Vascular Dysfunction in Black Individuals: Roles of Nitric Oxide and Endothelin-1

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SPECIFIC AIMS

Cardiovascular diseases (CVD) are the leading causes of mortality in the U.S., costing about $350 billion annually in healthcare[1]. However, while affecting a large portion of the U.S. population, the prevalence of CVD is disproportionately higher in Black (i.e., African American) men and women than in other racial groups[1,2]. Intensive research on the physiological basis for this elevated risk[3-5] has demonstrated a prominent role of deleterious alterations in vascular function starting early in life in Black individuals. Despite no symptoms of overt CVD, young Black individuals present less vasodilation[6,7], more vasoconstriction[7,8], and impaired vascular control of blood pressure[8,9] at rest and during exercise models[10-12] in comparison with White counterparts. These findings support the idea that **healthy young Black individuals must cope with subclinical impairments in vascular regulation that place continuous stress on the cardiovascular system and predispose to vascular remodeling and dysfunction**. Since vascular dysfunction is a predictor of CVD[15-18], research is needed to understand the mechanisms driving these racial differences and identify interventions that can improve vascular function, supporting future clinical practices to prevent CVD in the Black population.

Among multiple mechanisms, **nitric oxide (NO) and endothelin-1 (ET-1) appear as key targets for improving vascular regulation in Black individuals**. Treatments to increase NO bioavailability improve survival and health outcomes in Black patients with heart failure[19], demonstrating a critical role of NO signaling dysfunction on the CVD risk in this population. Indeed, studies indicate that **impaired vasodilation in Black individuals is largely due to low NO signaling**[20-25] compared with White counterparts. However, **interventions to increase NO signaling aimed at improving vasodilation in peripheral macro- and microvascular beds in Black individuals have not been tested**. Furthermore, NO mitigates the actions of ET-1, which is an acute vasoconstrictor and mediator of chronic vascular remodeling[26]. Accordingly, low NO signaling may lead to exaggerated ET-1 signaling in Black individuals. Indeed, ET-1 is associated with the pathophysiology of hypertension[27-29] and with impaired vascular control of blood pressure[30] in Black individuals, supporting a differential dysfunctional mechanism compared with White counterparts. Since ET-1 acutely restrains vasodilator responses[31-33], it is conceivable that **impaired vasodilation observed in Black individuals may be, in part, due to exaggerated ET-1 signaling** compared with White counterparts. **However, interventions to decrease ET-1 signaling and improve vasodilation in Black individuals have not been tested**.

We postulate that the peripheral micro- and microvasculature of Black individuals express constitutively lower NO signaling and higher ET-1 signaling than that of White individuals, which manifests as impaired vasodilation. Accordingly, acute interventions to increase NO signaling and decrease ET-1 signaling will greatly improve vasodilation in Black individuals but not in White individuals. Therefore, the research aims of this proposal are:

- **Specific Aim 1: To test whether an increase in NO signaling can increase vasodilator responses in young Black individuals**. We will use oral administrations of beetroot juice (Aim 1a), L-citrulline (Aim 1b), and Sildenafil (Aim 1c) to stimulate NO-mediated signaling. We hypothesize that each of these interventions will improve NO-mediated vasodilation in Black individuals but not in White individuals.
- **Specific Aim 2: To test whether a decrease in ET-1 signaling can increase vasodilator responses in young Black individuals**. We will use an acute oral administration of Bosentan to block ET_A and ET_B receptors. We hypothesize that this blockade of ET-1 signaling will reduce the vasoconstrictor tone and improve vasodilation in Black individuals but not in White individuals.

With this investigation, we strive to provide evidence on the roles of NO and ET-1 underlying the racial differences in vascular function and generate preliminary data on tailored novel interventions to improve vascular regulation in Black individuals, ultimately contributing to reduce mortality and improve cardiovascular health in this underrepresented group of the American population (Figure 1).

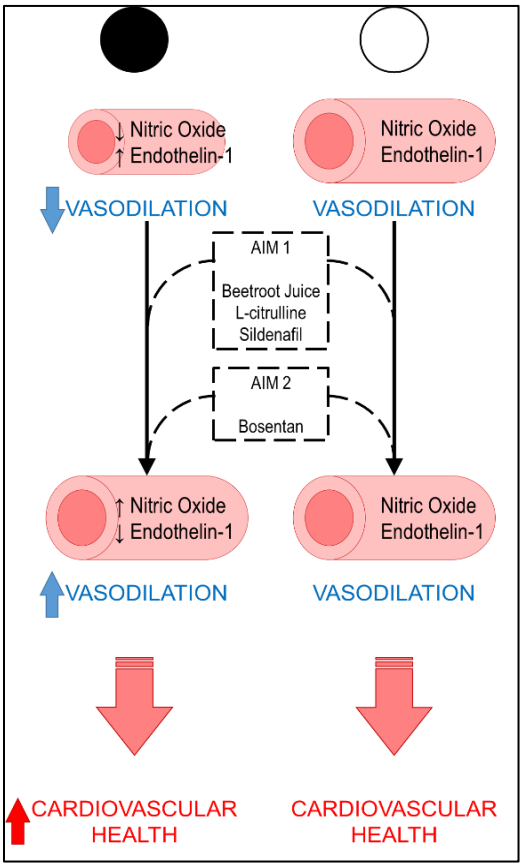

Figure 1. Proposed interventions and expected outcomes in Black and White individuals.

RESEARCH STRATEGY

Background

We and others have identified several subclinical deleterious alterations (e.g., impaired vascular control of blood pressure[8,9,34,35], reduced dilation[6,7,25], increased constriction[7,8], attenuated muscle contraction hyperemia[10-12], etc.), occurring at an early age before overt comorbidities in Black individuals in comparison with White counterparts. These findings indicate that the onset of CVD risk in Black individuals manifests early in life as subclinical impairments in vascular function. However, despite these well-documented alterations, our findings also suggest that some regulatory mechanisms (e.g., functional sympatholysis[11]) may be preserved in young Black individuals. Such unexpected results emphasize the need for identifying the exact physiological mechanisms underlying the racial differences in vascular function, aiming at developing tailored novel interventions to improve vascular regulation in Black individuals, a population at a higher risk of developing CVD than other racial groups in the U.S.[1,2].

