and infused at 2 $\mu L \cdot min^{-1}$ after a 30 s priming rate of 52 $\mu L \cdot min^{-1}$.

MICRODIALYSIS PROTOCOL

Following the microdialysis fiber insertion, at least 60 min with concomitant lactated Ringer's infusion was provided for trauma resolution. Following trauma resolution, the experimental drugs were infused for a 30 min wash-in period. After 30 min, the local temperature was increased to 33°C via the heaters to account for interindividual and intersite variability in skin temperature. After 10 min at 33°C, local skin temperature was elevated to, and subsequently clamped at, 39°C at a rate of $0.1°C \cdot s^{-1}$ to induce local

cutaneous vasodilation. 39°C was chosen as this temperature has been demonstrated to produce a dilation that is predominantly NO-mediated (33) and was used in our previous study noting differences in local thermal hyperemia between BW versus WW (11). Within the first 5 min, an initial rise in RBF occurs that is axon reflex-mediated, followed by a brief nadir and then sustained NO-mediated plateau in RBF after 30-40 min (33, 34). Following a sustained plateau of at least 5 min, L-NAME was perfused through all sites for ~30 min until RBF stabilized to allow for the quantification of the NO contribution to vasodilation. Upon stabilization, SNP was perfused through all sites before the local temperature was increased to 43°C to elicit site-specific maximal vasodilation within 20-30 min (29, 31, 32).

DATA ANALYSIS

Brachial blood pressure was measured in triplicate and used to calculate mean arterial blood pressure (MAP; ⅓ systolic + ⅔ diastolic). RBF was evaluated as a stable 1 min average flux at the end of the 33°C baseline, the 39°C plateau, the L-NAME steady state, and the combined SNP and 43°C maximum. These values were then calculated as cutaneous vascular conductance (CVC; $RBF \cdot MAP^{-1}$) and each value was normalized as a percent of site-specific maximal vasodilation ($\%CVC_{max}$).

STATISTICAL ANALYSIS

Data for the microdialysis protocol were analyzed at each stage using a two-way, mixed-model ANOVA with the factors of race (BW and WW) and drug site (control, L-arginine, BQ-123, BQ-788). When a significant main effect or interaction was observed, post-hoc

Holm-Sidak corrections were applied. All data were analyzed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA) and presented as mean ± SD. The level of statistical significance was set *a priori* at $\alpha = 0.05$. An *a priori* power analysis ($\alpha = 0.05$, $\beta = 0.80$) indicated that a minimum total sample size of 12 participants (i.e., 6 per group) would be needed to detect a large treatment effect (35), which aligns with previous comparisons made from our group (11).

Results

The participants in this study were well matched for age, BMI, waist and hip circumference, baseline blood pressure, and most blood biochemistry (Table 1). The BW were, on average, ~4 cm shorter than the WW (P = 0.046), though this did not influence BMI. Additionally, serum triglyceride and very low-density lipoprotein concentrations were lower in the BW versus WW (P = 0.003 for both). Despite these differences, the blood biochemistry profiles in both groups were within a normal range. Due to the nature of the local microdialysis infusions, we did not expect a change in MAP during the protocol. Indeed, no main effects of group or phase were observed, nor was a group-by-phase interaction noted for MAP (P > 0.115 for all; Table 2).

At baseline, no differences were noted in the absolute CVC for either group or drug site main comparisons (P > 0.137; Table 3). After normalizing to the site-specific maximum, however, a main effect of group (P = 0.034; Figure 2A) was noted such that the BW expressed a reduced relative CVC (i.e., $\%CVC_{max}$) compared to the WW. There was no main effect of drug (P = 0.758; Figure 2A) nor a group-by-drug interaction (P = 0.666; Figure 2A; Table 4), suggesting that the intervention drugs had no effect on baseline CVC.

During the initial dilation phase following local heating to 39°C, no main effects of group (P = 0.843) or drug site (P = 0.453) were noted for absolute CVC (Table 3). This lack of difference extended to the group-by-drug site interaction (P = 0.355). Once normalized to the maximal dilation, the lack of main effects (group: P = 0.087; drug site: P = 0.223) and interaction (P = 0.223) persisted in the relative CVC response (Figure 2B; Table 4).

The 39°C plateau phase of the local heating response produced both main effects of group (P = 0.036) and drug site (P = 0.025) for absolute CVC (Table 3). During this

phase, the BW presented substantially less cutaneous vasodilation relative to the WW, though multiple comparisons testing revealed no intervention drug to have altered absolute CVC relative to the control site. Further, no interaction between group and drug site was noted for the absolute CVC during the 39°C plateau ($P = 0.396$). The main effects of group and drug site ($P = 0.012$ and $P = 0.048$, respectively) were also documented for $\%CVC_{max}$ during the 39°C plateau (Figure 2C; Table 4). As before, the BW had a significantly attenuated vasodilation during the local heating. Multiple comparisons testing revealed that the sites treated with BQ-123 produced greater $\%CVC_{max}$ than the control sites ($P = 0.032$), with no differences at the L-arginine or BQ-788 sites ($P > 0.835$ for both). No group-by-drug site interaction ($P = 0.414$) was noted for the 39°C plateau relative CVC.

Comparable to the 39°C plateau, statistically significant main effects of group ($P = 0.027$) and drug site ($P = 0.039$) were noted for the NO-mediated contribution to absolute ΔCVC (i.e., the difference between the 39°C plateau and the response to L-NAME; Table 3). An interaction between group and drug site was not found ($P = 0.219$). The BW again had a reduced NO-mediated contribution to dilation relative to the WW. Unlike the 39°C plateau, though, multiple comparisons testing confirmed that BQ-123 significantly increased ($P = 0.035$) the absolute NO contribution to dilation relative to the control condition. Equally important are the findings for the $\Delta\%CVC_{max}$ (Figure 2D; Table 4). Indeed, main effects of both group and drug site ($P = 0.010$ and $P = 0.019$, respectively) were noted. In this regard, the BW again presented blunted NO-mediated dilation relative to the WW. Further, BQ-123 increased NO-mediated dilation during local heating relative to the control site ($P = 0.007$), with no effect at the L-arginine or BQ-788 sites ($P > 0.371$ for both). As before, no group-by-drug site interaction was noted for the relative CVC for the NO contribution to dilation ($P = 0.219$).

As the CVC during each phase of local heating is normalized to the site-specific maximum (i.e., CVC during combined SNP and 43°C heating; Table 3), differences in the maximal dilation can complicate interpretations of any submaximal data. In this regard, no differences were noted between group (main effect $P = 0.282$) for maximal dilation during the local heating protocol. Furthermore, no differences were noted across drug sites (main effect $P = 0.626$) or for the group-by-drug site interaction ($P = 0.808$).

Discussion

The present study aimed to better understand the underlying mechanisms of the previously observed (11) reduced vasodilatory responsiveness in young, BW versus WW. The main findings are threefold: 1) parallel to our previous report (11), we demonstrated that young, BW present reduced vasodilation to local heating relative to WW; 2) ET_AR antagonism increased the vasodilatory response to local heating, through a predominantly NO-mediated fashion, in both BW and WW, while ET_BR antagonism did not produce any changes to cutaneous hyperemia; and 3) supplemental L-arginine did not modify cutaneous vasodilation to local heating in either group. Collectively, these findings indicate that reduced vasodilatory responsiveness remains present in young, BW and that activation of ET_AR at least partially contributes to this response.

The increased risk for the development of CVD, hypertension, and other related chronic health disorders remains a prevalent issue within the BL population (1). Importantly, BL individuals also develop prehypertension and hypertension earlier than other racial/ethnic groups (36), which may contribute to the greater hypertension-related morbidity and mortality observed in this group (37). While the genesis of CVD and hypertension remains a multifactorial process, differences in vascular function may contribute to this important biological process. Indeed, this may be of particular importance in the BL population, as reduced vascular function has been observed in the macro- (38, 39) and microvasculature (9-12, 40) of BL individuals. While blunted vascular function often manifests during advancing age or with the development of overt disease (41-43), many of these studies included young, otherwise healthy BL individuals (9-11, 39), highlighting that vascular changes can occur before the typical onset of CVD (e.g., midlife) and in the absence of other traditional risk factors.

Previous reports from our group have aimed to illuminate the contributors to blunted microvascular function in the BL population. In a study from Hurr *et al.* (9), the observed reductions in cutaneous microvascular function during a standard local heating protocol were attributed to augmented oxidative stress. In this regard, the local administration of tempol, a superoxide dismutase mimetic, via intradermal microdialysis attenuated the blunted vasodilation in the BL participants, without altering the response in the WH participants. Interestingly, the local infusion of ascorbic acid did not modify the vasodilatory response to local heating, suggesting that additional factors may control the localization and production of this oxidative stress. While women were included in these data, direct sex comparisons were not made. A subsequent study by Patik *et al*. (11) aimed to further delineate the sources of the oxidative stress-related reductions in vascular function in the BL population. Using apocynin, a NOX antagonist, and allopurinol, an XO antagonist, they demonstrated that NOX- and XO-derived superoxide contributes to the reduced cutaneous thermal hyperemia in young, BL individuals. Remarkably, inhibition of these sources of superoxide almost completely ameliorated the difference in vasodilation between the BL and WH participants. Accounting for the lack of sex differences in the study by Hurr *et al.* (9), Patik *et al*. (11) made direct sex comparisons to better understand differences in microvascular function in young, BW and BM. Indeed, while the BW and BM in the study expressed similarly blunted cutaneous vasodilation to local heating, the BW did not respond to NOX and XO antagonism. Accordingly, these data would suggest that superoxide-derived oxidative stress, at least from NOX and XO, does not contribute to blunted cutaneous vasodilation in young, BL women.

The present study aimed to address these sex differences by elucidating additional candidate mechanisms that may contribute to the reductions in cutaneous

vasodilation. In parallel with previous studies from our group (9-11), we observed a race-specific reduction in vasodilation to local heating (e.g., 39°C), such that the BW presented with lower absolute and relative CVC compared to WH women. One potential factor that may improve vasodilation in these BL women is L-arginine supplementation. While intracellular L-arginine may be sufficient to drive the production of NO, L-arginine supplementation can still augment this enzymatic process (44), potentially overcoming any intracellular limitations to vasodilation. Indeed, a previous report assessing coronary endothelial function in cardiac catheterization patients noted that intracoronary L-arginine infusion augmented the coronary blood flow response to acetylcholine in BL, but not WH, participants (27). Further, intradermal infusions of L-arginine have been demonstrated to partially restore CVC during 42°C local heating in young, BL versus WH participants (10). In these previous studies women were included, however, sex differences were not directly elucidated. Accordingly, the present study aimed to better understand the role of L-arginine in BL and WH women. Despite the previously discussed findings (10, 27) and the efficacy of L-arginine in other at-risk populations (7, 26, 28), we did not find that intradermal L-arginine improved vasodilation, particularly in the BW.

The reasons for the lack of improvements in vasodilatory responsiveness with L-arginine are unclear. One of the key competitors to endothelial NOS for L-arginine is the enzyme arginase (44). In this regard, the inhibition of arginase improves vasodilator function in populations at-risk for CVD or with overt disease (28, 45-47), though these results are equivocal (10, 43). In a previous study by Kim *et al.* (10), arginase inhibition did not modify CVC in the BL participants, though sex differences in arginase activity in the BL population remain unknown. Accordingly, while arginase may reduce the physiological pool of L-arginine, further data are needed to understand how arginase may

play a role in disparate vascular function in BW. Beyond arginase, increased asymmetric dimethylarginine (ADMA) and creatine kinase activity may also alter L-arginine kinetics and vasodilation. ADMA, which competes with L-arginine for binding sites on NOS, is upregulated in BM and correlates with impaired vasodilation (48), though the impact in BW is unknown. Creatine kinase, on the other hand, may uncouple eNOS by reducing L-arginine concentrations in favor of creatine synthesis (49). Accordingly, the reduced NO production may reduce vasodilator function concomitantly with a rise in creatine and enhanced vascular contractility (49). The exact role of creatine kinase in reduced vascular function in young, BW, however, remains ambiguous.

As a vasoconstrictive peptide, ET-1 may impair vasodilation through either an increase in ET_AR/ET_BR mediated vasoconstriction, a reduction in ET_BR mediated vasodilation, or a combination of both. In this regard, hypertensive BL individuals present greater circulating ET-1 (19, 20, 50) and alterations in receptor expression, with apparent shifts that seem to favor vasoconstrictive phenotypes (20, 51). These differences extend to younger, BL individuals, as Treiber *et al.* (22) demonstrated greater circulating ET-1 in BL versus WH adolescents during mental stress, though this phenomenon was driven predominantly by male adolescents. Considering these findings, sex differences may be readily apparent in the vascular interactions with ET-1 in the BL population. Indeed, while BL men may have greater circulating ET-1, alterations in the ET-1 receptor interaction may be of greater consequence in BL women. Work by du Plooy *et al.* (23) noted that relations between ET-1, blood pressure, and arterial stiffness only existed in BL women, which may be secondary to augmentations in inflammation. As this study utilized a population from South Africa, however, direct interpretations of these data for the U.S. BL

population warrant caution given potential differences in the social determinants of health in these groups.

In the present study, we observed that ET_AR antagonism with BQ-123 increased cutaneous vasodilation. The significant effect of BQ-123 for 39°C $\%CVC_{max}$ demonstrates that ET-1 may restrain cutaneous vasodilation through an ET_AR-related pathway. More interestingly, though, are the findings that ET_AR antagonism increased the NO contribution to dilation. Upon blockade of ET_AR, ET_BR serve as the remaining binding site for ET-1, which may act in a vasoconstrictive manner if located on the vascular smooth muscle or in a vasodilator fashion through NO if located on the endothelium (52-54). Indeed, given the increase in NO-mediated dilation during ET_AR antagonism, it would appear that endothelial ET_BR activation was upregulated, subsequently augmenting vasodilation. Alternatively, ET_AR mediated vasoconstriction may substantially attenuate NO-mediated dilation in these young, BW and WW, and thereby augment dilation upon its removal.

Though the BW and WW in the present study responded similarly to ET_AR antagonism, the change in the 39°C plateau and NO contribution from the control site may help further illuminate these findings. In particular, the difference in $\%CVC_{max}$ from the control site to the BQ-123 site during the 39°C plateau was 9.1% in the BW and 9.5% in the WW. The difference in NO contribution from the control site to the BQ-123 site, on the other hand, was 13.2% in the BW and 6.5% in the WW, suggesting that other underlying mechanisms may modulate the response to ET_AR activation and inhibition differently between populations. These changes may be at the level of altered contractility through intracellular calcium kinetics and myosin light chain activation, or through altered NOS activation by ET_BR receptors in the presence and absence of ET_AR activity (55). Although the BW and WW did not respond differently to the BQ-123 treatment, the

findings are no less significant, as these findings open additional avenues of inquiry to better understand differences in vascular function in BW.

Contrary to the ET_AR antagonism, blockade of ET_BR in the current study did not produce any differences in the vasodilatory response to local heating. Previous studies have demonstrated disparate responses to ET_BR antagonism in other groups of women at risk for CVD (29, 30, 56), such that ET_BR antagonism has a favorable outcome in these populations. Further, a shift in ET_BR localization from the endothelium to the vascular smooth muscle has been noted in BW relative to WW (51). Accordingly, we expected improved vasodilation following ET_BR blockade in the BW. Our findings, however, did not reflect a change in the vascular responses to local heating during BQ-788 infusion, suggesting that ET_BR control of vascular tone does not contribute substantially during this experimental paradigm in these young, BW and WW.

During ET_BR antagonism, more ET-1 should be available to bind with ET_AR as ET_BR typically serve as physiological "sinks" for ET-1 clearance (55). Under circumstances in which ET_BR elicit greater vasodilation than vasoconstriction, we may also expect additional vasoconstriction during ET_BR antagonism following subsequent ET_AR activation. This interaction did not seem to be the case, however, as a reduction in %CVC-$_{max}$ was not observed in either the BW or WW during combined local heating and ET_BR antagonism. Accordingly, these findings may suggest that ET_BR binding may be minimal under these conditions and thus any displaced ET-1 was not of meaningful enough quantity to augment ET_AR-mediated constriction. Moreover, the additive dilator effect of ET_BR may only be realized in situations whereby ET_BR is the sole ET-1 receptor available. Alternatively, these data may suggest that the shift in ET_BR localization (51), and thus vascular effects, may be unique to older, BL individuals with overt CVD or CVD risk

factors. In this case, the combined findings from the present study may suggest that ET-1 receptor density and localization changes across the lifespan in BW, bearing their own consequences.

While the current study further elucidates the mechanisms of disparate cutaneous vascular function in young, BW, a few experimental considerations are worth discussing. First, while women were the primary focus of this study, the participants were only tested during the low-hormone phase of the menstrual cycle or during withdrawal bleeding in an effort to control for the effects of sex hormones. As sex hormones can strongly influence endothelial dependent vascular function (57, 58), our results may have differed had these women been studied in the mid-luteal phase, for instance. Indeed, research suggests that the menstrual cycle modifies ET-1 receptor responsiveness in young women (59), which may lead to more pronounced improvements in vascular function with the administration of our interventional drugs. Second, we did not include a combined BQ-123+BQ-788 infusion site. This decision was a byproduct of technical considerations limiting the current study to four infusion sites. Accordingly, future research should utilize a combined blockade site to better understand the contributions of ET_AR and ET_BR to vascular function during local heating. Third, this study used the cutaneous microcirculation rather than a different vascular bed. While the cutaneous microvasculature has been proposed as a model of generalized microvascular function (60), the findings in the present study may differ in other vascular beds (e.g., skeletal muscle, coronary). Fourth, we did not perform direct measures of ET-1 receptor density or intracellular L-arginine bioavailability. As these measures may not be homogenous throughout the systemic vasculature, understanding differences in these measures may provide additional insight into the efficacy of some treatment sites, while drawing conclusions to other vascular beds. Last, we did not

investigate the possible interplay between the mechanistic results and possible contributions of the various social determinants of health (e.g., socioeconomic status, perceived discrimination). As chronic elevations in social stressors can lead to detrimental changes in different biological mechanisms that may influence vascular function (61, 62), better understanding of how these social factors influence microvascular function is warranted. This may be of further importance when assessing sex differences, as BM and BW seem to experience social stressors differently (63). In this regard, understanding the connection between the social determinants of health and the physiological mechanisms may further explain our past (9, 11) and present findings. Accordingly, additional research investigating disparate microvascular function in BL individuals should integrate measures of the social determinants of health with the mechanistic investigations.

In conclusion, we present findings that further establish blunted cutaneous microvascular function in young, BW versus young, WW. Additionally, these data indicate that ET_AR activity restrains submaximal vasodilator capacity during local heating, though neither ET_BR activity nor reduced L-arginine bioavailability seem to contribute to these reductions. Accordingly, these data help our understanding of the mechanisms of reduced vascular function that remain unique in BW, though further research is needed to fully elucidate the remaining mechanistic contributors and social determinants to these functional disparities.