NO is a signaling molecule with a well-known role in promoting vasodilation in several conditions[36,37]. Research has associated low NO signaling with impaired vasodilation in Black individuals compared with White counterparts[24,25], which is concerning because low NO-dependent vasodilation is a mark of CVD[38,39]. Some experimental models using local infusions of L-arginine[6,38] support the hypothesis that interventions to increase NO signaling can improve vascular responses in Black individuals in comparison with White counterparts. However, systemic interventions to test this hypothesis have not been performed.

ET-1 is a vasoconstrictor peptide produced by the endothelium that binds to ET_A and ET_B receptors on smooth muscle[14]. NO inhibits ET-1 actions[26] by lowering its expression[40], release[41], and binding to receptors[42]. It is possible that low NO signaling in Black individuals may lead to exaggerated ET-1 signaling, which is indeed supported by an association of ET-1 with the pathophysiology of hypertension[27-29] and with impaired vascular control of blood pressure[30] in Black individuals. This is concerning because, besides attenuating acute vasodilator responses[31-33], prolonged exposure to exaggerated ET-1 signaling can induce irreversible vascular remodeling and dysfunction[26,43]. Nevertheless, systemic interventions to lower ET-1 signaling and improve vasodilation in Black individuals have not been investigated.

Significance

- Our research focuses on Black individuals, an understudied American population despite being at a higher risk for developing CVD than other racial groups.
- Our study includes healthy young individuals to allow for better identification of CVD risk factors associated with race while avoiding comorbidities and functional deterioration due to aging. We will shed light on the role of early specific subclinical alterations in young Black individuals that can, later on, lead to life-threatening diseases in this population.
- Low NO signaling appears to be a major contributor to the impaired vasodilation observed in Black individuals. Thus, we will directly target different steps of the NO pathway (Figure 2) to elucidate molecular aspects that may be particularly dysfunctional in Black individuals.
- Studies suggest that poor vascular control of blood pressure in Black individuals may be associated with exaggerated ET-1 signaling, but no direct evidence linking ET-1 with impaired vasodilator responses has been shown. We will directly examine a potential differential role of ET-1 on acute vascular regulation between Black and White individuals (Figure 3).
- In addition to elucidating molecular mechanisms, our research will likely generate supporting evidence on systemic interventions for the improvement of vascular regulation in Black individuals.

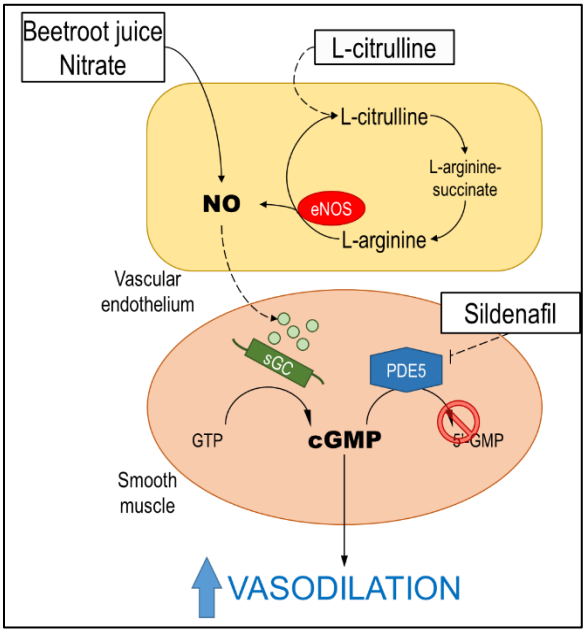

Figure 2. NO signaling[13] as a mechanism of racial differences in vascular function. By using oral administrations of beetroot juice (Aim 1a), L-citrulline (Aim 1b), and Sildenafil (Aim 1c), we expect to increase vasodilator responses in Black individuals, attenuating the impairment in comparison with White individuals.

Innovation

CONCEPTUAL:

- We will identify acute physiological benefits with oral drugs and supplements in young Black individuals, an understudied group with early impairments in vascular function.
- We will establish that such initial vascular alterations represent a novel research target for CVD prediction and early prevention in Black individuals and possibly in other at-risk populations.
- We will define exaggerated ET-1 signaling as a potential mechanism of the impaired vasodilator responses in young Black individuals.
- Our research model will show the feasibility of oral interventions and may set the stage for future clinical trials to evaluate novel treatments for this at-risk population.

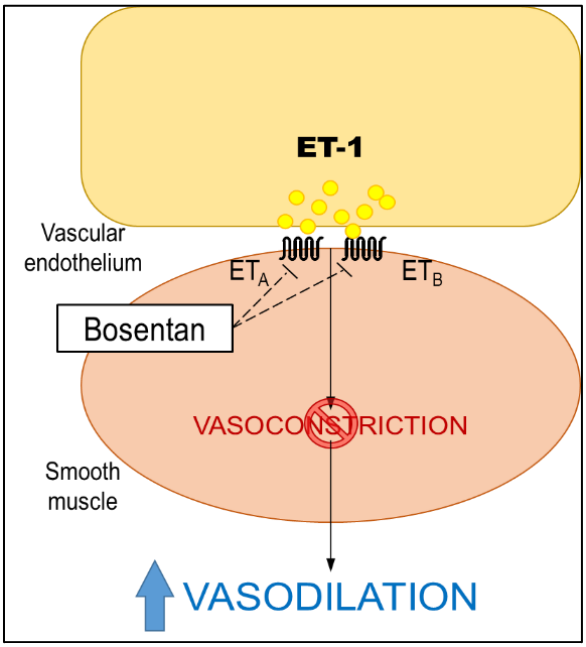

Figure 3. ET-1 signaling[14] as a mechanism of racial differences in vascular function. By using oral administration of Bosentan (Aim 2), we expect to reduce the vasoconstrictor tone and increase vasodilator responses in Black individuals, attenuating the impairment in comparison with White individuals.

TECHNICAL:

- We will use various techniques to stimulate and examine vasodilator responses, achieving several goals:
  - Previous research on racial differences has almost exclusively examined upper limb vasculature, although impairments in vascular function are typically more severe in the legs than in the arms[44-48]. In fact, lower limb vascular diseases enhance the risk for various CVD[49,50] and are more prevalent in the Black population than in other racial groups[1,51-53]. Our proposal will perform a comprehensive assessment of vascular function in the upper and lower limbs.
  - We will combine a variety of well-established procedures (flow-mediated dilation, passive leg movement, rhythmic handgrip exercise, knee extension exercise) with four interventions (oral administrations of beetroot juice, L-citrulline, Sildenafil, and Bosentan), and examination on two vascular beds (arm and leg). This approach will generate integrated information on the differential roles of NO and ET-1 on peripheral macro- and microvascular regulation between Black and White individuals.

Approach

PARTICIPANTS: Healthy young Black and White men and women of age between 18 and 40 years will be recruited from the University of Mississippi Medical Center (UMMC) and the city of Jackson community. Subjects will qualify for the study if they identify their race and the race of their biological parents as being only Black (i.e., African American) or only White (i.e., Caucasian American). Individuals whose parents are mixed Black and White, or of other races (Hispanic or Latino, Asian, Native American, etc.) will not qualify for the study. To avoid confounding factors associated with lifestyle, only individuals born and raised in the U.S. will be included. Exclusion criteria are any chronic or ongoing disease, prescribed pharmacological treatment, smoking or tobacco use, and obesity (BMI > 30 kg / m^2). Also, specific exclusion criteria will be considered before participation in the visits with the administration of Sildenafil or Bosentan. On a preliminary visit, subjects will sign the Informed Consent Form, answer questions about their medical history, be familiarized with the measurements and procedures, and perform a maximal knee extension exercise test.

MEASUREMENTS: Arterial diameter and blood velocity will be measured on the right leg or right arm via duplex Doppler ultrasound (Logiq P9, GE Medical Systems, Milwaukee, WI). A linear array transducer (12L-RS) will be placed on the popliteal artery immediately distal to the popliteal fossa, on the common femoral artery ~2 cm proximal to the bifurcation, or on the brachial artery ~5 cm proximal to the antecubital fossa. The position will be optimized for the visualization of arterial wall edges and sharpest blood velocity tracings. The skin will be marked and photographed to ensure probe placement in the same location throughout the study visits. The arteries will be imaged at a frequency of 10-13 MHz. Velocity will be measured at a frequency of 5 MHz, corrected to an insonation angle of 60°, with the sample volume encompassing the entire lumen without extending beyond the arterial wall edges. Ultrasound data will be recorded and saved on a computer system (Cardiovascular Suite,

Quipu, Pisa, Italy). Arterial blood flow will be calculated as [(diameter/2)^2 × velocity × π]. Beat-to-beat mean arterial pressure (MAP) will be measured using finger photoplethysmography (Human NIBP, ADInstruments, Colorado Springs CO) on the left hand. Conductance will be calculated as [blood flow / MAP]. Heart rate (HR) will be determined using a standard lead II electrocardiogram (Bioamp, ADInstruments).

Blood samples will be collected from a venous catheter in the antecubital area. For nitrate and nitrite concentrations, blood samples will be separated into Eppendorf tubes containing heparin and centrifuged. Plasma will be pipetted into separate Eppendorf tubes, flash-frozen in liquid nitrogen, and stored at -80°C. Analyses will be made via chemiluminescence with a NO analyzer (NOA 280i; Sievers Instruments, Boulder, CO) by addition to potassium iodide in acetic acid at room temperature, and to vanadium chloride in hydrochloric acid at 95°C[54]. For L-arginine and L-citrulline concentrations, aliquots of plasma will be spiked with stable isotope-labeled L-arginine and L-citrulline, which will serve as internal standards. Protein will be precipitated, filtrated, derivatized, and analyzed by liquid chromatography-tandem mass spectrometry[55]. For ET-1 concentrations, blood samples collected using EDTA as an anticoagulant will be centrifuged, aliquoted, and stored at -20°C for later assessment by quantitative enzyme immunoassay (R&D Systems, Minneapolis, Minnesota, USA)[56].

STUDY DESIGN: Studies will be conducted in the Clinical Research and Trials Unit at UMMC. Medical doctors and clinical researchers will provide oversight, technical assistance, and safety monitoring. All study visits will follow the same procedures (see Experimental Visits), except for the drug or supplement being administered.

For Aim 1, double-blind, randomized, placebo-controlled crossover designs will be used to examine the effects of increasing NO and cGMP bioavailability on vascular function of young Black and White individuals. Three sets of two visits will be performed as follows:

- Aim 1a: At the beginning of each visit, participants will ingest either a nitrate-rich beetroot juice or a nitrate-depleted beetroot juice (serving as placebo) in their liquid commercial form, which has a distinct color and flavor. The nitrates will be absorbed and reduced in the plasma to nitrite and NO, thereby increasing endothelium-independent NO bioavailability. The nitrate-rich beverage is commercially available as a sports supplement, while the nitrate-depleted beverage is manufactured-on-demand for research.
- Aim 1b: Participants will receive a 7-day supplement of L-citrulline or placebo before each study visit, with a washout period of 7 days between visits. The activity of the eNOS converts L-arginine into NO and L-citrulline, which is normally recycled back into L-arginine[57]. Unlike L-arginine, oral ingestion of L-citrulline is not catabolized in the gut, nor is it extracted from systemic circulation through hepatic first-pass metabolism. Thus, ingested L-citrulline becomes available in large quantities in the plasma for enzymatic conversion into L-arginine[55], thereby increasing endothelium-dependent NO bioavailability. The 7-day L-citrulline administration is effective to increase L-arginine plasma levels and NO signaling in a dose-dependent manner[55], while a single administration has failed to do so[58].
- Aim 1c: At the beginning of each visit, participants will ingest a liquid mixture prepared by Investigational Drug Services at UMMC containing Sildenafil or placebo. Sildenafil inhibits phosphodiesterase 5 (PDE5), an enzyme that degrades cGMP in the vascular smooth muscle cells inactivating the NO-mediated signal; thus, Sildenafil will prolong the availability of cGMP, enhancing the NO-mediated intracellular cascade.