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Tables and Figures

Table 4.1: Participant Characteristics. Blood biochemistry was taken from serum samples. AU: Arbitrary Units; BMI: Body Mass Index; TC: Total Cholesterol; HDL: High-Density Lipoprotein Cholesterol; LDL: Low-Density Lipoprotein Cholesterol; VLDL: Very Low-Density Lipoprotein Cholesterol; (*): $P < 0.05$; (§): $P < 0.01$

	White Women	*Black Women*	*P-Value*
	$n = 9$	$n = 9$	
Age (y)	22 ± 2	20 ± 2	0.125
Height (cm)	166.5 ± 3.5	162.3 ± 4.6	0.046
Mass (kg)	61.2 ± 7.9	58.1 ± 7.6	0.402
BMI ($kg \cdot m^{-2}$)	22.0 ± 2.2	22.0 ± 2.7	0.999
Waist Circ. (cm)	74.2 ± 5.3	72.4 ± 7.4	0.563
Hip Circ. (cm)	96.6 ± 5.2	95.6 ± 6.2	0.692
Waist-Hip Ratio (AU)	0.77 ± 0.04	0.76 ± 0.05	0.608
Baseline SBP (mmHg)	114 ± 6	112 ± 7	0.573
Baseline DBP (mmHg)	69 ± 4	69 ± 4	0.910
Glucose ($mg \cdot dL^{-1}$)	83 ± 8	87 ± 5	0.283
Sodium (mM)	141 ± 2	141 ± 2	0.721
Potassium (mM)	5.0 ± 0.5	4.8 ± 0.4	0.302
TC ($mg \cdot dL^{-1}$)	169 ± 31	150 ± 20	0.147
Triglycerides ($mg \cdot dL^{-1}$)	64 ± 15	42 ± 10	0.003
HDL ($mg \cdot dL^{-1}$)	64 ± 15	60 ± 17	0.539
VLDL ($mg \cdot dL^{-1}$)	13 ± 3	9 ± 2	0.003
LDL ($mg \cdot dL^{-1}$)	92 ± 19	81 ± 15	0.237

Table 4.2: Mean arterial pressure (MAP; mmHg) collected during each phase of the microdialysis protocol. L-NAME: N^{ω}-nitro-L-arginine methyl ester. White Women: $n = 9$ for all except initial dilation where $n = 8$; Black Women: $n = 9$ for all. Main effect of race: $P = 0.630$; main effect of phase: $P = 0.115$; race-by-phase interaction: $P = 0.925$

	White Women	*Black Women*
Baseline	82 ± 6	82 ± 5
Initial Dilation	81 ± 5	83 ± 5
39°C Plateau	81 ± 5	82 ± 5
L-NAME	80 ± 6	82 ± 5
Maximal Dilation	80 ± 6	82 ± 5

Table 4.3: Absolute cutaneous vascular conductance (CVC) at each microdialysis site. White Women: $n = 9$ for all except initial dilation where $n = 8$; Black Women: $n = 9$ for all. PU: Perfusion Units; NO: Nitric Oxide. (*): $P < 0.05$ for Control vs. BQ-123 multiple comparison.

Phase	Drug Site				P-Value		
	Control	L-arginine	BQ-123	BQ-788	Group	Drug Site	Interaction
Baseline CVC (PU ・mmHg^{-1})							
White Women	0.34 ± 0.10	0.38 ± 0.25	0.39 ± 0.11	0.34 ± 0.16	0.137	0.498	0.879
Black Women	0.28 ± 0.13	0.33 ± 0.21	0.29 ± 0.21	0.23 ± 0.11			
Initial Dilation CVC (PU ・mmHg^{-1})							
White Women	1.79 ± 0.42	1.54 ± 0.79	1.94 ± 0.61	2.00 ± 0.65	0.843	0.453	0.355
Black Women	1.85 ± 0.71	1.75 ± 0.19	1.87 ± 0.37	1.64 ± 0.71			
39°C Plateau CVC (PU ・mmHg^{-1})							
White Women	1.60 ± 0.59	1.76 ± 0.88	1.98 ± 0.90	1.80 ± 0.60	0.036	0.025	0.396
Black Women	1.16 ± 0.45	1.22 ± 0.47	1.44 ± 0.43	0.98 ± 0.42			
NO Contribution CVC (ΔPU ・mmHg^{-1})							
White Women	1.22 ± 0.47	1.28 ± 0.71	1.48 ± 0.84*	1.31 ± 0.45	0.027	0.039	0.577
Black Women	0.72 ± 0.28	0.91 ± 0.39	1.06 ± 0.35*	0.64 ± 0.35			
Maximal Dilation CVC (PU ・mmHg^{-1})							
White Women	3.10 ± 0.95	3.08 ± 1.10	3.06 ± 0.62	2.96 ± 0.71	0.282	0.626	0.808
Black Women	3.70 ± 1.12	3.36 ± 0.43	3.24 ± 0.83	3.34 ± 1.11			

Table 4.4: Relative cutaneous vascular conductance (%CVC_{max}) at each microdialysis site. White Women: $n = 9$ for all except initial dilation where $n = 8$; Black Women: $n = 9$ for all. PU: Perfusion Units; NO: Nitric Oxide. (*): $P < 0.05$ for Control vs. BQ-123 multiple comparison; (§): $P < 0.01$ for Control vs. BQ-123 multiple comparison.

Phase	Drug Site				P-Value		
	Control	L-arginine	BQ-123	BQ-788	Group	Drug Site	Interaction
Baseline CVC (%CVC_{max})							
White Women	11.6 ± 4.2	12.5 ± 6.5	12.6 ± 1.9	11.9 ± 5.2	0.034	0.758	0.866
Black Women	8.9 ± 7.7	10.2 ± 6.7	8.6 ± 4.4	7.2 ± 3.5			
Initial Dilation CVC (%CVC_{max})							
White Women	59.9 ± 11.1	52.6 ± 21.6	63.1 ± 12.4	66.9 ± 9.2	0.087	0.223	0.223
Black Women	50.9 ± 13.4	53.1 ± 10.3	60.7 ± 17.5	49.8 ± 14.2			
39°C Plateau CVC (%CVC_{max})							
White Women	54.4 ± 20.6	56.5 ± 16.3	63.9 ± 20.0*	61.4 ± 13.7	0.012	0.048	0.414
Black Women	36.5 ± 24.2	37.2 ± 16.6	45.6 ± 12.4*	33.3 ± 18.8			
NO Contribution CVC (Δ%CVC_{max})							
White Women	39.2 ± 16.6	39.3 ± 13.6	45.7 ± 19.0§	43.6 ± 12.5	0.010	0.019	0.219
Black Women	20.3 ± 8.9	28.1 ± 14.2	33.5 ± 10.5§	21.1 ± 13.2			

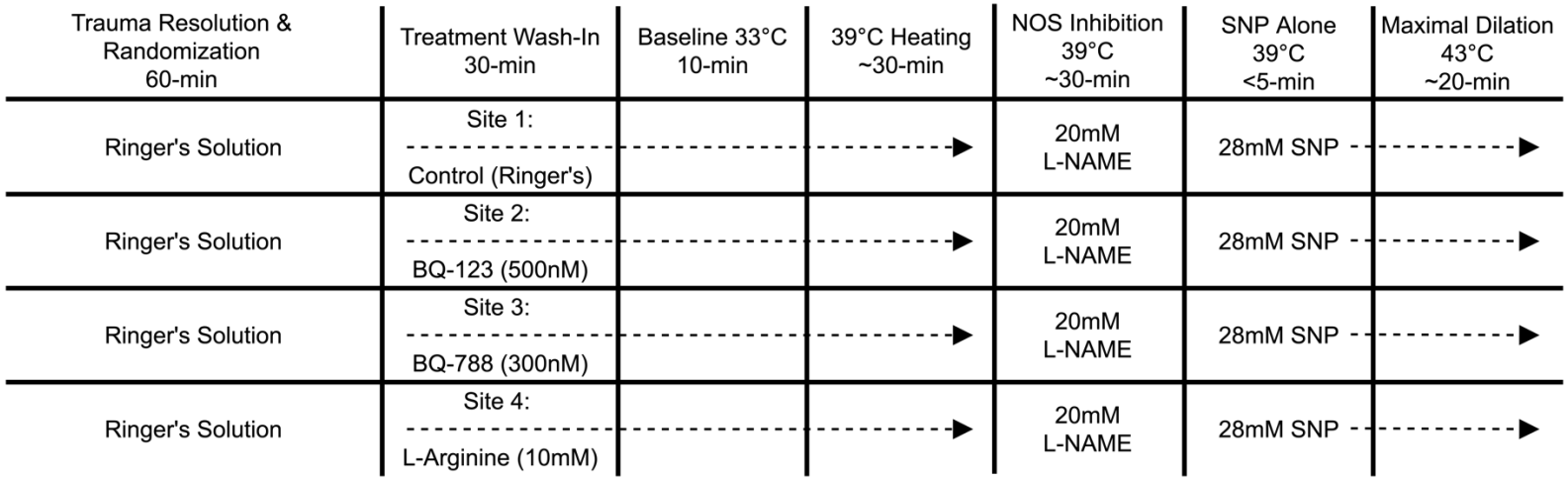

Figure 4.1: Protocol schematic outlining the timeframe and infusion scheme for each microdialysis phase. Dashed arrows indicate that the noted solution was infused during that timeframe. NOS: nitric oxide synthase; L-NAME: N^{ω}-nitro-L-arginine methyl ester; SNP: sodium nitroprusside dihydrate

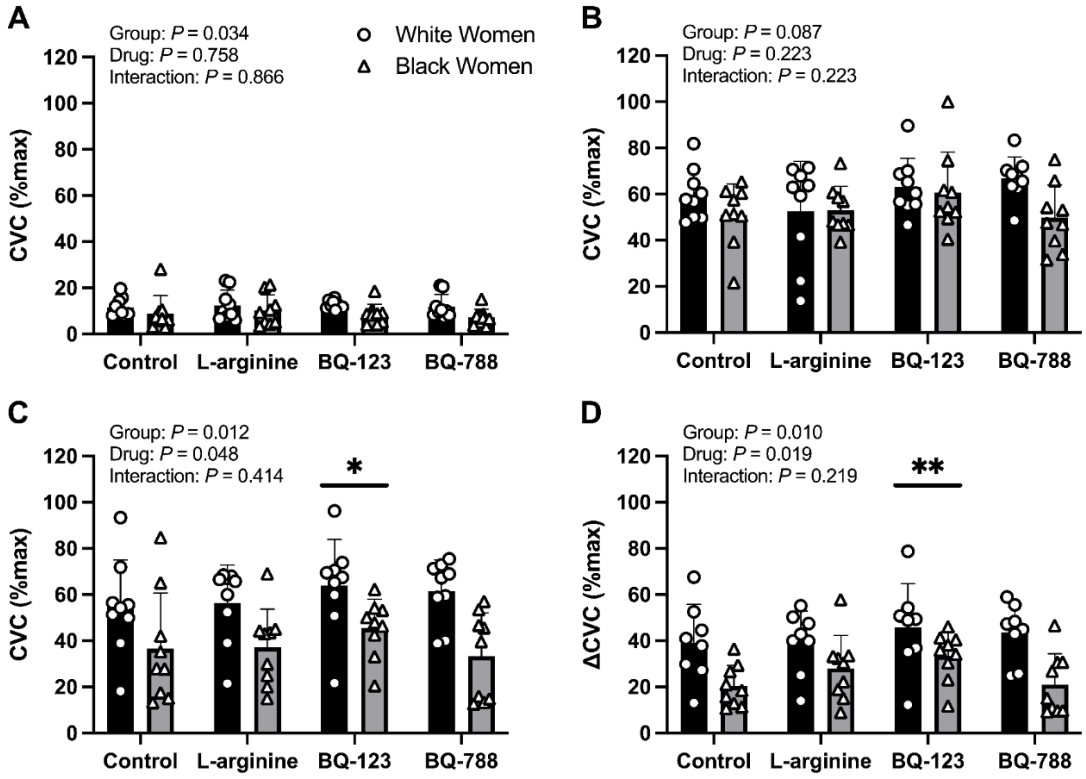

Figure 4.2: Group-by-drug site comparisons for black women (BW) and white women (WW). (*A*): baseline cutaneous vascular conductance (CVC; expressed as a %max); (*B*): initial dilation following the onset of 39°C local heating; (*C*): plateau in CVC following ~30-40 minutes of 39°C local heating; (*D*): nitric oxide (NO) contribution to heating (*): $P < 0.05$ between BQ-123 and Control sites; WW: $n = 9$ for all except initial dilation where $n = 8$; BW: $n = 9$ for all. (**): $P < 0.01$ between BQ-123 and Control sites.

CHAPTER 5: BLUNTED HYPEREMIC RESPONSE TO MENTAL STRESS IN YOUNG, NON-HISPANIC BLACK MEN IS NOT IMPACTED BY ACUTE DIETARY NITRATE SUPPLEMENTATION

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Abstract

Non-Hispanic black individuals suffer from an elevated prevalence of hypertension and cardiovascular disease (CVD) relative to other populations. This elevated disease risk is, in large part, related to impaired vascular function, secondary to reduced nitric oxide (NO) bioavailability. Emerging evidence suggests that dietary nitrate supplementation improves several cardiovascular parameters, including vascular function, in part by increased NO bioavailability. However, whether these findings extend to a population of black individuals is unknown. This study tested the hypothesis that forearm blood flow responses in young, non-Hispanic, black (BL) men during a mental stress challenge would be blunted relative to young, non-Hispanic, white (WH) men. We further hypothesized that acute dietary nitrate supplementation would improve this response in BL men. This study was comprised of two parts (Phase 1 and Phase 2). Phase 1 investigated the difference in blood flow responses between young, BL and WH men. In contrast, Phase 2 investigated the effect of acute nitrate supplementation on the responses in a subset of the BL men from Phase 1. Eleven (9 for Phase 2) BL and 8 WH men (23 ± 3 vs. 24 ± 4 y, respectively) participated in this double-blind, placebo-controlled, randomized, crossover study. During each visit, hemodynamic responses during 3 min of mental stress were assessed in the brachial artery using duplex Doppler ultrasound. Phase 1 was completed in one visit, while Phase 2 was completed over two visits separated by ~1-wk. During Phase 2, data were collected before and 2-h post-consumption of a beverage either high in nitrate content or nitrate depleted. In Phase 1, peak forearm blood flow (FBF; $P < 0.001$), total FBF ($P < 0.01$), and forearm vascular conductance (FVC; $P < 0.001$) were blunted in the BL. During Phase 2, pre-beverage responses were similar to Phase 1 and were unaffected following beverage consumption ($P > 0.05$ vs. pre-beverage for all variables). These data indicate

that young, BL men have blunted microvascular vasodilatory responses to acute mental stress, which may not be altered following acute nitrate supplementation.

Introduction

In the United States, the non-Hispanic black (BL) population experiences ~30% greater mortality from cardiovascular disease (CVD) and ~40% higher total CVD prevalence relative to other populations, including the non-Hispanic white (WH) population (2). This racial disparity is also present in the primary risk factors for CVD, as ~45% of BL adults have hypertension, which accounts for ~20% of deaths in this population and twice as many as in the WH population (2). These differences manifest earlier than typically expected with greater observed blood pressure in BL vs. WH adolescents (2, 3), and the associated detrimental side-effects of hypertension, including end-organ damage, are greater in the BL population as well (4, 5).

While the reasons for this elevated CVD risk in BL are multifactorial, impaired vascular function is a primary contributor. This impairment is evidenced by blunted vasodilatory responsiveness in the macro- and microcirculations to a variety of stimuli, including administration of endothelial-dependent and independent vasodilators (6-10), flow-mediated dilation (10, 11), carbon dioxide rebreathing (12), and during a standard local heating protocol (13, 14). Interestingly, most of these studies were conducted in young (< 30 y) subjects across numerous techniques, again highlighting racial differences in vascular function at a relatively early age prior to the onset of overt CVD. Mechanistically, these blunted vasodilatory responses are, in part, related to reduced nitric oxide (NO) bioavailability and/or responsiveness to NO (15). While many of the noted stimuli above require specialty equipment or invasive techniques, mental stress (e.g., Stroop color-word task, mental arithmetic) is an easily administered perturbation that elicits reproducible hemodynamic responses (16-18) and correlates well with responses to real-life acute stressors (19). Mental stress elicits a transient increase in forearm blood flow (FBF) that is predominantly mediated via NO (16, 17) and β-adrenergic mediated

mechanisms (18, 20, 21). Further, evidence demonstrates blunted hemodynamic responses to mental stress in middle-aged, BL men compared to middle-aged, WH men, which is attributable to reduced NO-mediated vasodilation (22). Whether this relation holds in young, BL men, a population with reduced vascular function (13, 14, 23), is currently unknown.

Understanding the relation between hemodynamics and mental stress is essential in BL individuals as this population experiences a higher prevalence of stress and racial discrimination (24), which may be contributing to the observed vascular dysfunction and elevated CVD risk. Indeed, both acute (e.g., mental arithmetic, anger stimulation) and chronic (e.g., socioeconomic status, occupational) stressors can invoke pro-oxidant (25, 26) and pro-inflammatory responses (27-29). These responses ultimately contribute to reduced vascular function through reductions in NO bioavailability (13, 30, 31). Therefore, a combination of acute and chronic stressors may be contributing to the observed increase in inflammation and oxidative stress (15, 32, 33) and subsequent reductions in NO bioavailability in BL individuals (13-15).

With the vascular dysfunction previously observed, including from our laboratory, it is plausible that therapeutic strategies aimed to improve NO bioavailability in BL may improve the vasodilatory responsiveness to mental stress. Accordingly, one such strategy is increased dietary nitrate (NO_3^-) consumption. Acute and chronic NO_3^- consumption is beneficial for numerous physiological outcomes, including endothelial function (34-37), vascular stiffness (34, 35, 37), and blood pressure (34, 38, 39). This improvement in vascular function is due, in large part, to the increase in circulating NO_3^-, nitrite (NO_2^-) and, subsequently, NO bioavailability (40, 41). The use of dietary NO_3^- presents a different approach to ameliorating vascular dysfunction compared to previously successful interventions by our group (13, 14, 42, 43). While these previous studies found positive

results, most were minimally invasive. Dietary NO_3^-, however, serves as an easily administered lifestyle intervention with demonstrated physiological benefit.

Based on these previous findings, we tested the hypotheses that 1) the forearm hyperemic response to mental stress would be blunted in young, BL men compared to young, WH men and 2) that acute dietary NO_3^- supplementation would ameliorate these blunted hyperemic responses to mental stress in the BL men.

Methods

ETHICAL APPROVAL

All procedures for this study were approved by the Institutional Review Board at the University of Texas at Arlington (UTA IRB #2017-0856). Participants were given a verbal description of all procedures, purposes, and risks involved before providing their informed, written consent. The study conformed to the standards set by the Declaration of Helsinki (apart from registration in a database).

PHASE 1

Participant Characteristics

8 young, WH, and 11 young, BL men participated in Phase 1 (Table 1). To determine eligibility, participants self-reported their racial identity and that of their parents. Individuals of mixed-race were excluded from the overall study to prevent a mixed racial background interfering with the interpretation of the results. Additional exclusion criteria included smokers and competitive athletes. All participants were normotensive during screening and free of overt cardiovascular, metabolic, and neurological disease and not currently taking vitamin/mineral-based supplements or medications. Participants were asked to fast overnight and abstain from caffeine, alcohol, and strenuous exercise for 24 h before each experimental visit. Upon arrival to the laboratory, participants assumed a supine position on the patient bed for 30 min to allow for equilibration, which was followed by baseline measures of hemodynamic variables and assessment of peripheral vascular function. All data were collected in a temperature-controlled laboratory (~23°C and 40% relative humidity).

Instrumentation

Each participant had their height and weight measured using a digital scale and stadiometer (Seca 769, Seca North America, Chino, CA). Waist and hip circumferences were assessed with a flexible tape measure at the level of the umbilicus and the largest circumference around the glutes, respectively. While lying supine, which was maintained in each period of data collection, each participant was instrumented for the continuous measurement of heart rate, via electrocardiography (CardioCard, Nasiff Associates, Central Square, NY), and intermittent blood pressure, via electrosphygmomanometry (Tango+, SunTech, Raleigh, NC).

Experimental Protocol

All measurements of brachial artery blood flow (i.e., FBF) were conducted using high-resolution, duplex Doppler ultrasound (GE Logiq P5, Milwaukee, WI) using recommendations previously described (44). Briefly, an adjustable (7-12 MHz) linear array transducer was selected for optimal B-mode signals of the brachial artery and held in a stereotactic clamp 5-10 cm proximal to the antecubital space. Once an image was obtained and optimized for a clear delineation between the lumen and vessel walls, duplex mode (at a pulsed frequency of 5 MHz) was utilized for the simultaneous measurement of brachial artery diameter and blood velocity. The sample volume was set to encompass the entirety of the lumen, without extending into vascular tissue, at an insonation angle of 60°. All images were recorded using a commercially available screen capture software (Elgato Video Capture, Elgato, San Francisco, CA). Each image was analyzed using continuous edge-detection software (Cardiovascular Suite, Quipu, Italy) along sections of the artery

with clearly defined vessel walls while second-by-second blood velocity was taken as the full velocity envelope.