For Aim 2, a double-blind, randomized, placebo-controlled crossover design will be used to examine the role of ET-1 on the vascular function of young Black and White individuals. Two visits will be performed, with a minimum of 7 days between them. At the beginning of each visit, participants will ingest a liquid mixture prepared by Investigational Drug Services at UMMC containing Bosentan or placebo. Bosentan blocks ET_A and ET_B receptors, leading to a reduction in vasoconstrictor tone and a greater magnitude of vasodilator responses.

EXPERIMENTAL VISITS: Figure 4 displays the design of the experimental visits. Participants will abstain from alcohol consumption, caffeine, and exercise for 24 h before all study visits, and will come after fasting overnight. Experiments will be conducted in a room with temperature tightly controlled at 20-21°C to minimize the influence of skin blood flow on the Doppler ultrasound measures. Women will participate in the study during the early follicular phase of the ovarian cycle to minimize variability in physiological responses across the menstrual cycle.

For Aim 1a, upon arriving at the laboratory, subjects will ingest 140 mL of a beetroot juice containing either a high (~12.8 mmol) or a low concentration (~0.0055 mmol) of nitrates (James White Drinks, Suffolk, UK)[59,60], in random order over two consecutive visits. Ninety minutes will be allowed after beverage ingestion to start

instrumentation to ensure that data collection occurs in the period of peak nitrate plasma concentration (i.e., 120 min)[60].

For Aim 1b, participants will receive pills containing 3 g of L-citrulline (Superior Labs, Park City, UT) or placebo, to be taken twice daily for 7 days each, before each study visit. The washout period will be 7 days. On the day of the study visit, subjects will ingest the last supplement or placebo pill upon arriving at the laboratory. Sixty minutes will be allowed before start instrumentation to ensure that data collection occurs in the period of peak L-arginine plasma concentration (i.e., 90 min)[55].

For Aim 1c, upon arriving at the laboratory, subjects will ingest a liquid mixture containing the PDE5 inhibitor Sildenafil (100 mg)[61], or placebo in random order. Thirty minutes will be allowed after drug ingestion to start instrumentation to ensure that data collection occurs in the period of peak plasma concentration of Sildenafil (i.e., 60 min)[62].

For Aim 2, upon arriving at the laboratory, subjects will ingest a liquid mixture containing the non-selective ET_A and ET_B receptors antagonist Bosentan (125 mg)[33], or placebo, in random order over two consecutive visits. Ninety minutes will be allowed after drug ingestion to start instrumentation to ensure that data collection occurs in the period of peak plasma concentration of the drug (i.e., 120 min)[63].

EXPERIMENTAL PROCEDURES: Subjects will receive instrumentation for HR, MAP, and blood sampling in the supine position with the right leg and arm supported for assessment of the popliteal and brachial artery. A rapid-inflation cuff (Hokanson, Bellevue, WA) will be placed ~5 cm distal to the right fibular head or ~3 distal to the right antecubital fossa. Subjects will rest for a minimum of 20 min, and blood samples will be collected. Then, **flow-mediated dilation (macrovascular function) and reactive hyperemia (microvascular function)** will be measured separately in the arm and leg as described previously[64,65]. Briefly, diameter and blood velocity will be recorded for 2 min, following which the cuff will be inflated (220 mmHg) for 5 min. At the last 30 s of cuff inflation, recordings will be resumed and continue for 3 min after cuff deflation. The macrovascular function will be measured as the percent change in arterial diameter after cuff deflation normalized to arterial shear rate. The microvascular function will be measured as the blood velocity area-under-the-curve from cuff deflation to return to resting values using the sum of trapezoids.

After that, a trial of **passive leg movement** will be performed as described previously[66,67]. Briefly, a researcher will move the subject's right lower leg (knee extension) through the range of motion of 90° to 180° and back to 90°, at a rate of 60 / min for 2 min.

Following that, subjects will perform a trial of **rhythmic handgrip exercise**[10] (Biometrics, Ladysmith, VA), consisting of 3 min of hand contractions at 30% maximal voluntary contraction with a duty cycle of 1 s contraction / 2 s relaxation. Measures will be recorded continuously for 2 min at rest and throughout the trial.

Subjects will then move to a seated position on a custom ergometer (Technavance, Austin, TX) with the arms supported by side tables, and receive instrumentation for HR, and MAP. The right foot will be attached to a boot connected to the ergometer[68]. Subjects will perform three trials of **knee extension exercise**, consisting of 3 min of voluntary knee extension at a rate of 50 / min at 15%, 30%, and 45% of their maximal workload. Measures will be recorded continuously for 2 min at rest and throughout each trial. All trials will be separated by 15 min intervals. After completing all trials, another set of blood samples will be collected.