Peripheral vascular function was assessed as the hyperemic response in the brachial artery during a mental stress challenge. Mental stress was induced through the use of rapid mental arithmetic. Participants were asked to quickly subtract a number (e.g., 7 or 13) from a three-digit number. To ensure adequate stress, participants were asked to perform the task at a rate of sixty subtractions per minute, as set by a metronome, while being repeatedly asked to perform the task faster. In the case of incorrect answers, participants were immediately informed they would need to repeat the calculation. Each mental stress test included a 2-min baseline, 3-min arithmetic portion, and 2-min recovery during which FBF data, as described above, were continuously recorded. A similar, albeit longer (5-min), protocol to elicit mental stress has been previously used and adequately demonstrated increases in sympathetic nerve activity, blood pressure, and heart rate, particularly within 2-min after the onset of the stressor (45). Accordingly, as steady-state hemodynamic responses were observed by 2-min, we elected to use a shorter, 3-min protocol which also elicits elevations in FBF (16, 22). During the mental stress test, finger photoplethysmography was utilized to assess beat-to-beat mean arterial blood pressure (MAP; Finometer Pro, Finapres Medical Systems, Netherlands). Following mental stress, each subject reported their perceived stress using a five point Likert scale (e.g., 0 being not stressed, 4 being the most stressed) (46).

PHASE 2

Based on the findings from Phase 1, this portion was designed to determine if the blunted vasodilatory responsiveness in the BL men could be restored following acute nitrate supplementation.

Participant Characteristics

Data were collected from 9 young, BL men (all of which also participated in Phase 1; 2 of the original 11 participants elected to not continue with Phase 2; Table 1). All eligibility criteria and visit conditions were similar to those previously described. Additionally, participants were asked to avoid antibacterial mouthwash for 72 h as the effects of the mouthwash markedly reduce the increase in plasma NO_2^- following dietary NO_3^- ingestion (47).

Instrumentation

Phase 2 was a double-blind, placebo-controlled, randomized, crossover study. Each visit was separate by at least 1 wk. Instrumentation for hemodynamic and peripheral vascular function data was similar to that previously described.

Nitrate/Nitrite Analysis

On each day, ~10 mL of blood was collected immediately before pre-beverage data collection (e.g., baseline) and 2 h post beverage consumption (see below for more detail).

Blood was collected into lithium heparin vacuum tubes via venipuncture and used for subsequent analysis of plasma NO_3^- and NO_2^- . Following collection, the blood samples were immediately centrifuged at 3,000g and 4°C for 5 min. The plasma was then aliquoted into microcentrifuge tubes before being flash-frozen in liquid nitrogen and stored at -80°C for future analysis. Plasma NO_3^- and NO_2^- were determined via ozone phase chemiluminescence, as previously described (48). Briefly, a reducing reagent (I_3^- for NO_2^- and vanadium(III) for NO_3^-) is applied to the samples within a purge vessel to release gaseous NO. Helium gas is then passed through the solution to force the NO out of solution whereby the NO is carried into an analyzer. The NO then rapidly reacts with ozone (O_3) to yield NO_2^* in an excited state. Upon returning to its ground state, the excited electron emits a photon, which is detected as chemiluminescence, whereby the magnitude of chemiluminescence detected above a standard curve represents the concentration of NO_3^- or NO_2^- in the sample.

Experimental Protocol

All pre-beverage data collection was identical to that described above for Phase 1. Upon completion of these baseline measures, participants were provided with 140 mL of either a NO_3^- -rich beverage (BR; Beet-It-Sport: ~12.8 mmol NO_3^- ; James White Drinks Ltd., Suffolk, UK) or a NO_3^- -depleted placebo (PL; ~0.0055 mmol NO_3^- ; James White Drinks Ltd., Suffolk, UK) that was taste-matched and indistinguishable. Participants were asked to consume the beverage as quickly as possible. Following ingestion, participants quietly rested for 2 h and then completed the same testing as pre-supplementation. This time course was selected as peak NO_2^- concentrations, and thus capacity to produce NO, occur as early as 2 h post NO_3^- consumption (49). While evidence suggests that plasma NO_2^-

concentrations can peak later (~3 h post NO_3^- consumption) (49, 50), starting instrumentation and data collection at 2 h post-consumption allowed us to capture our primary outcome variables in a window that encompassed high plasma NO_2^- concentrations.

DATA ANALYSIS

Brachial blood pressure was measured at rest and before each mental stress test on the arm contralateral to the Doppler ultrasound. Measurements were taken in triplicate and used to calculate MAP (⅓ systolic blood pressure [SBP] + ⅔ diastolic blood pressure [DBP]). Using brachial artery diameter (D) and mean blood velocity (V_{mean}; mean of anterograde and retrograde velocity) from the Doppler ultrasound, FBF was calculated as: $\pi \cdot (D \cdot 0.5)^2 \cdot V_{mean} \cdot 60$. Forearm vascular conductance (FVC) was calculated as $FBF \cdot MAP^{-1}$. Peak FBF was taken as the absolute greatest 1 s average FBF during mental stress. FBF area-under-the-curve (AUC) was taken as $\Sigma FBF \cdot 60^{-1}$ for the 3 min of mental stress.

STATISTICAL ANALYSIS

Hemodynamic responses to mental stress during Phase 1 were analyzed via unpaired samples t-tests between WH and BL. Hemodynamic responses to mental stress during Phase 2 were analyzed via two-way, repeated-measures ANOVA with the factors of treatment (BR and PL) and time (pre- and post-supplementation). In the case of significant interactions, post-hoc Holm-Sidak corrections were performed. Post-hoc effect sizes and power calculations were performed for Phase 1 and Phase 2 outcomes. All data were analyzed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA) and SPSS 24 (IBM Corp., Armonk, NY) and presented as mean ± SD. The level of statistical significance was set *a priori* at $\alpha = 0.05$.

Results

PHASE 1

The participants in this portion of the protocol were well matched for age, height, weight, body mass index, and baseline blood pressure (Table 1). Outcome variables for Phase 1 are presented in Table 2. Before mental stress, baseline FBF was similar between groups (Figure 2A; $P = 0.56$). However, during mental stress, peak FBF (Figure 2B; $P < 0.001$), and total FBF (Figure 2C; $P < 0.01$) were attenuated in the BL individuals. Despite a reduced pressor response from baseline in BL (Figure 2E; $P < 0.01$), peak FVC (Figure 2D; $P < 0.001$) remained blunted in the BL individuals. No difference was noted in the perceived stress between BL and WH ($P = 0.37$; Table 2).

PHASE 2

Testing in Phase 2 included 9 BL men who also participated in Phase 1 (Table 1). Due to technical difficulties, blood was not obtained from 2 participants. Therefore, the presented NO_3^- and NO_2^- data is from 7 participants. Plasma NO_3^- was unchanged from pre- to post-treatment in the PL condition (Pre: 61.90 ± 35.36 vs Post: 52.17 ± 31.88 μM; $P = 0.99$; Figure 3A), but increased following NO_3^- supplementation in the BR condition (Pre: 45.04 ± 19.13 vs Post: 661.01 ± 192.47 μM; $P < 0.001$; Figure 3A). Additionally, baseline NO_3^- was not different between BR and PL ($P = 0.17$) and the post-supplementation NO_3^- was significantly greater in BR than PL ($P < 0.001$). Plasma NO_2^- was unaffected by the treatment in the PL condition (Pre: 48.8 ± 21.2 vs Post: 35.4 ± 10.9 nM; $P = 0.98$; Figure 3B) but was augmented in the BR condition (Pre: 51.9 ± 23.0 vs Post: 304.1 ± 174.4 nM; $P < 0.01$; Figure 3B). Furthermore, plasma NO_2^- was not different between BR and PL at baseline ($P = 0.83$), but significantly elevated at the post-treatment time-point in BR versus

PL ($P < 0.01$). No effect of the NO_3^- supplementation was observed on resting systolic or diastolic blood pressure ($P > 0.09$ for both).

During mental stress (Table 3), no interactions were observed for the baseline FBF responses between BR and PL groups from pre- to post-treatment (Figure 4A; $P = 0.37$). Similar to baseline FBF, no interactions were noted for peak FBF (Figure 4B; $P = 0.24$), peak blood velocity (Figure 4C; $P = 0.08$), or total blood flow (Figure 4D; $P = 0.10$) during mental stress pre- to post-treatment in BR or PL. Further, no differences were found between pre- to post-treatment in either BR or PL for peak FVC (Figure 4E; $P = 0.15$) and the maximum blood pressure change from baseline (Figure 4F; $P = 0.69$). Further, no interactions were found for the perceived stress response to mental stress (Table 3; $P = 0.76$)

Discussion

This study investigated whether disparities exist in the hemodynamic responses to mental stress between young, WH and BL men and whether acute NO_3^- supplementation could improve these responses in the BL men. The two primary findings are: 1) young, BL men had a blunted brachial blood flow and vascular conductance response to acute mental stress compared to young, WH men, and 2) acute supplementation with dietary NO_3^- did not change this response to mental stress. This first observation corroborates previous findings in both young (51) and middle-aged (22) BL and WH men, but using high-resolution, duplex Doppler ultrasound, rather than venous occlusion plethysmography. Accordingly, the present data, in tandem with previous observations (13, 14, 23), suggest that, at a minimum, differences in function occur across multiple vascular beds, and may contribute to the elevated rates of prehypertension and hypertension in young, BL populations (2). However, contrary to our hypothesis, acute NO_3^- supplementation did not ameliorate the blunted mental stress response, suggesting that this acute intervention may not be sufficient to modify blood flow in young, BL men during this perturbation.

The BL population is at an elevated risk for CVD, hypertension, several other chronic health disorders (2). While the progression of CVD and hypertension is multifactorial, vascular dysfunction is a contributing factor (11, 23, 52). In particular, BL, relative to WH, exhibit impaired function in the cerebral vasculature (12), conduit arteries (9-11, 22), and microcirculation (9, 13, 14, 23, 43). The present data add to these previous observations by demonstrating a difference in vascular control between BL and WH men, such that BL men exhibit attenuated hemodynamic and pressor responses during similar degrees of mental stress. The observed relation between ethnicity/race and the mental stress response is of particular importance given the increased prevalence of stress and discrimination in the BL population (24). While the mental stress in the present study may

not be fully representative of such stressors related to finances or race-relations, for instance, evidence suggests that laboratory stressors can accurately reflect real-life stress (19). Additionally, the immediate and cumulative effects of stress can lead to increased CVD risk (53) following inappropriate or exaggerated allostatic responses (54). Accordingly, understanding the mechanisms behind the observed differences in vascular function in BL individuals and subsequent approaches to modify these responses are critical.

Interestingly, as the mental stress task provides a stimulus that does not require active involvement of the arm, the rise in FBF during the stressor is paradoxical. However, mental stress elicits a highly reproducible increase in heart rate, cardiac output, and blood pressure (16, 18, 20-22, 55), which ultimately increases blood flow to the periphery. This increase in blood flow elicits an increase in vascular shear stress in the arm and forearm and thus augments NO-mediated vasodilation (56). Indeed, research indicates that mental stress-induced vasodilation is NO-mediated (16, 17), which may be of particular importance during sustained increases in blood flow during mental stress. Interestingly, Cardillo, *et al.* (22) noted a hyperemic response during mental stress in middle-aged BL relative to WH men that was blunted similarly to the present study. When NO production was inhibited via infusion of L-NMMA, the hyperemic response was only reduced in the WH men without any effect in the BL men such that the observed differences between the BL and WH men during the control condition were abolished (22). These data suggest that blunted NO-mediated dilation in BL men contributes to the divergent FBF responses to mental stress. Similar observations have been made in hypertensive patients (55), suggesting that blunted NO-mediated vasodilation to mental stress exists in clinical populations and may contribute to enhanced vascular injury. In this regard, previous studies from our group and others have indicated blunted NO bioavailability in BL (7, 9, 10, 13-

15), which contributes to vascular dysfunction and may ultimately be causing the largely attenuated increase in FBF in BL individuals in the present investigation.

Beyond NO-mediated vasodilation, β-adrenergic cardiovascular adjustments have also been implicated in the hemodynamic responses to mental stress. The typical β_1-adrenoreceptor mediated increase in cardiac output during mental stress (18) is depressed in BL (57), which may derive from blunted β_1-adrenoreceptor sensitivity compared to WH (57, 58). Additionally, during mental stress, combined inhibition of β_1- and β_2-adrenoreceptors elicits a more pronounced increase and maintenance of systemic vascular resistance than β_1-adrenoreceptor antagonism alone (18, 21), implicating a direct role of β_2-adrenoreceptor mediated dilation. These data are corroborated by Halliwill *et al.* (59) who demonstrate that the drop in vascular resistance and rise in vascular conductance during mental stress are attenuated following propranolol infusion. Research also demonstrates blunted increases in FBF to intra-arterial isoproterenol (6, 8) and substantially smaller increases in vascular resistance to intravenous propranolol during mental stress (57) indicating reduced β_2-adrenoreceptor mediated dilation in BL versus WH participants. This reduced β_2-adrenoreceptor sensitivity may partially explain the reduced hyperemic responses to mental stress in BL.

The reduced pressor response in the BL men in the present study is in agreement with previous studies using mental stress (45, 51). Interestingly, the interaction of depressed β-adrenergic changes in cardiac output and vascular tone during mental stress may be an influencing factor in the reduced pressor response in the BL men. Traditionally, acute mental stress (e.g., mental arithmetic) induces a rise in blood pressure secondary to a rise in cardiac output, with little to no change in peripheral resistance (60-62). However, during a series of psychological stressors, WH exhibit a greater reliance on cardiac output with substantial peripheral dilation in the maintenance of blood pressure, while increases

in cardiac output and decreases in peripheral resistance are each attenuated in BL (57, 63). Following the infusion of propranolol, the differences between races disappear (57), suggesting that racial differences in the pressor responses to mental stress may be β-adrenergic mediated.

As the BL men in this study exhibited blunted cardiovascular reactivity (e.g., blood flow and pressure) to mental stress, these responses may, at first, seem cardioprotective. Typically, exaggerated cardiovascular reactivity to a stressor is often indicative of unfavorable cardiovascular health outcomes (64-68). However, many of these studies used stressors that may favor heavily vascular responses, particularly in BL (57). A few studies that did use mental stress either did not include BL participants (65) or used a mental stressor other than mental arithmetic (68) which may produce a different physiological response than mental arithmetic. As mental stress typically increases blood pressure through a myocardial (e.g., β-adrenergic) rather than a vascular (e.g., α-adrenergic) mechanism acutely (60), the expression of a predominantly vascular response to mental stress in BL men (57, 63) may confer an increased risk of cardiovascular disease. Indeed, the shift from predominantly myocardial to predominantly vascular mechanisms for blood pressure regulation is indicated in disease states (69) and may also be secondary to endothelial dysfunction (70). Blunted cardiovascular reactivity may also be predicated in the development of chronic disease (64, 71), suggesting that the reduced hemodynamic responses to mental stress in the BL men in this study may have long term implications. Alternatively, the differences in vascular function between the WH and BL men in the present study may not be pathophysiological in nature and may simply represent differences in physiological responses to an external stressor between populations. However, it is important to note that previous studies (9, 22, 57) have demonstrated similar

findings during mental stress in BL vs. WH men, underscoring the presence of racial differences in vascular function during this perturbation.

As mentioned, while several possible factors contribute to differences in vascular function, reductions in NO bioavailability in BL individuals contribute to these differences and have been observed across a variety of vascular beds (11, 13, 14, 22, 43). Further, various interventional strategies aimed at improving NO bioavailability have yielded favorable results in improving vascular function in this population, although these strategies are less accessible and more invasive (13, 14, 43). In the scope of the present investigation, finding methods to easily improve NO bioavailability are critical as the response to mental stress is predominantly NO-dependent (16) with racial differences also reported (22). Therefore, improving NO bioavailability presents a target to favorably change the blood flow responses to mental stress.

In the present study, acute dietary NO_3^- supplementation was used to augment circulating NO_3^- /NO_2^- and thereby the physiological pools of NO (40, 41). Upon ingestion, NO_3^- is absorbed into the bloodstream from the intestine and eventually transported back to the oral cavity where it is concentrated in the saliva (41). Commensal facultative anaerobic bacteria then reduce NO_3^- to NO_2^-, which is subsequently brought back to the gut. A portion of this NO_2^- is further reduced to NO, via gastric acid, while the remainder absorbs into the bloodstream (41). Bloodborne NO_2^- subsequently reduces to NO via interactions with deoxyhemoglobin, myoglobin, and xanthine oxidoreductase, among other pathways (41, 72). In this regard, both acute and chronic increases in dietary NO_3^- (e.g., leafy greens and beetroot) demonstrate positive impacts on cardiovascular health, including decreased blood pressure, decreased vascular stiffness, and augmented endothelial function (34-39, 73, 74). Further, improvements in blood pressure in young, BL women following acute dietary NO_3^- supplementation suggests treatment viability in

a young, BL population (74). However, despite the relatively quick onset of plasma NO_3^- / NO_2^- following treatment (73) and demonstrated efficacy in young, BL women (74), our present data do not suggest that acute NO_3^- supplementation improves hemodynamic responses in young, BL men in response to mental stress. This may be due to the lack of hypoxia, which is a predominant mechanism by which plasma NO_2^- is reduced to NO (41, 72).

Many of the pathways that reduce NO_2^- to NO require the presence of low oxygen tension (41). Perhaps the most crucial regulators for NO_2^- reduction are deoxyhemoglobin and the NO_2^- reductase activity within the cell (41, 75, 76). Following deoxygenation, NO_2^- reductases in hemoglobin facilitate the reaction of NO_2^- with ferrous deoxyhemoglobin ($HbFe^{2+}$) and a proton (H^+) to form NO and methemoglobin ($HbFe^{3+}$) (41). The NO bound within the red blood cells is, therefore, more stable and bioavailable at the level of the hypoxic tissue (76). The degree of hypoxia also determines the rate at which this reaction occurs as the rate of NO_2^- reduction by deoxyhemoglobin increases as the fractional saturation of hemoglobin decreases to 40-60% of its maximum (75). This would suggest that at high oxygen tensions, the conversion of NO_2^- to NO would be minimal. Accordingly, in the case of mental stress, it is unlikely that enough of a hypoxic stimulus exists to drive the conversion of plasma NO_2^- to NO. Therefore, in the present conditions, it is unlikely that the augmented concentrations of NO_3^- /NO_2^- were utilized for acute vascular responses during mental stress. That being said, research demonstrates that acute NO_3^- supplementation improves other hemodynamic responses (39, 73, 74), endothelial function (77), and autonomic nervous system function (78) that presumably occur independently of hypoxia. Accordingly, the hemodynamic responses to mental stress may occur through different mechanisms than other noted physiological measures that improve following acute NO_3^- supplementation.

Though this study adds to previous observations and demonstrates that differences in vascular function in response to mental stress exist in young, BL men, there are limitations. First, as NO_2^- reduction can require hypoxic conditions, the lack of measurement of hemoglobin and deoxyhemoglobin concentrations or tissue oxygen saturation in this study prevents conclusions on whether the appropriate environment existed for the NO_2^- reduction. Second, as the acute NO_3^- supplementation was only tested in BL men, whether acute NO_3^- supplementation would elicit improvements in the hemodynamic response to mental stress in WH men is unknown, which may provide additional context to the responses in the BL men. Had the WH men responded to treatment, consistent with responses in previous literature, this may suggest that the NO_3^- dose used was not adequate for the BL men. Accordingly, a larger dose or longer dosing schedule (i.e., days rather than hours) of NO_3^- would be more appropriate. However, in the scope of this study, the purpose was to augment the hyperemic response in the BL men using NO_3^- supplementation. Determining whether WH men respond positively to this treatment would not have changed the null responses to NO_3^- supplementation in the BL men. Additionally, the scope of this study did not include a sex differences aim. As previous literature using NO_3^- supplementation suggests efficacy in young, BL women (74), the exclusion of women from this study limits the ability to conclude if NO_3^- supplementation is generally ineffective in young BL or if this is only in young, BL men. Therefore, further studies investigating the effects of mental stress and NO_3^- supplementation in young, BL women are warranted.