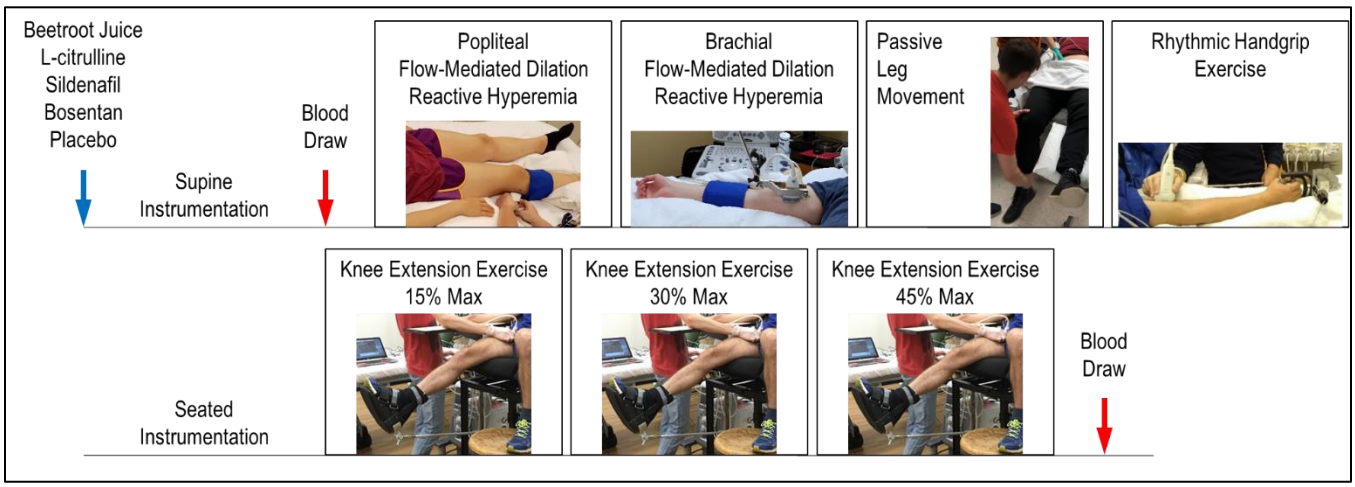

Figure 4. Protocol of the experimental visits. Visits will be identical except for the oral drug or supplement administration before any procedure.

STATISTICAL ANALYSES: Sample size estimation (statulator.com) is 11 individuals per group, based on the femoral artery blood flow response to knee extension exercise from our preliminary data (see below), using Student's *t*-test for independent samples, and considering a two-tailed alpha of 0.05, and power of 0.8 as parameters. This estimated sample size is adequate relative to our previous investigations[10,11]. Due to potential influence of sex and race on vascular function[69], we will duplicate the estimated sample size to recruit an equal number of men and women. To account for incompletion and exclusion of data during analyses, additional participants will be recruited, leading to an estimate of 30 individuals enrolled in each racial group. We will use a mixed-model ANOVA to compare vascular responses according to the effects of drug administration (Placebo vs. Drug), group (Black vs. White), and interaction. Subjects' characteristics will be compared using Student's t-test for independent samples and, in case of significant differences, such characteristics will be used as covariates in the ANOVA. Additionally, while men and women will be primarily grouped according to race, the large and balanced sample size will allow for sub-analyses comparing the effects of race on vascular responses separately in men and women.

PRELIMINARY STUDY: We recently compared common femoral artery blood flow (FABF, Doppler ultrasound) between White (n = 4) and Black (n = 8) healthy young men during knee extension exercise at 10 W, 20 W, and 30 W. Each exercise trial lasted for 3 min with a minimum rest interval of 5 min. Knee extension cadence was 50 repetitions per min dictated by a metronome and by real-time visual feedback. The participants performed an incremental knee extension exercise test to determine their maximal workload on a separate visit. The groups were of similar age (White men: 21 ± 2 years vs. Black men: 21 ± 1 years; P = 0.905), body mass index (White men: 21.3 ± 2.1 kg/m^2 vs. Black men: 23.2 ± 3.4 kg/m^2; P = 0.319), and achieved similar peak workloads in the maximal exercise test (Whites: 48 ± 15 W vs. Blacks: 52 ± 9 W; P = 0.542). FABF response to exercise was lower in Black men compared with White men across all workloads (Group P = 0.038; Figure 5). In this preliminary study, we have been able to demonstrate the necessary skills to establish the proposed comprehensive assessment of racial differences in vascular function and generated data to support our hypothesis of reduced leg blood flow response to knee extension exercise in Black men compared with White men.

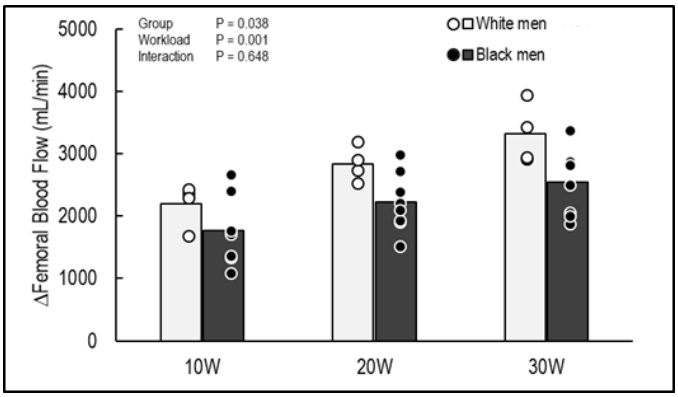

Figure 5. Individual and mean group changes in femoral artery blood flow from rest to knee extension exercise at 10W, 20W, and 30W in White men (light grey bars, white circles) and Black men (dark grey bars, black circles).

EXPECTED RESULTS AND FUTURE PLANS TO SECURE EXTRAMURAL FUNDING

Black individuals manifest impaired vascular responses compared with White individuals. We postulate that this is due, at least in part, to a low NO dilator signaling and a high ET-1 constrictor signaling. Accordingly, we anticipate that:

- Increases in conductance and arterial diameter in response to the experimental procedures (flow-mediated dilation, passive leg movement, rhythmic handgrip, and knee extension) will be lower in Black than in White individuals in the placebo visits.
- Increase in NO bioavailability with beetroot juice and L-citrulline, and the increase in cGMP with Sildenafil will augment the dilator signal and increase vascular responses in Black individuals, attenuating the impairment in comparison with White individuals.
- Reduction in the ET-1 constrictor signal with Bosentan will increase vascular responses in Black individuals, attenuating the impairment in comparison with White individuals.