Although a period of 72 h before each experimental visit without antibacterial mouthwash was included, it is possible that previous antibacterial mouthwash may have maintained a reduced bacterial presence in the oral cavity. While limited data exist to demonstrate the recolonization of oral bacteria, a previous study reported that, in a

crossover design separated by 24 h, there were no differences in the salivary nitrite production between a group that received the control first and a group that received the antibacterial mouthwash first (79). Additionally, we observed global increases in plasma NO_2^-, suggesting that this was not an issue. Last, previous literature demonstrates that brief (80) and prolonged (81) periods of sitting cause reductions in both macro- and microvascular function. Accordingly, as several hemodynamic variables during the intervention expressed main effects of time, any benefit from the NO_3^- supplementation may be masked by sedentary behavior. However, each participant stood and moved following pre-supplementation measurements and again before post-supplementation measurements, which should restore shear stress and conduit artery endothelial function (80). Additionally, literature suggests that prolonged sitting influences brachial artery function to a lesser degree than popliteal artery function (82), further minimizing the impact that prolonged sedentary behavior may have on the present results.

In conclusion, the present study highlights that young, BL men exhibit blunted hemodynamic and pressor responses to mental stress compared to young, WH men. Further, these observed differences in the responses to mental stress may not change following acute NO_3^- supplementation aimed at augmenting NO bioavailability. Although BL men exhibit an elevated prevalence of CVD and hypertension, possibly mediated by vascular dysfunction, these data suggest that acute treatment with dietary NO_3^- may not substantially alter vascular hemodynamics in response to mental stress. Combined with previous studies, these data reinforce that, at a minimum, young, BL men possess differences in vascular function that may contribute to the development of CVD. Additionally, while previous treatments have been successful in augmenting vascular function in this population, further consideration on dietary NO_3^- is necessary before definitive conclusions are made on its use as a therapeutic. In particular, understanding the

efficacy of dietary NO_3^- in other populations, such as WH men, is needed to conclude if BL men are unique in their nonresponse to the present intervention. Further studies should also be conducted to determine if an optimal intervention length or dietary NO_3^- dose exists to elicit hemodynamic changes. Accordingly, young, BL men exhibit different hemodynamic and pressor responses during mental stress compared to WH men and additional research is required to better understand the physiological relevance of these differences and potentially efficacious treatments in this population.

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Tables and Figures

Table 5.1: Participant Characteristics. BMI: Body Mass Index, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure

	White (n = 8)	Black	
		Phase I (n = 11)	Phase II (n = 9)
Age (y)	24 ± 4	23 ± 3	23 ± 3
Height (cm)	178.3 ± 7.3	178.1 ± 8.0	175.8 ± 6.1
Weight (kg)	76.1 ± 8.7	84.5 ± 12.6	82.7 ± 13.3
BMI ($kg \cdot m^{-2}$)	23.9 ± 1.6	26.7 ± 3.8	26.8 ± 4.2
Waist Circ. (cm)	80.8 ± 5.2	86.1 ± 11.6	86.6 ± 12.7
Hip Circ. (cm)	101.9 ± 6.0	105.3 ± 6.4	104.6 ± 6.8
SBP (mmHg)	117 ± 9	117 ± 10	118 ± 11
DBP (mmHg)	67 ± 4	68 ± 6	68 ± 6

Table 5.2: Responses to mental stress between white and black subjects during Phase 1 of testing. FBF: Forearm Blood Flow, FVC: Forearm Vascular Conductance, MAP: Mean Arterial Blood Pressure, AU: Arbitrary Units

	Group		P-Value	Effect Size (*d*)	Observed Power
	White	Black			
Baseline FBF ($mL \cdot min^{-1}$)	89 ± 38	76 ± 56	0.56	0.283	0.090
Peak FBF ($mL \cdot min^{-1}$)	333 ± 111	153 ± 58	**<0.001**	2.034	0.985
Total Blood Flow (mL)	490 ± 113	309 ± 119	**<0.01**	1.566	0.888
Peak FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	3.76 ± 1.13	1.79 ± 0.6	**<0.001**	2.178	0.993
ΔMAP (mmHg)	22 ± 4	16 ± 4	**<0.01**	1.461	0.842
Perceived Stress (AU)	2.4 ± 0.8	2.8 ± 1.2	0.37	0.439	0.230

Table 5.3: Responses to mental stress during Phase 2 of testing. SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, FBF: Forearm Blood Flow, FVC: Forearm Vascular Conductance, MAP: Mean Arterial Blood Pressure, AU: Arbitrary Units

	Placebo		Beetroot		*P*-Values			Effect Size	Observed
	Pre	Post	Pre	Post	Treatment	Time	Interaction	(*f*)	Power
Baseline SBP (mmHg)	116 ± 10	114 ± 11	115 ± 12	116 ± 15	0.977	0.689	0.099	0.659	0.376
Baseline DBP (mmHg)	67 ± 5	66 ± 6	66 ± 8	69 ± 6	0.800	0.497	0.126	0.604	0.325
Baseline FBF ($mL \cdot min^{-1}$)	93 ± 58	46 ± 31	125 ± 136	49 ± 25	0.353	0.081	0.332	0.366	0.150
Peak FBF ($mL \cdot min^{-1}$)	215 ± 125	135 ± 46	175 ± 106	145 ± 70	0.212	**0.046**	0.239	0.449	0.202
Peak Velocity ($cm \cdot sec^{-1}$)	31.9 ± 15.9	20.1 ± 8.2	23.2 ± 9.0	21.2 ± 8.5	0.080	**0.042**	0.075	0.723	0.436
3-Min Flow AUC (mL)	344 ± 148	199 ± 61	305 ± 112	229 ± 89	0.870	**0.023**	0.100	0.656	0.373
Peak FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	2.62 ± 1.38	1.48 ± 0.48	2.11 ± 1.22	1.63 ± 0.73	0.256	**0.025**	0.151	0.240	0.289
ΔMAP (mmHg)	18 ± 8	16 ± 9	20 ± 9	17 ± 10	0.477	0.062	0.691	0.146	0.065
Perceived Stress (AU)	2.7 ± 1.1	2.3 ± 1.0	2.4 ± 1.2	2.0 ± 1.1	0.120	0.134	0.760	0.110	0.059

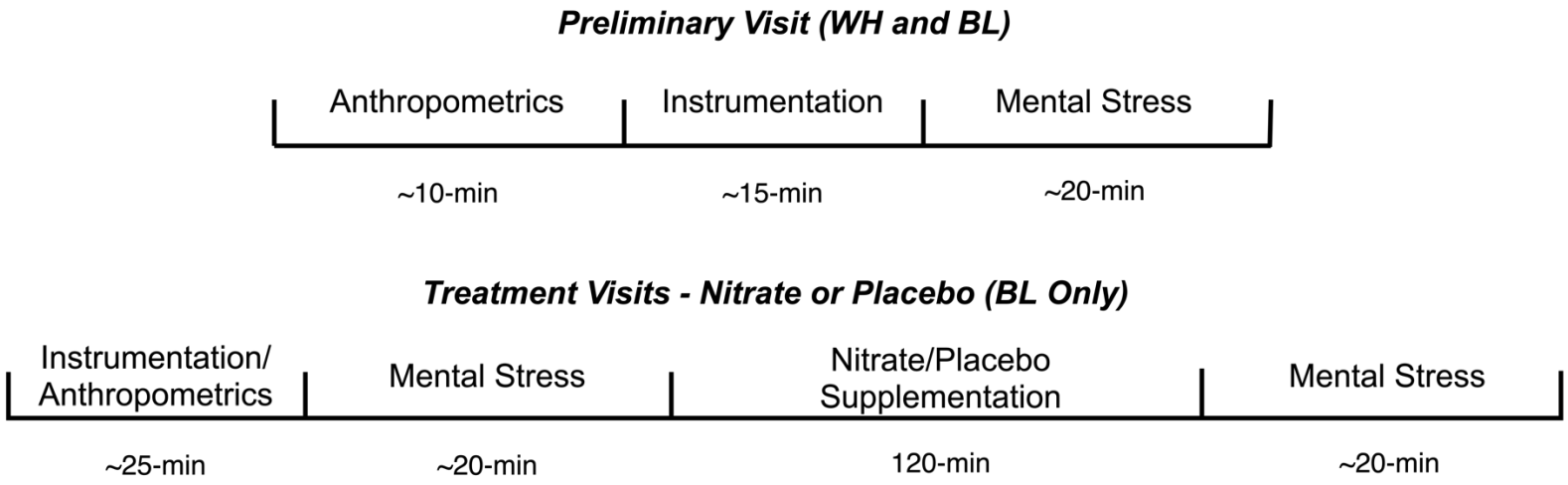

Figure 5.1: Schematic diagram of both the preliminary testing and treatment visits. Preliminary testing was conducted in WH and BL individuals to determine if hemodynamic responses differed between these groups. Treatment visits were conducted in BL only to determine if the blunted hemodynamic responses would be improved by acute nitrate supplementation. Additionally, nitrate and placebo visits were randomized and separated by at least 1 wk.

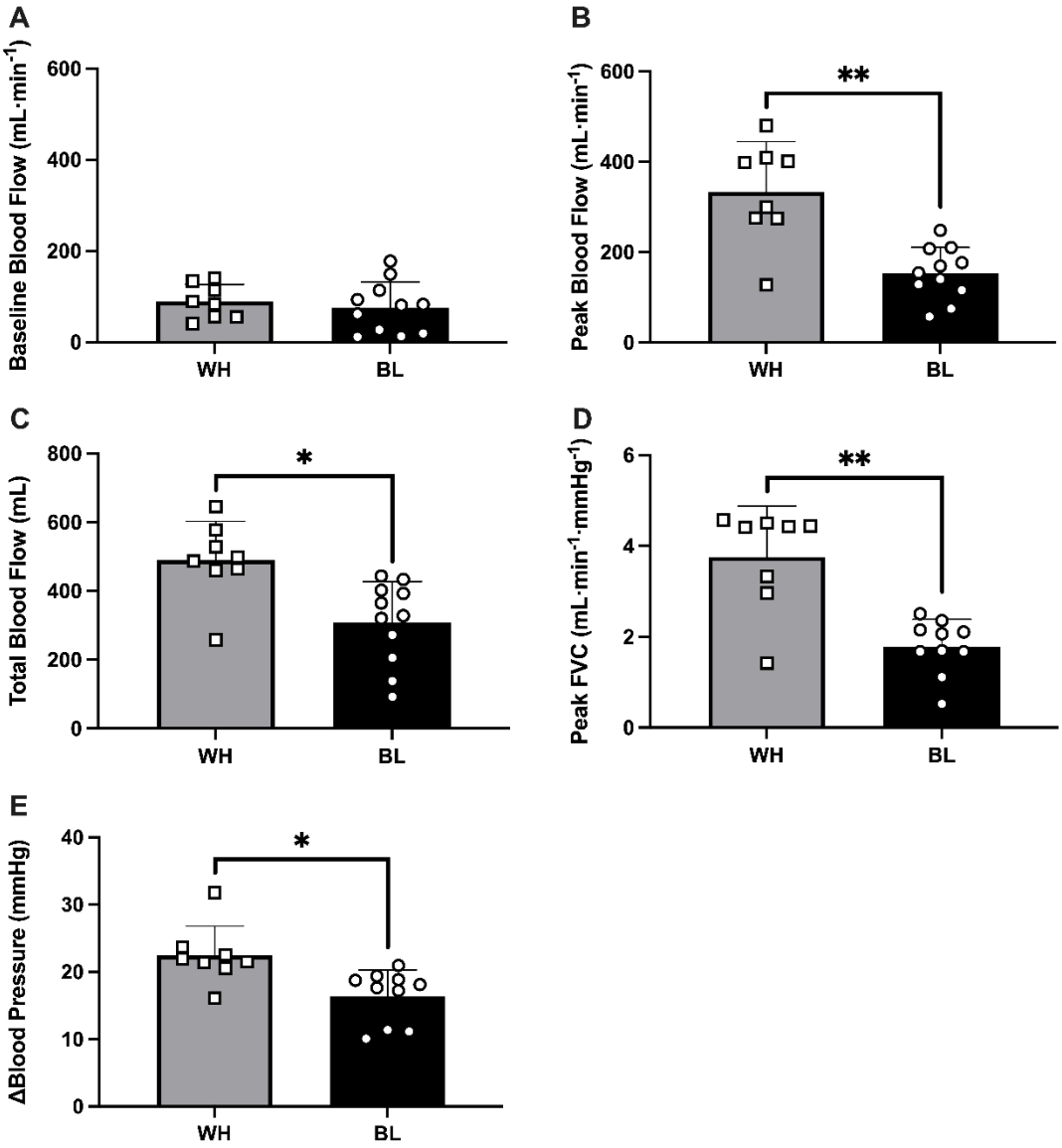

Figure 5.2: Forearm blood flow and vascular conductance responses during mental stress. Baseline blood flow (A), peak blood flow (B), 3-min total blood flow during mental stress (C), peak forearm vascular conductance (FVC; D), and delta mean arterial blood pressure (ΔMAP; E) responses to mental stress in both WH and BL participants. Due to technical issues, peak FVC and ΔMAP were only collected in 10 BL participants. (*): $P < 0.01$; (**): $P < 0.001$.

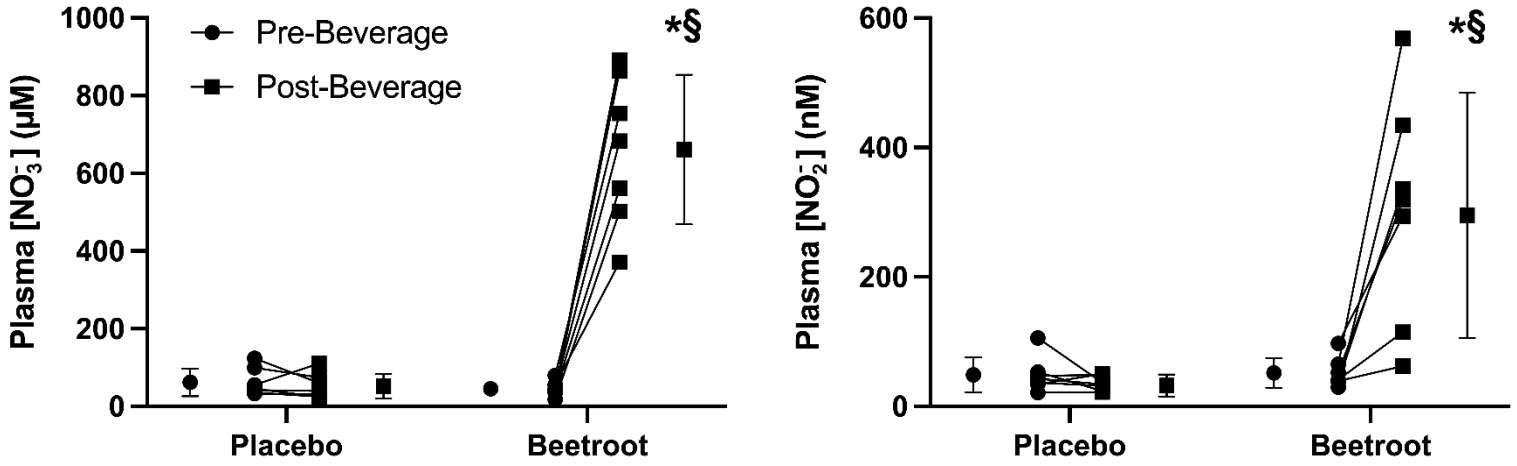

Figure 5.3: Group averages and individual responses for plasma nitrate (NO_3^- ; A) and nitrite (NO_2^- ; B) concentrations pre- and post-treatment for both the PL and BR conditions. (*): $P < 0.01$ for pre- to post-treatment change during BR for NO_3^- and NO_2^- ; (§): $P < 0.01$ for post-treatment comparison between PL and BR for NO_3^- and NO_2^- .

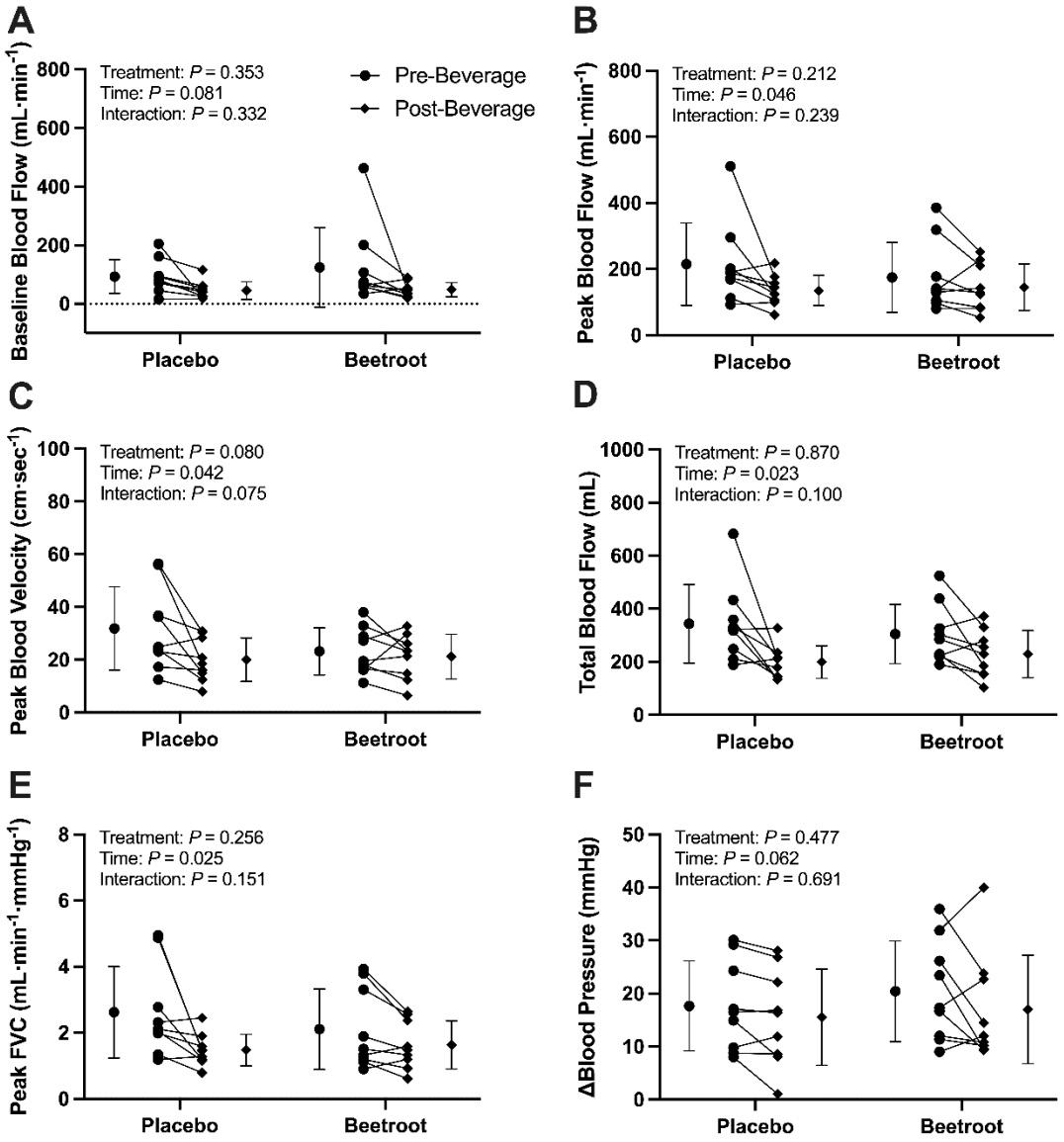

Figure 5.4: Responses to mental stress for baseline blood flow (A), peak blood flow (B), peak blood velocity (C), total blood flow in 3-min (D), peak forearm vascular conductance (FVC; E), and peak change in blood pressure from baseline during mental stress (F) pre- and post-treatment. No statistically significant interactions were observed for any variable; however, a significant main effect of time was noted for peak blood flow, peak blood velocity, total blood flow, and peak FVC.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

Cardiovascular diseases (CVD) remain some of the most burdensome health conditions in the United States non-Hispanic black (BL) population and may only continue to grow worse over the coming years (1). While many factors contribute to the etiology of CVD, blunted vascular function remains a hallmark of CVD genesis (2-6) through direct modulation of vascular tone and induction of vascular remodeling (7). Indeed, preclinical reductions in vascular function can predict the development and onset of conditions such as hypertension (8). Blunted vascular function is often present in the non-Hispanic black population in the macro- and microcirculations in response to a variety of stimuli, including administration of endothelial-dependent and independent vasodilators (9-13), flow-mediated dilation (13, 14), carbon dioxide rebreathing (15), and during a standard local heating protocol (16, 17). Accordingly, understanding the breadth of altered vascular function, its contributors and mechanisms, and potential therapeutic interventions remains of utmost importance to alleviate the burden of CVD in the BL population.

The studies described herein represent a targeted and systematic approach to continue expanding our current scientific understanding. Indeed, for the first time, this work demonstrates the influence of sex on peripheral and cerebral vascular reactivity in young, BL individuals. Further, this work highlights the influence of endothelin-1 (ET-1) receptor type A (ET_AR) on vasodilatory restraint in the cutaneous microcirculation during local heating in BL women and identifies this pathway as a possible interventional target. Last, the described research identifies that acute nitrate supplementation may not adequately augment vascular function in young, BL men, setting up different interventional strategies in future research.

Despite the key findings of this research, several important future directions remain to be studied. In Chapter 3, we observed that BL women present with greater cerebral and

peripheral macrovascular function relative to BL men, yet similar-to-blunted peripheral microvascular function. During this study, we only included measures in the women during the low hormone phase of the menstrual cycle. As the menstrual cycle can strongly influence vascular function (18, 19), these measurements should be repeated at several time points across the cycle to determine the effect of different concentrations of sex hormones on these measures. Indeed, if the BL women did not receive the typical "boost" in vascular reactivity during the mid-luteal phase, that may present an interesting conundrum and may explain the abnormally augmented CVD prevalence in BL women. This study also focused on the use of transcranial Doppler (TCD) ultrasound and did not use other imaging modalities that can directly account for blood flow. Given the inherent limitation of being unable to measure blood flow directly, our measures of TCD may be underreporting the actual differences in vascular function between the BL men and women. Accordingly, future studies should employ traditional Doppler ultrasound and measure changes in blood flow through the internal carotid and vertebral arteries.

Regarding imaging modalities, consideration of tissue composition and volume should be made. Indeed, given the inherent size differences between the BL men and women in this study, some, if not all, of the difference in post-occlusive reactive hyperemia may be explained by lean tissue volume and desaturation rates. Accordingly, lean forearm volume and tissue oxygenation measures should be made to control for the stimulus driving reactive hyperemia. Last, this study focused primarily on physiological observations, without much consideration of other factors. Indeed, the social determinants of health (e.g., socioeconomic status, perceived discrimination) may significantly influence the vascular function in these groups. In this regard, chronic elevations in stress may contribute to augmentations in oxidative stress and inflammation (20, 21), which are key contributors to NO-related reductions in vascular function (16, 22) and are highly prevalent in the BL

population (20, 23, 24). Future research thus needs to make appropriate considerations for the social determinants of health and how these factors interact with underlying physiology in CVD development.

Based on previous work from our lab identifying the roles of oxidative stress in impaired microcirculatory function in young, BL men, but not women, Chapter 4 aimed to better characterize additional candidate mechanisms for this reduced function. Indeed, ET_AR seem to restrain vasodilatory function during cutaneous thermal hyperemia, but neither ET_BR nor L-arginine deficiencies seem to contribute to this dysfunction in BL women. As previously mentioned, the menstrual cycle and sex hormones exert strong influences on vascular function (18, 19). In this regard, we only studied women during a low hormone phase, which may cause underreporting of the effects of our interventional drugs. For instance, ET-1 receptor responsiveness changes across the menstrual cycle (25), which may lead to more pronounced augmentations in cutaneous thermal reactivity during ET-1 receptor antagonism in young, BL women. Future research should also investigate the role of dual ET-1 receptor blockade as antagonism of one receptor alone may not fully elucidate the role of this highly potent vasoconstrictor peptide in regulating vascular tone in young, BL women.

Additionally, some of the background literature on these topics focused on the efficacy of L-arginine supplementation (26) or ET-1 peptide/receptor dynamics (27, 28) in patient populations, which may obfuscate our scientific rationale. Accordingly, measures of intracellular L-arginine and ET-1 concentrations/receptor densities and localizations would help clarify the extent to which these mechanisms may contribute to the altered microvascular function we observed in the young, BL women. Finally, we only studied these physiological responses in women, primarily because Patik *et al.* (16) identified that the mechanisms of reduced cutaneous microvascular function in BL individuals remained

mostly unexplained in the BL women. The findings in this previous study combined with the methodological approach in the current study, however, do not diminish the importance of understanding the roles of L-arginine and ET-1 in BL men. To some extent, the former has been answered (29), though the role of ET-1 in blunted cutaneous microvascular function in BL men remains unclear. In this regard, other candidate mechanisms should be targeted. Asymmetric dimethylarginine (ADMA) and creatine kinase both represent attractive targets that may contribute to the observed reductions in vascular function in BL women. Indeed, ADMA competes with L-arginine for binding sites on nitric oxide (NO) synthase (NOS), reducing vascular function, and is upregulated in the BL population (30), though the role of ADMA in BL women remains unknown. Further, creatine kinase may uncouple NOS production of NO by reducing L-arginine bioavailability in favor of creatine synthesis (31), though the effects of this enzyme in BL women are uncertain.

Finally, Chapter 5 aimed to explore possible treatment options to improve the hemodynamic and cardiovascular responses to mental stress in BL men. While these data suggest that BL men present substantially smaller increases in brachial artery blood flow and blood pressure relative to well-matched, non-Hispanic white (WH) men, acute nitrate supplementation, via beetroot juice, was not an adequate stimulus to induce changes in the responses to mental stress in the BL men. These findings open several new lines of inquiry. First, the underlying mechanisms by which BL men produce different hemodynamic responses to mental stress are unknown. While we speculate that NO (32) and β-adrenoreceptor activity (33) may be key contributors to this altered response in the BL men, these mechanisms have not been confirmed in young, BL men to an appropriate extent. Second, as young, BL men produce attenuated rises in blood flow and pressure during mental stress, it remains important to determine if these responses are pathophysiological. Given the greater prevalence of stress and racial discrimination in this

population (34), presenting a detrimental response to an acute stressor may indicate a severely heightened risk for cardiovascular damage in young, BL men. Third while this study measured the response to mental stress in young, BL and WH men, there was no arm investigating sex differences. Given the sex differences in vascular function across different circulations in Chapter 3, understanding the role of mental stress and acute nitrate supplementation in BL women remains an important follow-up question for this line of inquiry. Finally, the relative ineffectiveness of the acute nitrate treatment leads to questions regarding optimal efficacy. In this case, a greater dose (e.g., higher total amount of nitrate) or a longer supplementation window (e.g., weeks or months) may be needed to observe meaningful physiological changes. Alternatively, a supplementation method that may be better at delivering nitrate or nitrite to the vasculature may be warranted.

The studies presented in this dissertation help to further illuminate the breadth of reduced vascular function in the BL population, particularly between BL men and women. Further, these studies elucidate different mechanisms and interventional tactics that may help future scientists and clinicians develop strategies to delay the onset of or reverse the progression of CVD in BL individuals. Collectively, this dissertation provides greater insight and context into blunted vascular function in the BL population, such that future research may continue to reduce the disparate burden of CVD in the BL population.

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APPENDIX: ETHICS DOCUMENTS

October 23, 2017

Dr. Matthew Brothers
Kinesiology
The University of Texas at Arlington
Box 19259

IRB No.: 2016-0847
Title: *The Effect of an Antioxidant Therapy on Vascular Function in Caucasiansand African Americans*

Original Approval Date: September 20, 2016
Modification Approval Date: October 19, 2017
Expiration Date: September 13, 2018

EXPEDITED MODIFICATION APPROVAL

The UT Arlington Institutional Review Board (UTA IRB) Chair (or designee) reviewed and approved the modification(s) to this full board protocol on **October 19, 2017** in accordance withTitle 45 CFR 46. 110(b)(2). Therefore, you are authorized to conduct your research. The modification(s), indicated below, was/were deemed minor and appropriate for expedited review:

- Add procedures for collecting Doppler blood flow measures on the femoral artery ofthe leg in addition to the existing Doppler procedures for the brachial artery of the arm; the Doppler ultrasound device will be placed on the upper thigh and a blood pressure cuff placed just above the knee to collect these additional measurements.
- Revise the Form 1 and Informed Consent Document to reflect the above changes

MODIFICATION TO AN APPROVED PROTOCOL:
Pursuant to Title 45 CFR 46.103(b)(4)(iii), investigators are required to, "promptly report to the IRB ***any*** proposed changes in the research activity, and to ensure that such changes in approved research, during the period for which IRB approval has already been given, are **not initiated without prior IRB review and approval** except when necessary to eliminate apparent immediate hazards to the subject." Modifications include but are not limited to: Changes in protocol personnel, number of approved participants, and/or updates to the protocol procedures or instruments. All proposed changes must be submitted via the electronic submission system prior to implementation. Failure to obtain prior approval for modifications is considered an issueof non-compliance and will be subject to review and deliberation by the IRB

which could result in the suspension/termination of the protocol.

INFORMED CONSENT DOCUMENT:
The IRB approved version of the informed consent document (ICD) must be used when prospectively enrolling volunteer participants into the study. All signed consent forms must besecurely maintained on the UT Arlington campus for the duration of the study plus a minimumof three years after the completion of all study procedures (including data analysis). The complete study record is subject to inspection and/or audit during this time period by entities including but not limited to the UT Arlington IRB, Regulatory Services staff, OHRP, FDA, andby study sponsors (if the study is funded).

ADVERSE EVENTS:
Please be advised that as the principal investigator, you are required to report local adverse(unanticipated) events to The UT Arlington Office of Research Administration; RegulatoryServices within 24 hours of the occurrence or upon acknowledgement of the occurrence.

TRAINING AND CONFLICT OF INTEREST DISCLOSURES:
All investigators and key personnel identified in the protocol must have documented HumanSubjects Protection (HSP) training on file AND must have filed a current Conflict of InterestDisclosure (COI) with The UT Arlington Office of Research Administration; Regulatory Services. HSP completion certificates are valid for 3 years from the completion date.

COLLABORATION:
If applicable, approval by the appropriate authority at a collaborating facility is required prior tosubject enrollment. If the collaborating facility is *engaged in the research*, an OHRP approved Federalwide Assurance (FWA) may be required for the facility (prior to their participation in research-related activities). To determine whether the collaborating facility is engaged in research, go to: http://www.hhs.gov/ohrp/humansubjects/assurance/engage.htm

CONTACT FOR QUESTIONS:
The UT Arlington Office of Research Administration; Regulatory Services appreciates your continuing commitment to the protection of human research subjects. Should you have questionsor require further assistance, please contact Regulatory Services at regulatoryservices@uta.edu or 817-272-3723.

Sincerely,

Digitally signed by Deborah BehanPhD, RN-BC
DN: cn=Deborah Behan PhD, RN-BC,o=UTA, ou=IRB Chair, email=dgreen@uta.edu, c=US
Date: 2017.10.24 17:06:30 -05'00'

Deborah Behan PhD, RN-BC

Deborah Behan, PhD

Associate Clinical Professor, Nursing
UT Arlington IRB Chair

PRINCIPAL INVESTIGATOR / FACULTY ADVISOR
R. Matthew Brothers, Ph.D. Department of
Kinesiology. Email:Matthew.Brothers@uta.edu. Office:
817-272-3288

TITLE OF PROJECT
The Effect of an Antioxidant Therapy on Vascular Function in Caucasians and AfricanAmericans

INTRODUCTION
You have been asked to participate in a research study about the effect of antioxidants on blood flow in your brain, arm, and skin. Your participation is voluntary. Refusal to participate or discontinuing your participation at any time will involve no penalty or loss of benefits to which you are otherwise entitled. Please ask questions if there is anything you do not understand.

You can participate in this study if you are between the ages of 18 -45, are not pregnant, are not a smoker, have no cardiovascular disease (i.e. high blood pressure), metabolic illnesses such as diabetes or metabolic syndrome, or have a condition that affects your blood vessels including Reynaud's disease, cold-induced urticaria, cryoglobulinemia, etc. In addition, subjects currently taking medications known to influence the autonomic nervous system such as, but not limited to, Albuterol, Adderall, Cardura (alpha- adrenergic receptor blocker), Tenormin (beta-adrenergic receptor blocker), Salagen (cholinergic agonist), Inversine (anti-hypertensive) etc. will be excluded. Individuals withfood allergies of any kind will also be excluded from this study. This study will take 2 days to complete with approximately 3 – 10 days in between visits. Testing on each day will last about 4.5 hours.

Your participation is voluntary. If you do not want to participate or you want to stop participating at any time you can. You will not be punished in anyway if this happens. Please ask questions if there is anything you do not understand.

PURPOSE
African Americans have a higher risk for several diseases that involve reduced blood vessel function including high blood pressure and diabetes. Reactive oxygen species are substances that can build up in your body, and can cause a greater chance to have problems with your heart and blood vessels. However, an increase in antioxidants (things that can lower reactive oxygen species) can improve blood flow and blood vessel function. We want to look at the relationship between reduction in reactive oxygenspecies stress using antioxidant therapy and control of blood vessels in the brain, arm,and skin.

DURATION
You will visit the laboratory 2 times. During the first visit we will have you answer some questions about how healthy you are to see if you can be in the study. We will also show you the different things that will be done to you later on this visit and also on the other visit. After we show you the different things we will then do the blood flow measurements before and after taking either a placebo or antioxidant therapy by mouth. The antioxidant supplements will consist of 2 pills of 1 g Vitamin C each (Sundown Naturals, Boca Raton, FL 33487) and 1 pill containing 100 mg Co-enzyme Q10 and 150 IU Vitamin E (Ultra CoQ10, Qunol™, Fairfield, NJ 07004). The placebo will be sugar pills of similar size, color and taste.

Each visit will last about 4.5 hours for a total of about 9 hours between the two visits. These 2 visits can be separated by 3 - 10 days.

NUMBER OF SUBJECTS
The number of anticipated subjects in this research study is 50.

PROCEDURES
Study Day 1 – step-by-step protocol:

1. Arrival at laboratory, health history questionnaire
2. Measures of body height, weight, waist and hip circumference
3. Blood draw from a vein in your non-dominant arm
4. Measures of blood pressure, heart rate, brain blood flow, and arm/leg blood flowduring ~1 hours of resting quietly.
5. Take either the placebo or antioxidant pills
6. Repeat measures of blood pressure, heart rate, brain blood flow, and arm/legblood flow during ~1 hours of resting quietly.
7. Blood draw from a vein in your non-dominant arm
    - ***Total time:*** ~4.5 hour

Study Day 2 – step-by-step protocol:

1. Arrival at laboratory, health history questionnaire
2. Measures of body height, weight, waist and hip circumference
3. Blood draw from a vein in your non-dominant arm
4. Measures of blood pressure, heart rate, brain blood flow, and arm/leg blood flowduring ~1 hours of resting quietly.
5. Take either the placebo or antioxidant pills (the one not taken on Day 1)
6. Repeat measures of blood pressure, heart rate, brain blood flow, and arm/legblood flow during ~1 hours of resting quietly.
7. Blood draw from a vein in your non-dominant arm
    - ***Total time:*** ~4.5 hour

A detailed list of procedures is described below. These will be done on both study

days.If at any time you wish to discuss the information above or any other risks you may experience, you may ask questions now or call the Principal Investigators listed on the frontpage of this form. You do not have to do any of the following procedures if you do not want to. In addition, the research team may decide to not do all of the procedures. If this isthe case we will circle the procedures below that we will perform if you agree.

Cardiac Rhythm / Heart Rate:

- **Description of Procedure:** Sticky patches will be applied to your skin to measurethe heart's electrical signals.
- **Duration of Procedure:** The electrocardiogram will be measured during theentire experiment.

Finger Blood Pressure:

- **Description of Procedure**: In order to continuously measure your blood pressure a small cuff will be placed on one of your fingers. The cuff will lightly squeeze your finger during the experiment. Sometimes the squeezing can make people feel uncomfortable after a while. If this happens with you, we can easily stop the squeezing to give the finger a rest. There is no known risk to this procedure.
- **Duration of Procedure:** This cuff will be on periodically throughout the experiment. The longest duration each time will be about 10 minutes.

Blood pressure (upper arm):

- **Description of Procedure:** Your blood pressure will also be monitored using a cuff placed on your upper arm that is inflated and deflated periodically.
- **Duration of Procedure:** The cuff will be on your upper arm during the entire experiment. We will take blood pressure measurements at different time points during the experiment. Each measurement will last approximately 30 seconds.

Venous blood sample:

- **Description of Procedure:** Blood draw will be done either by a needle stick that is immediately removed or by a thin plastic tube (catheter) in a vein in your arm(a little above your elbow). The amount of blood to be sampled will be 15 ml; about 2 tablespoons. The total blood drawn over all of the 2 visits will not be greater than 100 mls or about 14 tablespoons. Blood will be used to identify potential blood markers that may contribute to elevating blood pressure and decreasing blood flow in obese individuals (i.e. amount of sugar or other substances that

cause your blood vessels to narrow). All blood will be kept in containers in a freezer located in the lab until we do the measurements. We will label the containers in a way that only we will be able to identify them.

- **Duration of Procedure:** We will take up to three blood samples during eachvisit.

Arm and Leg Blood Flow:

- **Description of Procedure:** A picture of a vessel in your arm and/or leg will be taken by an ultrasound camera, similar to standard ultrasound tests done to examine the health of fetuses prior to birth and to the "sonar" used by fishermanto locate fish underwater.
- **Duration of Procedure:** The echocardiogram pictures will be taken approximately 3 different times. Each set of pictures will require approximately 10 minutes to take.

Flow Mediated Dilation / Blood Vessel Responsiveness:

- **Description of Procedure:** A blood pressure cuff will be placed around your forearm and/or thigh. This cuff will be inflated, as is done when your blood pressure is being measured, but instead of deflating the cuff immediately it will remain inflated for 5 minutes.
- **Duration of the Procedure:** This procedure will take approximately 8 minutes.

Cerebral blood flow:

- **Description of Procedure:** A gel covered probe will be placed against the templeof your head. This probe will record blood flow inside your brain, similar to standard ultrasound tests described above.
- **Duration of Procedure:** We will measure your brain blood flow during the entireexperiment.

End-Carbon Dioxide Concentration ($PETCO_2$):

- **Description of Procedure:** Your body is constantly producing carbon dioxide which then leaves the body when you breathe out. This procedure will provide a measurement of how much carbon dioxide is expired each time you breathe out. To do this you will breathe normally into a mouthpiece with a tube into the mouthpiece to measure carbon dioxide. This measurement will be unnoticeable to you during the protocol.
- **Duration of Procedure:** This measurement will only be made during the cerebralvasomotor reactivity procedure (see below).

Respiratory Excursions (pneumobelt):

- **Description of Procedure:** A small belt will be placed around your upperstomach to monitor when you breathe in and out.
- **Duration of Procedure:** This measurement will be made during the duration ofthe study visit.

Cerebral Vasomotor Reactivity:

- **Description of Procedure:** This measurement is to look at how blood flow in your brain changes to changes in carbon dioxide (a gas that your gets rid of when you blow out). After breathing room air for 6 minutes, you will be asked to brieflyincrease your pace of breathing for 30 seconds after which you will breathe into and out of a bag through a mouthpiece. During the time when you are breathing into and out of a bag we will provide extra oxygen to maintain normal oxygen concentrations in the blood. You will breathe faster than normal for 30 seconds and breathe into and out of the bag for no more than 4 minutes. During this time we will be measuring blood pressure, heart rate, brain blood flow, and carbon dioxide (all of which are described above).
- **Duration of the Procedure:** This procedure will be done twice and will take approximately 8 minutes each time.