Some alternative approaches to be attempted in future studies include:

- Direct intra-arterial infusions of vasoactive compounds (e.g., L-arginine, L-NMMA, selective blockers of ET_A and ET_B receptors, etc.) to evoke quick local responses with lower concentrations, preventing systemic cardiovascular effects[70], and allowing for deeper mechanistic insight.
- Chronic oral administration of beetroot juice, L-citrulline, Sildenafil, and Bosentan with multiple follow-up assessments, to evaluate the persistence of improvements on vascular responses, whether maladaptive mechanisms become expressed and prevent vascular improvements, effects on blood pressure control, etc.
- Harvesting of endothelial cells via catheterization to establish correlations between vascular responses and cellular ET-1 levels (higher specificity than plasma measurement), expression of ET_B receptors (known to evoke NO production in the endothelium), eNOS, etc.
- Use of the stored blood samples collected in this study to examine molecular markers of other vasoactive mechanisms (e.g., reactive oxygen species, ADMA, ATP, angiotensin II, catecholamines, etc.) to establish correlations with the vascular responses, as well as for the design of novel hypotheses and future studies.
- The large and balanced sample size of men and women will allow for secondary analyses that may corroborate, as previously suggested[69], a differential effect of race on vascular function between men and women.

External funding opportunities will be pursued (see below) to support the completion of the current specific aims, as well as new studies in the field as new questions arise:

- NIH Research Project Grant (R01): Parent Funding Opportunity Announcement (PA-19-091), submitted to National Institute on Minority Health and Health Disparities (NIMHD).
- NIH Notice of Special Interest (NOT-HL-19-711): To support epidemiological research to assess cardiovascular health during childhood and its relationship to adult disease in underrepresented populations. Submitted through the R01 mechanism (PA-19-056) to National Heart, Lung, and Blood Institute (NHLBI).
- AHA Career Development Award: To support highly promising academic professionals in the first professional appointment to explore innovative questions or pilot studies that will provide preliminary data and training.
- AHA-VIVA Physicians Research Award: A research partnership program to offer training and mentorship to potential future leaders in the domain of vascular research. Submitted through the AHA Career Development Award mechanism.

I am an author of 5 articles[8-12] about vascular physiology in young Black individuals. The current proposal will advance our previous findings by examining the differential roles of two molecular mechanisms on the macro- and microvasculature of Black individuals. Furthermore, by examining arms and legs with several techniques, we will establish that such subclinical alterations affect multiple vascular beds in Black individuals and, consequently, impose early and continuous stress on the cardiovascular system and a health risk over time. The administration of commercially-available drugs and supplements via the oral route will support and facilitate the design of future clinical trials aiming at evaluating long-term responses. By providing a deeper and broader knowledge of vascular function in young Black individuals, elucidating CVD risk factors occurring early in life, and offering an insight into potentially available treatments, this proposal will build a foundation for the design of greater clinical studies with longer observations.

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DHHS FWA # 00003630
IORG #0000043
IRB 1 Registration #00000061
IRB 2 Registration #00005033

Approval Notice
Initial Application

09/01/2020

Thales Barbosa, MD
SOM – Physiology and Biophysics
University of Mississippi Medical Center
2500 North State Street
Jackson, MS 392164505

RE: IRB File #2020V0181
Racial Differences in Cardiovascular Function: The Role of Endothelin-1

Your Initial Application was reviewed and approved by the Expedited Review (revisions required) process on 8/28/2020 after the initial review by the Convened Board on 8/11/2020. You may begin this research.

Please note the following information about your approved research protocol:

- Protocol Approval period: 09/01/2020 – 08/31/2021
- Approved Enrollment #: 50
- Performance Sites: UMMC, Clinical Research and Trials Unit
- Funding: NIH

Documents/ Materials:

Type	Description	Version #	Date
Protocol	TCB_Racial Differences ET-1_Study Protocol	1	09/01/2020

Consent	TCB_Racial Differences ET-1_Informed Consent Form	1	09/01/2020
Recruitment Flyer	TCB_Racial Differences ET-1_Recruitment Flyer	1	09/01/2020
Data Collection Sheet	Medical History Form	1	09/01/2020

Please remember to:

- Use the IRB file number (2020V0181) on all documents or correspondence with the IRB concerning your research protocol.
- Review and comply with all requirements on the enclosure, UMMC Investigator Responsibilities, Protection of Human Research Participants.

The IRB has the prerogative and authority to ask additional questions, request further information, require additional revisions, and monitor the conduct of your research and the consent process.

Please note, if this study involves an intervention (whether or not it involves a drug or device) you (or the "responsible party") must register the study before enrollment begins and report results within 12 months of study closure through Clinicaltrials.gov http://www.clinicaltrials.gov/. Penalties for responsible parties who fail to register applicable clinical studies are significant and include civil monetary penalties and, for federally-funded studies, withholding or recovery of grant funds. For additional information please go to http://irb.umc.edu/GuidanceInfo/ ClinTrialRegistry.htm.

We wish you the best as you conduct your research. If you have questions or need additional information, please contact the Human Research Office at (601) 984-2815.

IRB 1

Enclosure(s): (1) Investigator Responsibilities, Protection of Human Research Participants

UMMC Investigator Responsibilities
Protection of Human Research Participants

The IRB reviews research to ensure that the federal regulations for protecting human research participants outlined in UMMC policy, the Department of Health and Human Services (DHHS) regulations (45 CFR 46) and the Food and Drug Administration (FDA) regulations (21 CFR Parts 50 & 56), as well as other requirements, are met. The University of Mississippi Medical Center's Federalwide Assurance (FWA), FWA# 00003630, awarded by the Office for Human Research Protections (OHRP) at DHHS, is a written pledge to follow federal guidelines for protecting human research participants in accordance with the principles of the Belmont Report. **All investigators must read both the Belmont Report and the UMMC FWA to understand their responsibilities in conducting research involving human participants**. Both documents are available on the Human Research Office webpage, http://irb.umc.edu/, and in hard copy by request from the Human Research Office. Some of the responsibilities investigators have when conducting research involving human participants are listed below.