Central Aortic Blood Pressure and Pulse Wave Velocity Measurement:

- **Description of Procedure:** A blood pressure cuff will be placed on your upper arm and will be connected to a device that measures your central (heart level) and peripheral (arm) blood pressure. In order to measure your pulse wave we will alsoplace a blood pressure cuff on the upper thigh of your left leg. You may feel a small amount of pressure as we feel for your pulse at the top of your leg (femoral)and your neck (carotid) to locate both arteries. After locating each artery we will make measurements between the respective arteries. Finally, we will gently place a small probe on the skin over the artery in your neck while at the same time the blood pressure cuff on your leg will inflate. The carotid and femoral arterywaveforms are detected and used to calculate carotid-femoral pulse wave velocity (a measure of the stiffness of your blood vessels).
- **Duration of the Procedure:** This procedure will be done twice and will take approximately 10 minutes each time.

Cerebral Autoregulation / Bilateral leg cuff inflation/deflation:

- **Description of Procedure:** A large cuff (similar to the blood pressure cuff) will beplaced around the upper part (thigh) of each of your legs. The cuffs will be will beinflated, as is done when your blood pressure is being measured in your arm, but instead of deflating the cuff

immediately it will remain inflated for 5 minutes.

- **Duration of the Procedure:** This procedure will be done twice and will take approximately 8 minutes each time.

Sympathetic Nerve Activity / Microneurography

- **Description of Procedure:** A tiny microelectrode will be placed in a nerve inyour right leg located just below your knee on the outer part of the leg. Alternatively, the median nerve located at the inside of the elbow will be used. First, the course of the nerve will be determined by electrical stimulation of the nerve with a pencil shaped electrode. When the nerve is stimulated, involuntary twitching and/or tingling sensations of the foot or hand will occur. The twitching or tingling will disappear when the stimulation is stopped. Once the nerve is found, two tiny, sterile, microelectrodes will be inserted through the skin. One is areference electrode placed just above the nerve site (2 cm) and the other is the recording electrode. The recording electrode is advanced into the nerve. When thetip of the electrode enters the nerve, you may briefly notice either pressure or tingling sensations. At this point, minor adjustments in the position of the electrode will be made until we begin to record the nerve signals.

- **Duration of the Procedure:** We will measure your brain blood flow during the entire experiment.

Cold Pressor Test

- **Description of Procedure:** You will be asked to place your hand in ice water for 2 minutes. This procedure will be used to cause brief changes in your heart rate and blood pressure.
- **Duration of the Procedure:** You will have your hand in ice water for about 2 minutes.

POSSIBLE RISKS/DISCOMFORTS

The principal investigator and laboratory team are experienced with all procedures outlined in this study in both healthy and diseased populations. Nevertheless, as with all studies involving human subjects, there are risks associated with experimentation that are currently unknown. All possible risks associated with this study are explained below. To reduce these risks, you will only be able to join the study if you are healthy and you will have to complete a health history questionnaire as a precautionary measure to ensure the study poses no additional risks. Furthermore, potential risks will also be minimized by:

(a) using only safe, well-established procedures; (b) constant, personal monitoring of each experimental session by the investigators and staff; and (c) knowledge that you can request to stop at any point during the procedure. In

addition, you should know that the principal investigator may also terminate the testing procedure at any time. All experimental procedures will conform to the procedures for this study as approved by the University of Texas at Arlington Institutional Review Board (IRB). An IRB reviews and approves research for the protection of human subjects.

Blood pressure:

- Other than some potential discomfort associated with cuff inflation there is no riskto this procedure.

Venous blood sample:

- There is a risk of infection, bruising, and occasional light-headedness during the blood draw. These minor risks are minimized by using clean conditions andpeople who are experienced and trained with blood collection.

Cerebral Vasomotor Reactivity:

- You might feel dizzy and/or short of breath. If this occurs we will stop and you will immediately breathe normal room air which will make this feeling stopimmediately. This measurement is often done in many labs and doctor's offices. We have done this measurement in about 300 people over the last 10 years withno problems.

Cerebral Autoregulation - Bilateral leg cuff inflation/deflation

- This is a research technique used to study changes in blood pressure and brain blood flow. There is minimal risk during this procedure. At the onset of the occlusion, you will feel the cuff inflate around their legs. During the occlusion, which can last up to five minutes, you may feel some pain, numbness, and/or tingling in your legs; however, this discomfort will subside within 30 seconds of deflating the cuff. If this procedure is overly uncomfortable, the test will be ended immediately.

Sympathetic Nerve Activity / Microneurography

- The nerve recording procedure occasionally may result in the leg muscles feelingtired. Also, you may have a pins-and-needles feeling or a greater sensitivity to touch in the leg. However, these side effects rarely occur and do not usually last more than a couple of minutes. Please refrain from exercising for 24 hours, and rubbing/massaging the site of the nerve measurement. A member of the research team will contact you 24 hours after you leave the laboratory to make sure there are no affects.

Cold Pressor Test

- You might experience minor discomfort during the procedure however, you willbe allowed to remove your hand from the water if you feel it intolerable.

These vitamins are known to interact with some medications, so you are encouraged tocontact your physician or pharmacist before agreeing to participate

COMPENSATION
You will be compensated for time spent in the laboratory. The rate of compensation will be $50.00 per study visit. If you complete both study day's it will be a total of $100 ($50 for each visit).

It is important that you report any illness or injury to the research team listed in this form immediately. Compensation for an injury resulting from your participation in this research is NOT available from The University of Texas at Arlington. In the event that you suffer a research-related injury, your medical expenses will be your responsibility or that of your third-party payer, although you retain your legal rights during your participation in this research.

The Internal Revenue Service (IRS) considers all payments made to research subjects to be taxable income. Your personal information, including your name, address, and social security number, may be acquired from you and provided to UT Arlington's accounting office for the purpose of payment. If your total payments for the year exceed $600.00, UTArlington will report this information to the IRS as income and you will receive a Form 1099 at the end of the year. If you receive less than $600.00 total for payments in a year, you are personally responsible for reporting the payments to the IRS.

ALTERNATIVE PROCEDURES
There are no alternative procedures offered for this study. However, you can elect not to participate in the study or quit at any time at no consequence.

VOLUNTARY PARTICIPATION
Participation in this research study is voluntary. You have the right to decline participation in any or all study procedures or quit at any time at no consequence. Should you choose not to complete all study procedures, you will still receive compensation for the time spent participating following consent.
During the course of the study, you may decide not to participate in some experimental measurements or procedures and therefore, this portion of the protocol will not be completed. However, all other measurements will be performed. This will not affect the scientific value of your participation as each experimental measurement and procedure provides important and in most cases

independent information. In addition, the principal investigator and research staff may decide to re-enroll you in the study, if you agree, if previous testing was unsuccessful or certain experimental measurements and procedures were not initially performed. The re-enrollment has no additional safety risks other than those already stated.

CONFIDENTIALITY

Every attempt will be made to see that your study results are kept confidential. A copy of this signed consent form and all data collected from this study will be stored in the investigator's lab (electronic data) and office (paper files) for at least three (3) years after the end of this research. The results of this study may be published and/or presented at meetings without naming you as a subject. Additional research studies could come from the information you have provided, but your information will not be linked to you in anyway (i.e., it will be coded in a manner that others cannot identify you). Some of the blood we sample will be labeled with your initials and date of birth and will be sent to a lab outside of UT Arlington for analysis. The remaining blood analysis will be performedin our laboratory at UTA and will be coded in a manner that others cannot identify you. Although your rights and privacy will be maintained, the Secretary of the Department of Health and Human Services, the UTA IRB, and personnel particular to this research have access to the study records for inspectional purposes. Your records will be kept completely confidential according to current legal requirements. They will not be revealed unless required by law, or as noted above. The IRB at UTA has reviewed and approved this study and the information within this consent form. If in the unlikely event it becomes necessary for the IRB to review your research records, the University ofTexas at Arlington will protect the confidentiality of those records to the extent permittedby law.

CONTACT FOR QUESTIONS

Questions about this research study may be directed to Dr. Matt Brothers (817-272-3151, matthew.brothers@uta.edu). If you believe that you had any discomfort or risks as aresult of the study, you should also contact Dr. Matt Brothers.

Any questions you may have about your rights as a research subject or a research-related injury may be directed to the Office of Research Administration; Regulatory Services at 817-272-3723 or regulatoryservices@uta.edu.

As a representative of this study, I have explained the purpose, the procedures, the benefits, and the risks that are involved in this research study:

Signature and printed name of principal investigator or person obtaining consent
Date

CONSENT

By signing below, you confirm that you are 18 years of age or older and have read or had this document read to you. You have been informed about this study's purpose, procedures, possible benefits and risks, and you have received a copy of this form. You have been given the opportunity to ask questions before you sign, and you have been toldthat you can ask other questions at any time.

I voluntarily agree to participate in this study. By signing this form, I understand that I amnot waiving any of my legal rights. Refusal to participate will involve no penalty or loss ofbenefits to which I am otherwise entitled. I may discontinue participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

SIGNATURE OF VOLUNTEER DATE

September 7, 2017

Dr. Robert Matthew Brothers
Kinesiology
The University of Texas at Arlington
Box 19259

EXPEDITED APPROVAL OF HUMAN SUBJECT RESEARCH

IRB No.: 2017-0856
TITLE: *The Effect of Nitrate Supplementation on Vascular function in Caucasians andAfrican Americans*
Approval Date: September 7, 2017
Expiration Date: September 7, 2018

Approved Number of Participants: 50 (Do not exceed without prior IRB approval)

The University of Texas Arlington Institutional Review Board (UTA IRB) has made the determinationthat this research protocol involving human subjects is eligible for expedited review in accordance withTitle 45 CFR 46.110(a)-(b)(1), 63 FR 60364 and 63 FR 60353, categories (2), (4) and (7). The IRB Chairperson (or designee) approved this protocol effective **September 7, 2017.** IRB approval for the research shall continue until **September 7, 2018.**

APPROVED NUMBER OF PARTICIPANTS:
This protocol has been approved for enrollment of a maximum of **50** participants and is not to exceed thisnumber. The IRB considers a subject to be enrolled once s/he consents to participate in the study. If additional data are needed, the researcher must submit a modification request to increase the number of approved participants **before** the additional data are collected. Exceeding the number of approved participants is considered an issue of non-compliance and will be subject to deliberation set forth by the IRB and the Vice President for Research.

INFORMED CONSENT DOCUMENT:
The IRB approved version of the informed consent document (ICD) must be used when prospectively enrolling volunteer participants into the study. All signed consent forms must be securely maintained on the UT Arlington campus for the duration of the study plus a minimum of three years after the completionof all study procedures (including data analysis). The complete study record is subject to inspection and/or audit during this time period by entities including but not limited to the UT Arlington IRB, Regulatory Services staff, OHRP, FDA, and by study sponsors (if the study is funded).

MODIFICATION TO AN APPROVED PROTOCOL:
Pursuant to Title 45 CFR 46.103(b)(4)(iii), investigators are required to, "promptly report to the IRB ***any***proposed changes in the research activity, and to ensure that such changes in

approved research, during the period for which IRB approval has already been given, are **not initiated without prior IRB review and approval** except when necessary to eliminate apparent immediate hazards to the subject." Modifications include but are not limited to: Changes in protocol personnel, number of approved participants, and/or updates to the protocol procedures or instruments. All proposed changes must be submitted via the electronic submission system prior to implementation. Failure to obtain prior approval for modifications is considered an issue of non-compliance and will be subject to review and deliberationby the IRB which could result in the suspension/termination of the protocol.

ANNUAL CONTINUING REVIEW:
In order for the research to continue beyond the first year, the Principal Investigator must submit a Continuing Review for approval via the online submission system within 30 days preceding the date of expiration indicated above. Continuing review of the protocol serves as a progress report and provides theresearcher with an opportunity to make updates to the originally approved protocol. Failure to obtain approval for a continuing review will result in automatic ***expiration of the protocol*** all activities involvinghuman subjects must cease immediately. The research will not be allowed to commence by any protocol personnel until a new protocol has been submitted, reviewed, and approved by the IRB. Per federal regulations and UTA's Federalwide Assurance (FWA), there are no exceptions and no extensions of approval granted by the IRB. The continuation of study procedures after the expiration of a protocol is considered to be an issue of non-compliance and a violation of federal regulations. Such violations couldresult in termination of external and University funding and/or disciplinary action.

ADVERSE EVENTS:
Please be advised that as the Principal Investigator, you are required to report local adverse (unanticipated) events to The UT Arlington Office of Research Administration; Regulatory Serviceswithin 24 hours of the occurrence or upon acknowledgement of the occurrence.

HUMAN SUBJECTS TRAINING AND CONFLICTS OF INTEREST DISCLOSURES:
All investigators and key personnel identified in the protocol must have documented Human Subjects Protection (HSP) training on file and must have filed a current Conflict of Interest Disclosure (COI) withThe UT Arlington Office of Research Administration; Regulatory Services. HSP completion certificatesare valid for 3 years from completion date.

COLLABORATION:
If applicable, approval by the appropriate authority at a collaborating facility is required prior to subjectenrollment. If the collaborating facility is *engaged in the research*, an OHRP approved Federalwide Assurance (FWA) may be required for the facility (prior to their participation in research-related activities). To determine whether the collaborating facility is engaged in research, go to:
http://www.hhs.gov/ohrp/humansubjects/assurance/engage.htm

CONTACT FOR QUESTIONS:
The UT Arlington Office of Research Administration; Regulatory Services appreciates your continuing commitment to the protection of human research subjects. Should you have questions or require further assistance, please contact Regulatory Services at

regulatoryservices@uta.edu or 817-272-2105.

Sincerely,

Deborah Behan PhD, RN-BC

Digitally signed by Deborah BehanPhD, RN-BC
DN: cn=Deborah Behan PhD, RN-BC,o=UTA, ou=IRB Chair, email=dgreen@uta.edu, c=US
Date: 2017.09.07 17:37:23 -05'00'

Deborah Behan, PhD

Associate Clinical Professor, Nursing
UT Arlington IRB Chair

PRINCIPAL INVESTIGATOR / FACULTY ADVISOR
R. Matthew Brothers, Ph.D. Department of
Kinesiology. Email:Matthew.Brothers@uta.edu. Office:
817-272-3288

TITLE OF PROJECT
The Effect of Beet Root Juice on Vascular Function in Caucasians and AfricanAmericans

INTRODUCTION
You have been asked to participate in a research study about the effect of beet root juice on blood flow in your brain, arm, and leg. Your participation is voluntary. Refusal to participate or discontinuing your participation at any time will involve no penalty or loss of benefits to which you are otherwise entitled. Please ask questions if there is anything you do not understand.

You can participate in this study if you are between the ages of 18 - 45, are not pregnant, are not a smoker, have no cardiovascular disease such as high blood pressure and/or diabetes, do not have food allergies, and are not taking any prescription medications. Thisstudy include one initial screening / familiarization visit and an additional 2 days to complete with approximately 3 – 10 days in between visits. The screening / familiarizationvisit will be approximately 1.5 hours and each study visit will last about 5 hrs.

Your participation is voluntary. If you do not want to participate or you want to stop participating at any time you can. You will not be punished in anyway if this happens. Please ask questions if there is anything you do not understand.

PURPOSE
African Americans have a higher risk for several diseases that involve reduced blood vesselfunction including high blood pressure and diabetes. Reduce blood vessel function refers to the ability of the vascular system to relax or constrict to control blood flow throughout the body. The consumption of beet root juice has been shown to increase blood plasma concentrations of nitric oxide, (a compound that can potentially widen blood vessels) whichmay improve blood flow and blood vessel function. We want to look at the relationship between the ingestion of a high nitrate concentrated juice and control of blood vessels in the brain, arm, skin, and leg.

DURATION
You will visit the laboratory 3 times. During the first visit we will have you answer some questions about how healthy you are to see if you can be in the study. We will also show you the different things that will be done to you on the next two visits.

The initial screening / familiarization visit will last about 1.5 hours. Each study visit will last about 5 hours for a total of about 11.5 hours for the entire study. The two study visits can be separated by 3 - 10 days.

NUMBER OF SUBJECTS
The number of anticipated subjects in this research study is 50.

PROCEDURES
Screening / Familiarization Day – step-by-step protocol:

1. Arrival at laboratory, verbal description of the protocol, sign consent form andhealth history questionnaire
2. Measures of body height, weight, waist and hip circumference
3. Familiarization with the blood flow measurements
   - ***Total time:*** ~1.5 hour

Study Day 1 – step-by-step protocol:

1. Arrival at laboratory
2. Measures of body height, weight, waist and hip circumference
3. If you are a woman of child-bearing age, you will be asked to provide a urinesample for a pregnancy test.
4. Blood draw from a vein in your non-dominant arm
5. Consume either the placebo or beetroot juice
6. About 3 hours after you have the treatment we will obtain another blood draw andthen perform measures of blood pressure, heart rate, brain blood flow, arm blood flow, skin blood flow, and leg blood flow.
   - ***Total time:*** ~5 hour

Study Day 2 – step-by-step protocol:

1. Arrival at laboratory
2. Measures of body height, weight, waist and hip circumference
3. If you are a woman of child-bearing age, you will be asked to provide a urinesample for a pregnancy test.
4. Blood draw from a vein in your non-dominant arm
5. Consume either the placebo or beetroot juice (the one not taken on Day 1)
6. About 3 hours after you have the treatment we will repeat the blood draw and measures of blood pressure, heart rate, brain blood flow, arm blood flow, skin bloodflow, and leg blood flow.
   - ***Total time:*** ~5 hour

A detailed list of procedures is described below. The investigator has checked the procedures you will have <u>today</u>, please initial each procedure checked by the investigator to confirm each item has been explained to you and that you have had enough time to askquestions. These will be done on both study days. If at any time

you wish to discuss the information above or any other risks you may experience, you may ask questions now or callthe Principal Investigators listed on the front page of this form. You do not have to do any ofthe following procedures if you do not want to. If this is the case we can still do the other procedures if you agree.

☐ INITIAL:_____

Height and Weight:

- Description of Procedure: We will measure your height and weight. There is no risk associated with these measurements.

☐ INITIAL:_____

Electrocardiogram:

- **Description of Procedure:** Sticky patches will be applied to your skin to measurethe heart's electrical signals.

☐ INITIAL:_____

Blood pressure:

- **Description of Procedure:** Your blood pressure will also be monitored using a cuff placed on your upper arm that is inflated and deflated periodically. Also, a small cuff that fits on one finger will be used for continuous blood pressure measurements.

☐ INITIAL:_____

Venous blood sample:

- Description of Procedure: A small thin, plastic tube (catheter) will be placed in avein in your arm (a little above your elbow) for blood sampling. The amount of blood to be sampled on each study visit will be about 50 ml: about 7 table spoons (on each day). Therefore, the total blood drawn will not be greater than 100 mls or about 15 tablespoons. Blood will be used to identify potential blood markers that may contribute to elevating blood pressure and decreasing blood flow in obese individuals (i.e. amount of sugar or other substances that cause your blood vessels to narrow). Some of the blood will be labeled with your initials and date of birth and will be sent to a lab outside of UT Arlington for analysis. The lab result will be ready to pick up two days after the experiment. If you are interested to get the result, you can let us know and we will make the arrangement. The remaining blood will be kept in containers in a freezer located in the lab until we do the measurements. We will label the containers in a way that only we will be able to identify them.

☐ INITIAL:_____

Doppler ultrasound:

- **Description of Procedure:** A picture of a vessel in your arm, leg, or neck will be taken by an ultrasound camera, similar to standard ultrasound tests done to examinethe health of fetuses prior to birth and to the "sonar" used by fisherman to locate fish underwater.

☐ INITIAL:_____

Blood Vessel Response:

- **Description of Procedure:** A blood pressure cuff will be placed around your forearm or leg. This cuff will be inflated, as is done when your blood pressure is being measured, but instead of deflating the cuff immediately it will remain inflatedfor 5 minutes.

☐ INITIAL:_____

Brain blood flow:

- **Description of Procedure:** A gel covered probe will be placed against the templeof your head. This probe will record blood flow inside your brain, similar to standard ultrasound tests described above.

☐ INITIAL:_____

Carbon Dioxide Concentration ($PETCO_2$):

- **Description of Procedure:** Your body is constantly producing carbon dioxide which then leaves the body when you breathe out. This procedure will provide a measurement of how much carbon dioxide is expired each time you breathe out. To do this you will breathe normally into a mouthpiece with a tube into the mouthpiece to measure carbon dioxide. This measurement will be unnoticeable to you during the protocol.

☐ INITIAL:_____

Respiration:

- **Description of Procedure:** A small belt will be placed around your upper stomachto monitor when you breathe in and out.

☐ INITIAL:_____

Cerebral Vasomotor Reactivity:

- **Description of Procedure:** This measurement is to look at how blood flow in yourbrain changes to changes in carbon dioxide (a gas that your

gets rid of when you blow out). After breathing room air for 6 minutes, you will be asked to briefly increase your pace of breathing for 30 seconds after which you will breathe into and out of a bag through a mouthpiece. During the time when you are breathing into and out of a bag we will provide extra oxygen to maintain normal oxygen concentrations in the blood. You will breathe faster than normal for 30 seconds andbreathe into and out of the bag for no more than 4 minutes. During this time we willbe measuring blood pressure, heart rate, brain blood flow, and carbon dioxide (all of which are described above).

☐ INITIAL:_____

Central and Peripheral Blood Pressures and Pulse Wave Measurement:

- **Description of Procedure:** A blood pressure cuff will be placed on your upper armand will be connected to a device that measures your central (heart level) and peripheral (arm) blood pressure. In order to measure your pulse wave we will alsoplace a blood pressure cuff on the upper thigh of your left leg. You may feel a smallamount of pressure as we feel for your pulse at the top of your leg (femoral) and your neck (carotid) to locate both arteries. After locating each artery we will makemeasurements between the respective arteries. Finally, we will gently place a smallprobe on the skin over the artery in your neck while at the same time the blood pressure cuff on your leg will inflate. The carotid and femoral artery waveforms aredetected and used to calculate carotid-femoral pulse wave velocity (a measure of the stiffness of your blood vessels).

☐ INITIAL:_____

Active knee extension exercise:

- **Description of Procedure:** You will be asked to perform knee extension exerciseusing only a single leg at a rate of 60 times a minute. The intensity of the exercise will be quite low and will only slightly increase your heart rate, similar to if you were walking at a medium speed.

☐ INITIAL:_____

Passive knee extension exercise:

- **Description of Procedure:** A member of the research team will move your leg from a bent position to a straight position. The range of motion is similar to knee extension exercise. The difference is that we will ask you to remain as still as possible and let the researcher do the work for you. The intensity of the exercise will be quite low and will

only slightly increase your heart rate, similar to if you were walking at a medium speed.

☐ INITIAL:_____

Skin blood flow:

- Description of Procedure: skin blood flow will be measured at different places onyour forearm. This measurement will be done using laser-Doppler devices. These devices use very low power laser light to measure how fast blood is flowing in theblood vessel in your skin. Up to four small probes will be placed on one of your forearms. There are no risk associated with this procedure.

☐ INITIAL:_____

Manipulation of skin temperature:

- Description of Procedure: Control of lacal skin temperature is accomplished by locally heating the site where skin blood flow is measured. The highest temperature we will use is 109° F for 30 minutes.

☐ INITIAL:_____

Mental Subtraction:

- **Description of Procedure:** You will be asked to continually subtract either the number 6 or 7 from a number that is given to you by a member of the research team.We will ask you to do this as quickly as possible for a period of about 3 minutes.

☐ INITIAL:_____

Beet root juice/Placebo:

32. On the 2 study visit days you will consume 140 ml of beetroot juice containing either a high nitrate concentration (~12.8 mmol) or a low nitrate concentration (~0.0055 mmol) beverage. The beet root juice is soemthing that can be purchased online and at most natural grocery stores. In addition this amount of beetroot juice has been used inover 200 research studies. This amount of nitrate is similar to what would be consumedwith a meal of a large salad and steak. The low nitrate beverage is provided to us by the company who makes the beetroot juice beverage, James White Fine Pressed Fruit Juices. This beverage has also been used in over 200 research studies.

POSSIBLE RISKS/DISCOMFORTS

The principal investigator and laboratory team are experienced with all procedures outlinedin this study in both healthy and diseased populations. Nevertheless, as

with all studies involving human subjects, there are risks associated with experimentation that are currentlyunknown. All possible risks associated with this study are explained below. To reduce theserisks, you will only be able to join the study if you are healthy and you will have to completea health history questionnaire as a precautionary measure to ensure the study poses no additional risks. Furthermore, potential risks will also be minimized by: (a) using only safe,well-established procedures; (b) constant, personal monitoring of each experimental session by the investigators and staff; and (c) knowledge that you can request to stop at anypoint during the procedure. In addition, you should know that the principal investigator may also terminate the testing procedure at any time. All experimental procedures will conform to the procedures for this study as approved by the University of Texas at Arlington Institutional Review Board (IRB). An IRB reviews and approves research for the protection of human subjects.

Blood Pressure:

- Other than some potential discomfort associated with cuff inflation there is no riskto this procedure.

Blood Vessel Response:

- There will be a period of cuff inflation similar to the blood pressure measure described above. You may feel slight discomfort and possibly numbness at the fingertips during and after cuff inflation. If this happens it will go away in about30 seconds following cuff deflation.

Venous Blood Sample:

- There is a risk of infection, bruising, and occasional light-headedness during the blood draw. These minor risks are minimized by using clean conditions and peoplewho are experienced with blood collection.

Cerebral Vasomotor Reactivity:

- You might feel dizzy and/or short of breath. If this occurs we will stop and you willimmediately breathe normal room air which will make this feeling stop immediately. This measurement is often done in many labs and doctor's offices. We have done this measurement in about 300 people over the last 10 years with noproblems.

Beverage Consumption:

- There are no known risks involved with drinking either of the beverages.

In the case of an emergency, study personnel will contact the UTA Police Department. In case of injury related to study participation, please note that UTA has not set any funds aside for medical costs. In the case of injury, any medical

costs will be the responsibility of you or your insurance company.

COMPENSATION
You will be compensated for time spent in the laboratory. The rate of compensation will be $15.00 per hour and will be no more than a total of $120.00. The rate of compensationwill always be rounded to the nearest 1/3 hour.

The Internal Revenue Service (IRS) considers all payments made to research subjects to be taxable income. Your personal information, including your name, address, and social security number, may be acquired from you and provided to UT Arlington's accounting office for the purpose of payment. If your total payments for the year exceed $600.00, UTArlington will report this information to the IRS as income and you will receive a Form 1099 at the end of the year. If you receive less than $600.00 total for payments in a year, you are personally responsible for reporting the payments to the IRS.

ALTERNATIVE PROCEDURES
There are no alternative procedures offered for this study. However, you can elect not to participate in the study or quit at any time at no consequence.

VOLUNTARY PARTICIPATION
Participation in this research study is voluntary. You have the right to decline participation in any or all study procedures or quit at any time at no consequence. Should you choose not to complete all study procedures, you will still receive compensation for the time spent participating following consent.

During the course of the study, you may decide not to participate in some experimental measurements or procedures and therefore, this portion of the protocol will not be completed. However, all other measurements will be performed. This will not affect the scientific value of your participation as each experimental measurement and procedure provides important and in most cases independent information. In addition, the principal investigator and research staff may decide to re-enroll you in the study, if you agree, if previous testing was unsuccessful or certain experimental measurements and procedures were not initially performed. The re-enrollment has no additional safety risks other than those already stated.

CONFIDENTIALITY
Every attempt will be made to see that your study results are kept confidential. A copy of this signed consent form and all data collected from this study will be stored in the investigator's lab (electronic data) and office (paper files) for at least three (3) years after the end of this research. The results of this study may be published and/or presented at meetings without naming you as a subject. Additional research

studies could come from the information you have provided, but your information will not be linked to you in anyway (i.e., it will be coded in a manner that others cannot identify you). Likewise, at thistime we plan to do all of the blood analysis in our laboratory at UTA; however, if in the future we decide to send some samples to other labs for analysis all of the information provided will not be linked to you in anyway (i.e., it will be coded in a manner that others cannot identify you). Although your rights and privacy will be maintained, the Secretary of the Department of Health and Human Services, the UTA IRB, and personnel particular to this research have access to the study records for inspectional purposes. Your records will be kept completely confidential according to current legal requirements. They will notbe revealed unless required by law, or as noted above. The IRB at UTA has reviewed andapproved this study and the information within this consent form. If in the unlikely event it becomes necessary for the IRB to review your research records, the University of Texasat Arlington will protect the confidentiality of those records to the extent permitted by law.

CONTACT FOR QUESTIONS

Questions about this research study may be directed to Dr. Matt Brothers (817-272-3151, matthew.brothers@uta.edu). If you believe that you had any discomfort or risks as a resultof the study, you should also contact Dr. Matt Brothers.

Any questions you may have about your rights as a research subject or a research-related injury may be directed to the Office of Research Administration; Regulatory Services at 817-272-2105 or regulatoryservices@uta.edu.

As a representative of this study, I have explained the purpose, the procedures, thebenefits, and the risks that are involved in this research study:

_____

Signature and printed name of principal investigator or person obtaining consent
Date

CONSENT

By signing below, you confirm that you are 18 years of age or older and have read or had this document read to you. You have been informed about this study's purpose, procedures,possible benefits and risks, and you have received a copy of this form. You have been giventhe opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

I voluntarily agree to participate in this study. By signing this form, I understand that I amnot waiving any of my legal rights. Refusal to participate will involve no penalty or loss of benefits to which I am otherwise entitled. I may discontinue participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

SIGNATURE OF VOLUNTEER DATE

8/11/2020

IRB Approval of Full Board Continuing Review + Modification

PI: Robert Brothers
Department: Kinesiology
IRB Protocol #: 2018-0648.3
Study Title: *The Effect of Endothelin and L-Arginine on Racial Differences in Vasoconstriction*
Date of Convened Meeting: 08/11/2020

Effective Approval: 8/11/2020
In-person interactions with human subjects must comply with UTA's list of permitted researchactivities and the related requirements under COVID-19 limitations: https://resources.uta.edu/research/regulatory-services/human-subjects/news-and-announcements.php.

Protocol Details

- Original Protocol Approval Date: 10/4/2018
- Protocol Expiration Date: 8/14/2021
- FDA Regulated: IND
  - Subject to FDA Regulations 21 CFR parts 56.109(f)
    - Continuing Review required: Yes, standard

The IRB has approved the above referenced submission in accordance with applicable regulations and/or UTA's IRB Standard Operating Procedures. The approved modifications arelimited to:

- Removed Olvera, Despain, Kaur, and Richey from protocol personnel

Principal Investigator and Faculty Advisor Responsibilities

All personnel conducting human subject research must comply with UTA's IRB Standard OperatingProcedures and RA-PO4, Statement of Principles and Policies Regarding Human Subjects in Research. Important items for PIs and Faculty Advisors are as follows:

- **Notify Regulatory Services of proposed, new, or changing funding source**
- Fulfill research oversight responsibilities, IV.F and IV.G.
- Obtain approval prior to initiating changes in research or personnel, IX.B.
- Report Serious Adverse Events (SAEs) and Unanticipated Problems (UPs), IX.C.
- Fulfill Continuing Review requirements, if applicable, IX.A.
- Protect human subject data (XV.) and maintain records (XXI.C.).
- Maintain HSP (3 years), GCP (3 years), and RCR (4 years) training as applicable.

PRINCIPAL INVESTIGATOR / FACULTY ADVISOR
R. Matthew Brothers, Ph.D. Department of
Kinesiology. Email:Matthew.Brothers@uta.edu. Office:
817-272-3156

TITLE OF PROJECT
The Effect of Endothelin and L-Arginine on Racial Differences in Vasoconstriction

A description of this clinical trial will be available on
http://www.ClinicalTrials.gov , asrequired by U.S. Law. This Web site will not include information that can identify
you. At most, the Web site will include a summary of the results. You can search thisWeb site at any time.

INTRODUCTION
You have been asked to participate in a research study about the effect of various substances on blood flow in your arm and skin. Your participation is voluntary. Refusal toparticipate or discontinuing your participation at any time will involve no penalty or loss of benefits to which you are otherwise entitled. Please ask questions if there is anything you do not understand.

You can participate in this study if you:

- Are between the ages of 18 – 35
- Self-report parents as either both Caucasian or both African American
- Are not pregnant
- Are not a smoker
- Have no cardiovascular (e.g., high blood pressure), metabolic (e.g., diabetes),neurological (e.g., seizures, Alzheimer's), and/or kidney diseases
- Have no diseases of the microvasculature (e.g., Reynaud's, cold-induced urticaria,cyoglobulinemia)
- Have no food allergies or previous reactions to the study drugs
- Are not on any prescription medications or supplements

This study will take ~4-hours to complete in one visit. Typically, there is only one visit. However, in the event that not all of the measurements are complete on one day, you maybe asked to come back for additional visits. Your participation is voluntary. If you do not want to participate or you want to stop participating at any time you can. You will not be punished in anyway if this happens. Please ask questions if there is anything you do not understand.

PURPOSE

African Americans have a higher risk for several diseases that involve reduced blood vesselfunction including high blood pressure and diabetes. Endothelin is substance that is in yourbody that causes the blood vessels to narrow which can cause a greater chance to haveproblems with your heart and blood vessels. Also, L-Arginine is a natural substance in yourbody. Too little L-Arginine can also lead to problems with your heart and blood vessels. We want to look at the relationship between endothelin and L-Arginine and control ofblood vessels in the arm and skin.

DURATION
This study will last approximately 4-hours.

NUMBER OF SUBJECTS
The number of anticipated subjects in this research study is 70.

PROCEDURES

- Arrival at laboratory, informed consent, health history questionnaire
- Measures of body height, weight, waist and hip circumference
- Blood draw
- Placement of small microdialysis tubes – see below for more detail.
- Measures of blood pressure, heart rate, and skin blood flow/temperature during

~3-hours of quiet rest while drug infusions through the microdialysis tubesoccur

- ***Total time: ~ 4 hours***

A detailed list of procedures is described below. If at any time you wish to discuss the information above or any other risks you may experience you should feel free to do so. Youdo not have to do any of the following procedures if you do not want to. If this is the case wecan still do the other procedures if you agree.

☐ INITIAL:_____

Cardiac Rhythm / Heart Rate:

- **Description of Procedure:** Sticky patches will be applied to your skin to measurethe heart's electrical signals.
- **Duration of Procedure:** The electrocardiogram will be measured during the entireexperiment.

☐ INITIAL:_____

Blood pressure (upper arm):

- **Description of Procedure:** Your blood pressure will also be monitored using a cuff placed on your upper arm that is inflated and deflated periodically.
- **Duration of Procedure:** The cuff will be on your upper arm during the entire experiment. We will take blood pressure measurements at different time points during the experiment. Each measurement will last approximately 30 seconds.

☐ INITIAL:_____

Venous blood sample:

- **Description of Procedure:** A small thin, plastic tube (catheter) will be placed in avein in your arm (a little above your elbow) for blood sampling. The amount of blood to be sampled will be 33 ml; about 4 tablespoons. If you are asked to revisitthe laboratory for follow-up testing we will also take a single blood sample on these days. Therefore, the total blood drawn will not be greater than about 100 mls or about 12 tablespoons. Blood will be used to identify potential blood markers that may contribute to elevating blood pressure and decreasing blood flow in obese individuals (i.e. amount of sugar or other substances that cause yourblood vessels to narrow). Some of the blood will be labeled with your initials and date of birth and will be sent to a lab outside of UT Arlington for analysis. If this blood sample returns values outside the normal range, you will be contacted to let you know – this will occur by personal communication - over the phone or in person. We will not leave any information in a voice mail, text, or send in an email. In addition, we may perform a detailed analysis of the blood samples in order to detect differences in the levels of different proteins and other blood markers related to vascular health. Some of this analysis will be "RNA sequencing," however, this is not the same as the type of full analysis that is offered by companies like 23 & ME, and it will not provide the research team with full access to the participant's genetic information. The remaining blood willbe kept in containers in a freezer located on the first floor in the Science and Engineering Innovation and Research (SEIR) building until we do the measurements. We will label the containers in a way that only we will be able to identify them.
- **Duration of Procedure:** We will only obtain one blood sample at the beginning ofthe study.

☐ INITIAL:_____

Brachial Blood Flow:

- **Description of Procedure:** A picture of a vessel in your arm will be taken by an ultrasound camera, similar to standard ultrasound tests conducted on pregnant women and to the "sonar" used by fisherman to locate fish underwater.
- **Duration of Procedure:** Imaging will require approximately 10 minutes to take.

☐ INITIAL:_____

Flow Mediated Dilation/Blood Vessel Responsiveness:

- **Description of Procedure:** A blood pressure cuff will be placed around your forearm. This cuff will be inflated, as is done when your blood pressure is being measured, but instead of deflating the cuff immediately it will remain inflated for 5-minutes.
- **Duration of the Procedure:** This procedure will take approximately 10-minutes.

☐ INITIAL:_____

Skin blood flow:

- **Description of Procedure:** Skin blood flow will be measured at four different places on your forearm. This measurement will be done using laser-Dopplerdevices. These devices use very low power laser light to measure how fast blood isflowing in blood vessels in your skin. Four small probes will be placed on one of your forearms.
- **Duration of Procedure:** Skin blood flow will be measured during the entire experiment.

☐ INITIAL:_____

Manipulation of skin temperature:

- **Description of Procedure:** Control of local skin temperature is accomplished by locally heating using a small probe (~2.5-cm/1-in diameter) the site where skin blood flow is measured. This only heats the skin under the probe. The highest temperature we will use is 43°C (109.4°F) for approximately 15-minutes.
- **Duration of Procedure:** The total duration of local heating will be about 2-hours minutes.

☐ INITIAL:_____

Placement of Small Microdialysis Tubes:

- **Description of Procedure:** These tubes will be used to deliver small amounts of substances, commonly used in research studies like this one, into your skin. To dothis we will place 4 small microdialysis tubes into the skin of one of your forearms.

  The microdialysis tube is placed almost exactly like an "IV" in the hospital, exceptthat veins are not entered. First the skin is cleaned with alcohol. Then four very thin(25 gauge) needles will be placed just under the surface of your skin on one of yourforearms. Four special, sterile, thin tubes (about 1/100th of an inch in diameter; about the size of fishing line) with microscopic holes in it will be placed through the needle. Then the needle will be removed, leaving the special tubing just under the surface of your skin on your arm. Additional tubing (without holes) will be connected to the special tube in your skin and a sterile salt solution that is used in hospitals (Ringer's solution) will be pumped slowly through the tubing assembly. Only a tiny amount of the salt solution and drug will go into your skin. After the study ends, all tubes are removed.
- **Duration of Procedure:** These tubes will be in the skin of your forearm during theentire experiment.

☐ INITIAL:_____

Drug Solutions:

- **Description of Procedure:** Throughout the experiment, small amounts of commonly used drug solutions (see below for description of drug solutions) will bedelivered through the tubes. These drugs will be used to measure the effect of various substances on the ability of your blood vessels to relax. These drugs will bemixed in a solution called Ringer's solution (similar to a lightly salted solution thatis used in hospitals). The drugs to be used include BQ-123 (an endothelin receptor type A blocker), BQ-788 (an endothelin receptor type B blocker), L-Arginine (a naturally occurring substance in your body that helps blood vessels relax by producing nitric oxide – a substance that helps your blood vessels relax), L-NAMEa drug to temporarily block nitric oxide (a substance that helps your blood vessels relax) and sodium nitroprusside (SNP: a substance that helps your blood vessels relax). L-NAME, BQ-123, and L-Arginine are not approved by the Food and DrugAdministration. SNP is approved for clinical use to lower blood pressure and BQ-123 is approved for clinical use to treat high blood pressure in the lungs.
- **Duration of Procedure:** These tubes will be in your forearm during

the entire experiment. BQ-123, BQ788, and L-Arginine be delivered for a total of about 2 hours. L-NAME and SNP will be delivered for about 30 minutes each.