1. Conducting the Research: You are responsible for making sure that the research is conducted according to the IRB approved research protocol. **You are also responsible for the actions of the study's co-investigators and research staff**.

2. Participant Enrollment: You may not recruit or enroll participants prior to the IRB approval date or after the expiration date of IRB approval. All recruitment materials for any form of distribution or media use must be approved by the IRB prior to their use. If you need to recruit more participants than was noted in your IRB approval letter, you must submit an amendment requesting an increase in the number of participants.

3. Informed Consent: Informed consent is a process that begins with the initial contact and ends at some point after the study is complete. You are responsible for the conduct of the consent process, ensuring that effective informed consent is obtained and documented using **only** the IRB-approved and stamped consent document(s), and for ensuring that no human participants are involved in research prior to obtaining their informed consent. Whoever is presenting the consent document to the potential participant and conducting the consent discussion must have all pertinent information at hand, be knowledgeable about the study and the disease or condition involved, if any, and have the ability and experience to answer questions regarding the study and any treatment involved. Please give all participants a signed copy of each consent or assent document they sign, and keep the originals in your secured research files for at least six (6) years. When appropriate, you should place a copy of the consent document in the participant's medical record.

4. Continuing Review: The IRB must review and approve all IRB-approved research protocols at intervals appropriate to the degree of risk, but not less than once per year. **There is no grace period.** Prior to the date on which IRB approval of the research expires, the IRB will send you three reminders to submit a Continuing Review, 90, 60 and 30 days prior to expiration. Although reminders are sent, **it is ultimately your responsibility to submit the renewal in a timely fashion to ensure that a lapse in IRB approval does not occur.** If IRB approval of your research lapses, you must stop new participant enrollment, and contact the IRB immediately.

5. Amendments and Revisions: If you wish to amend or change any aspect of your research, including research design, interventions or procedures, number of participants, participant population, consent document, instruments, surveys or recruitment and retention material, you must submit the amendment or revisions to the IRB for review with a Request for Change. You **may not initiate** any amendments or changes to your research without first obtaining IRB review and written approval. The **only exception** is when the change is necessary to eliminate apparent immediate hazard to participants. In that case the IRB should be immediately informed of this necessity, but the change may be implemented before obtaining IRB approval.

6. Unanticipated Events: All adverse events that are unanticipated **(unanticipated means that the event is unexpected, possibly related and places participants at greater risk of harm than previously recognized)** and serious protocol deviations, must be reported to the IRB **within fourteen (14) days** of discovery of the incident. Any research related injury occurring at a UMMC performance site or to a UMMC study participant and participant complaints must also be reported, along with any instances of serious or continuing problems, or non-compliance with the IRB's requirements for protecting human research participants. The only exception to this policy is death - **the death of a UMMC research participant must be reported within 48 hours.** All reportable events should be submitted to the IRB with the Adverse Event/Unanticipated Problem Report form.

   Events that do not meet the definition of an unanticipated problem involving risk to participants or others, including research related injury occurring at a UMMC performance site or to a UMMC study participant, participant complaints, problems, minor protocol deviations and non-compliance with the IRB's requirements for protecting human research participants should be reported as follows: Minor deviations and problems should be submitted at the time of continuing review, as instructed on the form. All other events should be reported in writing via letter or email to the IRB with sufficient detail to allow the reviewer to understand the problem and any actions taken to prevent it from happening again.

7. Research Record Keeping: At a minimum, you must keep the following research related records in a secure location for at least six years: the IRB approved research protocol and all amendments; all versions of the investigator's brochure; all informed

consent documents; all recruiting materials; all renewal applications; all adverse or unanticipated event reports; all correspondence to and from the IRB; and all raw data.

8. Reports to FDA and Sponsor: When you submit the required annual report to the FDA or you submit required reports to your sponsor, you **must** provide a copy of that report to the IRB. You may submit the report with your IRB continuing review application.

9. Provision of Emergency Medical Care: When a physician provides emergency medical care to a participant without prior IRB review and approval, to the extent permitted by law, such activities will not be recognized as research and the data cannot be used in support of the research.

10. Final Reports: When you have completed the study, (no further participant enrollment, interactions, interventions or data analysis) or stopped work on it, you must submit a Final Report to the IRB using the Final Report form.

11. On-Site Evaluations, FDA Inspections, or Audits: If you are notified that your research will be reviewed or audited by the FDA, OHRP, the sponsor, any other external agency, or any internal group, you **must** inform the IRB immediately and submit all audit reports received as a result of the audit to the IRB.

If you have questions or need assistance, please contact the Human Research Office at 601 984-2815.