POSSIBLE RISKS/DISCOMFORTS

The principal investigator and laboratory team are experienced with all procedures outlinedin this study in both healthy and diseased populations. Nevertheless, as with all studies involving human subjects, there are risks associated with experimentation that are currentlyunknown. The list of known risks associated with this study are explained below. To reducethese risks, you will only be able to join the study if you are healthy and you will have to complete a health history questionnaire as a precautionary measure to ensure the study poses no additional risks. Furthermore, potential risks will also be minimized by: (a) using only safe, well-established procedures; (b) constant, personal monitoring of each experimental session by the investigators and staff; and (c) knowledge that you can requestto stop at any point during the procedure. In addition, you should know that the principal investigator may also terminate the testing procedure at any time. All experimentalprocedures will conform to the procedures for this study as approved by the University ofTexas at Arlington Institutional Review Board (IRB). An IRB reviews and approves research for the protection of human subjects.

Blood pressure:

- Other than some potential discomfort associated with cuff inflation there is no riskto this procedure.

Venous blood sample:

- There is a risk of infection, bruising, and occasional light-headedness during the blood draw. These minor risks are minimized by using clean conditions and peoplewho are experienced with blood collection.

Skin blood flow:

- There are very minimal risks associated with using these devices to measure skin blood flow; they are painless and harmless in all respects.

Manipulation of skin temperature:

- There is the risk that burns can be caused by temperatures as low as 44°C (111.2°F)when administered for long periods. The temperature used in this study will not exceed 43°C (109.4°F) and will only be administered for a maximum of 20- minutes.

Placement of Small Microdialysis Tubes:

- You may experience some discomfort and bleeding with the small needles used during the placement of the microdialysis tubes. There is

an even small chance of experiencing bruising, infection, and irritation. However, after the needles have been removed, the microdialysis tubes should not hurt and will be physically unnoticeable.

Drug Solutions:

- Due to the small concentration of the drugs delivered, there is very minimal risk associated with receiving these drugs. There is a minor risk of infection and allergicreaction. There are no known negative experiences in humans with the concentrations of each of the drugs. Also these drugs are delivered in such a smallamount that they will only effect an area about the size of a quarter on your arm (inother words they will not affect the rest of your body).
- If you notice any changes in skin color or pain after the study is completed you must contact Dr. Brothers immediately – 817-272-3156, matthew.brothers@uta.edu at which point you will likely be referred to visit theemergency room.

COMPENSATION

You will be compensated for time spent in the laboratory. The rate of compensation will be $15.00 per hour and will be no more than a total of $75.00. The rate of compensation will always be rounded to the nearest 1/3 hour.

It is important that you report any illness or injury to the research team listed in this form immediately. Compensation for an injury resulting from your participation in this researchis NOT available from The University of Texas at Arlington. In the event that you suffer aresearch-related injury, your medical expenses will be your responsibility or that of your third-party payer, although you retain your legal rights during your participation in this research.

The Internal Revenue Service (IRS) considers all payments made to research subjects to be taxable income. Your personal information, including your name, address, and social security number, may be acquired from you and provided to UT Arlington's accounting office for the purpose of payment. If your total payments for the year exceed $600.00, UTArlington will report this information to the IRS as income and you will receive a Form 1099 at the end of the year. If you receive less than $600.00 total for payments in a year, you are personally responsible for reporting the payments to the IRS.

ALTERNATIVE PROCEDURES

There are no alternative procedures offered for this study. However, you can elect not to participate in the study or quit at any time at no consequence.

VOLUNTARY PARTICIPATION

Participation in this research study is voluntary. You have the right to decline participationin any or all study procedures or quit at any time at no consequence. Should you choose not to complete all study procedures, you will still receive compensation for the time spentparticipating following consent.
During the course of the study, you may decide not to participate in some experimental measurements or procedures and therefore, this portion of the protocol will not be completed. However, all other measurements will be performed. This will not affect the scientific value of your participation as each experimental measurement and procedure provides important and in most cases independent information. In addition, the principal investigator and research staff may decide to re-enroll you in the study, if you agree, if previous testing was unsuccessful or certain experimental measurements and procedures were not initially performed. This may include another blood draw at the additional visit(s). The re-enrollment has no additional safety risks other than those already stated.

CONFIDENTIALITY

Every attempt will be made to see that your study results are kept confidential. A copy of this signed consent form and all data collected from this study will be stored in the investigator's lab (electronic data) and office (paper files) for at least three (3) years after the end of this research. The results of this study may be published and/or presented at meetings without naming you as a subject. Additional research studies could come from the information you have provided, but your information will not be linked to you in anyway (i.e., it will be coded in a manner that others cannot identify you). Some of the blood we sample will be labeled with your initials and date of birth and will be sent to a lab outside of UT Arlington for analysis. The remaining blood analysis will be performedin our laboratory at UTA and will be coded in a manner that others cannot identify you. Although your rights and privacy will be maintained, the Secretary of the Department of Health and Human Services, the Food and Drug Administration, the UTA IRB, and personnel particular to this research have access to the study records for inspectional purposes. Your records will be kept completely confidential according to current legal requirements. They will not be revealed unless required by law, or as noted above. The IRB at UTA has reviewed and approved this study and the information within this consentform. If in the unlikely event it becomes necessary for the IRB to review your research records, the University of Texas at Arlington will protect the confidentiality of those records to the extent permitted by law.

CONTACT FOR QUESTIONS

Questions about this research study may be directed to Dr. Matt Brothers (817-272-3156, matthew.brothers@uta.edu). If you believe that you had any discomfort or risks as a resultof the study, you should also contact Dr. Matt Brothers.
Any questions you may have about your rights as a research subject or a research-related injury may be directed to the Office of Research Administration; Regulatory

Services at 817-272-3723 or regulatoryservices@uta.edu.

As a representative of this study, I have explained the purpose, the procedures, thebenefits, and the risks that are involved in this research study:

_____

Signature and printed name of principal investigator or person obtaining consent
Date

CONSENT

By signing below, you confirm that you are 18 years of age or older and have read or had this document read to you. You have been informed about this study's purpose, procedures,possible benefits and risks, and you have received a copy of this form. You have been giventhe opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

I voluntarily agree to participate in this study. By signing this form, I understand that I amnot waiving any of my legal rights. Refusal to participate will involve no penalty or loss of benefits to which I am otherwise entitled. I may discontinue participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

_____

SIGNATURE OF VOLUNTEER DATE

6/17/2020

IRB Approval of Greater than Minimal Risk (GMR) Continuing Review

PI: Zachary Martin
Faculty Advisor: Robert Brothers
Department: Kinesiology
IRB Protocol #: 2019-0318.2
Study Title: *A Plant-Based Diet and Vascular Function in African Americans*
Date of Convened Meeting: 06/09/2020

Effective Approval: 6/17/2020
Face-to-face or in-person interactions with human subjects approved within this protocol may onlyproceed once restrictions are lifted related to COVID-19: https://resources.uta.edu/research/coronavirus/index.php

Protocol Details

- Original Protocol Approval Date: 6/20/2019
- Protocol Expiration Date: 6/20/2021
- Continuing Review required: Yes, standard

The IRB has approved the above referenced submission in accordance with applicableregulations and/or UTA's IRB Standard Operating Procedures.

Principal Investigator and Faculty Advisor Responsibilities

All personnel conducting human subject research must comply with UTA's IRB Standard OperatingProcedures and RA-PO4, Statement of Principles and Policies Regarding Human Subjects in Research. Important items for PIs and Faculty Advisors are as follows:

- **Notify Regulatory Services of proposed, new, or changing funding source**
- Fulfill research oversight responsibilities, IV.F and IV.G.
- Obtain approval prior to initiating changes in research or personnel, IX.B.
- Report Serious Adverse Events (SAEs) and Unanticipated Problems (UPs), IX.C.
- Fulfill Continuing Review requirements, if applicable, IX.A.
- Protect human subject data (XV.) and maintain records (XXI.C.).
- Maintain HSP (3 years), GCP (3 years), and RCR (4 years) training as applicable.

TITLE OF RESEARCH PROJECT
A Plant-Based Diet and Vascular Function in African Americans

RESEARCH TEAM
From the Department of Kinesiology
Zachary Martin, M.S.
(zachary.martin@uta.edu)
Matthew Brothers, Ph.D.
(matthew.brothers@uta.edu)
Rauchelle Richey, B.S.
Jeremiah Campbell, M.S.
John Akins, M.S.
Christian Villegas, B.A.

From the College of Nursing and Health Innovation
Yaewon Seo, Ph.D.
Jeanette Blankenship, M.S.

IMPORTANT INFORMATION ABOUT THIS RESEARCH PROJECT
The research team above is conducting a research study about diet and blood vessel health in African Americans. The purpose of this study is to compare the function of blood vessels and certain blood markers of health in African Americans following a plant-based diet and African Americans following a typical American Diet. If you agree to participate, you will be asked to come to the lab for two visits, which will last approximately 3 hours each. We will first explain the procedures to you, and you will fill out a consent form and a form about your health. We will also perform the measurements on this day (and during the second visit). If you qualify andagree to participate, you will be compensated for your time. You can choose to participate in this research study if you are healthy, between the ages of 18-40 years, and are not pregnant.

You might want to participate in this study if you want to help society learn more about the roleof diet in preventing cardiovascular disease. However, you might not want to participate in this study if you do not have the time to attend two approximately 3-hour study visits on the UT Arlington campus, do not like sitting still for long periods of time, or are uncomfortable with having your blood drawn.

This study has been reviewed and approved by an Institutional Review Board (IRB). An IRB is anethics committee that reviews research with the goal of protecting the rights and welfare of human research subjects. Your most important right as a

human subject is informed consent. You should take your time to consider the information provided by this form and the research team and ask questions about anything you do not fully understand before making your decision about participating.

TIME COMMITMENT

You will be asked to participate in 2 study visits on the UTA Campus in Arlington, Texas, andeach visit will last approximately 3 hours (no more than 4 hours) for a combined total of between 6 – 8 hours. The visits will be within a one-month period.

RESEARCH PROCEDURES

If you decide to participate in this research study, this is the list of activities (and any riskassociated with them) that we will ask you to perform as part of the research.

1. First, you will read through this **Informed Consent** form and talk with the researchteam to make sure that any questions you may have are answered. You will then make your choice about whether to participate.
    - There are no risks associated with this process.

2. If you agree to participate, you will be asked to allow a member of the researchteam to take your **height, weight, and waist and hip circumference**.
    - There are no risks associated with these measures.

3. You will then be asked to fill out several **surveys** regarding your diet and otherdemographic information.
    - Some of the surveys will ask questions about your perceptions of discrimination throughout your life while others may ask about where youlive and how much money you make.
    - This will help us interpret our physiological findings in the context of socialfactors that may have an impact on your health.
    - You may become slightly uncomfortable when answering some of the more personal questions; however, you may skip any questions or surveys that youdo not wish to answer.

4. You will lay on a table where a machine will scan your body to determine how muchfat and other tissues you have in your body. This

is called a **DXA scan**.

- The DXA test has minimal to no risk as described next. The test exposes you to a small amount of radiation where the X-ray beam crosses your body. This radiation exposure is not required for your medical care and is for research only. This project calls for a single total body scan. The dose for one total body scan is equal to a whole-body radiation dose of about 1.5 millirems. A millirem (mrem) is a unit of whole-body radiation dose. For example, the average person in the United States receives a radiation exposure of 300 mrem per year from natural background sources. These sources can include the sun, outer space, and natural radioactive substances that are found in the earth's air and soil. 1.5 mrem is less than you would receive from 2 days of natural background radiation. In order to receive a DXA scan, you must notbe pregnant, and must also not have any of the following:
    - Medical devices – ostomy devices, prosthetic devices, surgical devices, pacemaker leads, radioactive seeds, radiopaque catheters ortubes;
    - Jewelry or metal clothing – metal buttons, zippers, snaps, earrings,necklaces;
    - Foreign objects – shrapnel, buckshot, metal plates or pins.
    - X-ray procedures within the last 7 days which use iodine, barium, orother nuclear medicine isotopes.

5. You will perform a **maximal exercise test** on a stationary bike while you breathe intoa tube. This is called a VO2max test.
    - Your heart rate, blood pressure, and breathing rate will increase during this test – you may feel uncomfortable due to the short bout of vigorous exercisethat you will perform.
    - Because you will be carefully screened and we will closely monitor you during the test, there is minimal risk associated with performing a routinemaximal exercise test.
    - In the rare event that you have a medical complication during the exercisetest, the CPR-trained test administrators will care for you immediately.

6. You will have your **blood drawn** similar to what they do at the doctor's office or hospital. The amount of blood to be sampled will be 75 ml (about 5 tablespoons).

- Blood will be used to identify blood markers that may contribute to poorhealth and blood vessel function (i.e. the amount of sugar or other substances that cause your blood vessels to narrow).
- We will label the blood tubes in a way that only we will be able to identifythem.
- There is a slight risk of infection, bruising, and occasional light-headedness during the blood draw. These minor risks are minimized by only using cleanequipment and practices and only having people who are experienced withblood collection perform the procedure.

7. You will undergo a noninvasive **pulse wave analysis and pulse wave velocityassessment**.
    - A blood pressure cuff will be placed on your upper arm and will be connectedto a device that measures your central (heart level) and peripheral (arm) blood pressure.
    - In order to measure the speed of the blood flow in your body, we will alsoplace a blood pressure cuff on the upper thigh of your left leg.
    - You may feel a small amount of pressure as we feel for your pulse at the topof your leg (femoral) and your neck (carotid) to locate both arteries.
    - After locating each artery, we will make measurements between therespective arteries.
    - Finally, we will gently place a small probe on the skin over the artery in yourneck while at the same time the blood pressure cuff on your leg will inflate.
    - The carotid and femoral artery waveforms are detected and used to calculatecarotid-femoral pulse wave velocity (a measure of the stiffness of your blood vessels).
    - There are no risks associated with these measures.

8. You will undergo a test called the **cerebrovascular motor reactivity test**, which willlook at how blood flow in your brain changes to changes in carbon dioxide (a gas that your body gets rid of when you breathe out).
    - After breathing room air for 6 minutes, you will breathe into and out of arubber bag through a mouthpiece.

- During the time when you are breathing into and out of the bag, we willprovide extra oxygen to maintain normal oxygen concentrations in yourblood.
- You will breathe into and out of the bag for no more than 4 minutes. Duringthis time, we will be measuring blood pressure with an arm and finger cuff; breathing rate with a stretchy band around your belly; heart rate with stickyelectrodes; brain blood flow and neck blood flow with noninvasive ultrasound probes; and carbon dioxide with a nasal cannula.
- You might feel dizzy and/or short of breath during this procedure. If this occurs, we will stop, and you will immediately breathe normal room air which will make this feeling stop immediately. This measurement is often done in many labs and doctor's offices. We have done this measurement inhundreds of people over the last 10 years with no problems. The facemask and/or mouthpiece you use is cleaned with medical-grade disinfectant.

9. You will have **skin blood flow** measured at different places on your forearm.
    - This measurement will be done using laser-Doppler devices. These devices use very low power laser light to measure how fast blood is flowing in bloodvessels in your skin.
    - Up to four small probes will be placed on one of your forearms.
    - We will locally heat the site where skin blood flow is measured. The highesttemperature we will use is 111.2° F for 30 minutes.
    - Although your skin may feel very warm (and you may stop at any time), thereare no risks associated with this procedure.

10. To determine the responsiveness of the blood vessels in your arm, you will undergoa noninvasive test called **flow-mediated dilation**.
    - First, a picture of a vessel in your arm will be taken by an ultrasound camera, similar to standard ultrasound tests done to examine the health of fetuses prior to birth and to the "sonar" used by fisherman to locate fish underwater.
    - Then, a blood pressure cuff will be placed around your forearm. This cuff willbe inflated, as is done when your blood pressure is being measured, but instead of deflating the cuff immediately, it will remain inflated for 5 minutes. It is expected that you may feel slight discomfort when the cuff is inflated and feel "pins

and needles" in your hand and fingers. This feeling goes away shortly after the cuff is inflated.

- There are no risks associated with these measurements and procedures.

POSSIBLE BENEFITS

Once we publish our findings, this study may help society better understand the role of one'sdiet in blood vessel function. If you wish to receive some of the results from tests that you participate in, you may find that information beneficial. You are welcome to have the results from your laboratory blood tests, exercise test, and body composition scan.

POSSIBLE RISKS/DISCOMFORTS

The principal investigator and laboratory team are experienced with all procedures outlined in this study in both healthy and diseased populations. Nevertheless, as with all studies involving human subjects, there are risks associated with experimentation that are currently unknown. All possible risks associated with this study have been stated above in the explanation followingthat procedure. To reduce these risks, you will only be able to join the study if you are healthy and you will have to complete a health history questionnaire as a precautionary measure to ensure the study poses no additional risks. Furthermore, potential risks will also be minimized by (a) using only safe, well-established procedures; (b) constant, personal monitoring of each experimental session by the investigators and staff; and (c) knowledge that you can request to stop at any point during the procedure. In addition, you should know that the principal investigator may also terminate the testing procedure at any time. All experimental procedureswill conform to the procedures for this study as approved by the University of Texas at Arlington Institutional Review Board (IRB). An IRB reviews and approves research for the protection of human subjects. Again, remember that you have the right to quit any study procedures at any time without penalty, and may do so by informing the research team.

COMPENSATION

You will be compensated with cash for time spent in the laboratory at a rate of $15.00 per hour,rounded up to the next 1/3 hour. We will either give you a form and instructions for obtaining compensation and the MAC building on campus or obtain the compensation for you. If you choose not to complete all study procedures, you will still receive compensation for your time in the lab.

The Internal Revenue Service (IRS) considers all payments made to research subjects to be taxable income. Your personal information, including your name,

address, and social security number, may be acquired from you and provided to UTA's accounting office for the purpose ofpayment. If your total payments for the year exceed $600.00, UTA will report this information to the IRS as income and you will receive a Form 1099 at the end of the year. If you receive less than $600.00 total for payments in a year, you are personally responsible for reporting thepayments to the IRS.

ALTERNATIVE OPTIONS

There are no alternative options offered for this study.

CONFIDENTIALITY

The research team is committed to protecting your rights and privacy as a research subject. Allpaper and electronic data collected from this study will be stored in a secure location on the UTA campus and/or a secure UTA server for at least three (3) years after the end of this research.

The results of this study may be published and/or presented without naming you as a participant. The data collected about you for this study may be used for future research studiesthat are not described in this consent form. If that occurs, an IRB would first evaluate the use ofany information that is identifiable to you, and confidentiality protection would be maintained.

While absolute confidentiality cannot be guaranteed, the research team will make every effortto protect the confidentiality of your records as described here and to the extent permitted bylaw. In addition to the research team, the following entities may have access to your records, but only on a need-to-know basis: the U.S. Department of Health and Human Services and theFDA (federal regulating agencies), the reviewing IRB, and sponsors of the study.

CONTACT FOR QUESTIONS

Questions about this research study or reports regarding an injury or other problem may bedirected to R. Matthew Brothers, Ph.D. (matthew.brothers@uta.edu).

Questions about the radiation or DEXA procedure may be direction to Laura Warren, RadiationSafety Officer (817-272-2185, lwarren@uta.edu).

Any questions you may have about your rights as a research subject or complaints about the research may be directed to the Office of Research Administration; Regulatory Services at 817-272-3723 or regulatoryservices@uta.edu.

CONSENT

By signing this form, you are confirming that you understand the study's purpose, procedures, potential risks, and your rights as a research subject. By agreeing to participate, you are not waiving any of your legal rights. You can refuse to participate or discontinue participation at any time, with no penalty or loss of benefits that you would ordinarily have. Please sign below if youare at least 18 years of age and voluntarily agree to participate in this study.

SIGNATURE OF VOLUNTEER DATE

*If you agree to participate, please provide the signed copy of this consent form to the research team. They will provide you with a copy to keep for your records.