RESEARCH PROTOCOL

Title	Racial Differences in Cardiovascular Function: The Role of Endothelin-1
Principal Investigator	Thales C. Barbosa, Ph.D.
Co-Investigators	Licy L. Yanes Cardozo, M.D. Joshua S. Speed, Ph.D. Vishnu V. Garla, M.D. John E. Hall, Ph.D.
Abstract	The current study will examine the role of Endothelin-1 (a potent endogenous vasoconstrictor; ET-1) on brachial and popliteal artery flow-mediated dilation, reactive hyperemia, and blood flow responses to passive leg movement, rhythmic handgrip exercise, and rhythmic knee extension exercise in Black and White individuals. In two separate visits, we will administer an acute oral dose of Bosentan (antagonist of ET-1 receptors ET_A and ET_B) or Placebo (randomized double-blind cross-over design), followed by examination of brachial artery flow-mediated dilation and reactive hyperemia at rest, as well as blood flow responses to passive leg movement, low, moderate, and high-intensity rhythmic handgrip exercise and rhythmic knee extension exercise in Black and White individuals.
Background	Black (i.e. African American) individuals develop cardiovascular diseases earlier in life and at greater rates than other racial groups[1-3]. Because of this greater risk, studies assessing racial differences in cardiovascular physiology are needed. It has been demonstrated that, compared with White individuals (i.e. Caucasian American, the largest racial group in the U.S. population), healthy young Black individuals have impaired vascular responses to various stressors, characterized by augmented vasoconstriction and reduced vasodilation[4-8]. However, much remains unknown regarding the mechanisms by which these individuals demonstrate vascular dysfunction. Techniques to assess acute vascular function consist of applying stimuli to evoke dilation in the macro- and microvasculature. For example, the prolonged inflation and subsequent deflation of a pneumatic cuff on the forearm or calf evoke brachial or popliteal artery flow-mediated dilation and forearm reactive hyperemia, which have been associated with cardiovascular health[9]. Furthermore, the vasodilator responses to passive leg movement, rhythmic handgrip exercise, and rhythmic knee extension exercise are also considered sensitive for the detection of vascular dysfunction[10-12]. Multiple mechanisms interact and determine the magnitude of these dilator responses, with vasoconstrictor factors (ET-1, adrenergic receptors, angiotensin II, etc.) causing attenuation, and vasodilator factors (NO, intravascular ATP, adenosine, K^+ channels, EDHF, etc.) causing an increase in vascular responses[13].

Purpose	To begin to elucidate the alterations in vascular function in Black individuals, we chose to target ET-1, a peptide produced by the vascular endothelium that mediates vasoconstriction by binding ET_A and ET_B receptors on smooth muscle[14,15]. ET-1 seems to play a prominent role in the pathophysiology of hypertension specifically in Black patients[16,17]. Also, it is suggested that ET-1 may be associated with impaired vascular control of blood pressure in young normotensive Black men[18]. Vascular responsiveness to ET-1 is also upregulated in adults exposed to insults during their fetal life (e.g., poor maternal nutrition, chronic stress, no prenatal care), psychological stressors during childhood (e.g., physical and emotional abuse, household dysfunction), and high ethnic discrimination[19-24]—all of which are more prevalent among Black than White individuals, possibly contributing to racial disparities in vascular function[25-28]. We hypothesize that Black individuals have reduced vasodilator responses as a consequence of a greater vasoconstrictor tone mediated by ET-1. Therefore, we hypothesize that the antagonism of ET_A and ET_B receptors will reduce the vasoconstrictor tone and augment the vasodilator responses in Black but not in White individuals.
Specific Aim	We will examine the role of ET-1 on brachial and popliteal artery flow-mediated dilation, reactive hyperemia, and blood flow responses to passive leg movement, rhythmic handgrip exercise, and rhythmic knee extension exercise between Black and White individuals. Using an acute oral administration of Bosentan, we will antagonize ET_A and ET_B receptors to reduce the vasoconstrictor tone mediated by ET-1 and, consequently, allow for greater flow-mediated dilation, reactive hyperemia, and blood flow responses to passive leg movement, handgrip, and knee extension. We anticipate that this effect of Bosentan will be more prominent in Black individuals than in White individuals. In a separate visit, the participants will receive a Placebo to serve as a control. We will also examine prenatal and at-birth medical records, and apply questionnaires of adverse childhood experiences and perceived discrimination to assess whether these early-life risk factors are associated with racial disparities in vasodilation between Black and White individuals.
Study Period	3 years Anticipated start date: 09/01/2020 Anticipated end date: 09/01/2023
Data Generation Period	N/A

Study Design	The study will consist of three visits to the research clinic (Clinical Research and Trials Unit at the UMMC campus) over 4 months. If a participant misses an appointment, rescheduling will be attempted twice. Women will provide a urine sample for a pregnancy test and answer questions about the most recent menstrual period before any research measurement or procedure be performed in any visit. Visit 1: Informed Consent Form, written consent for access to their prenatal and at-birth medical records, assessment of medical history, application of the questionnaires "Adverse Childhood Experiences (ACE)" and "Scale of Ethnic Experiences (SEE)", collection of blood samples and endothelial cells from the antecubital vein, familiarization with measurements and procedures that will take place in the next visits, and a rhythmic knee extension maximal exercise test. Visit 1 will last for about 2 hours. Visit 2: Qualifying participants will participate in Visit 2 within 4 weeks after Visit 1. At the beginning of the visit, we will randomly administer either a single oral dose of Bosentan (125 mg) or Placebo. Two hours after administration, we will perform the research measurements and procedures as explained below. This visit will last for about 4 hours. Visit 3: Those who complete Visit 2 will participate in Visit 3, which will take place 1 to 12 weeks later. All research measurements and procedures will be identical, except for the drug administration. At the beginning of the visit, we will administer either Bosentan (125 mg) or Placebo, whichever was not administered in Visit 2. Summary of the Research Measurements: • Anthropometrics: Height, weight, waist, and hip for characterization. • Electrocardiogram: Adhesive patches placed on the chest and abdomen for heart rate. • Respiration: An elastic belt placed around the abdomen for respiratory movements. • Blood pressure: Brachial blood pressure will be measured throughout the study with an automated blood pressure monitor for validation of the finger blood pressure values. • Finger blood pressure: A small cuff placed around one of the fingers for continuous recording of beat-to-beat blood pressure. • Arm blood flow: Duplex Doppler ultrasound will be used for measurements of brachial artery diameter and blood velocity, used for calculation of blood flow. • Leg blood flow: Duplex Doppler ultrasound will be used for measurements of popliteal and femoral artery diameter and blood velocity, used for calculation of blood flow.

	• ET-1 expression: Plasma and endothelial lysates will be collected from the antecubital vein using a catheter and J-wires. ET-1 peptide will be measured using an ELISA kit. RNA from endothelial cells will be isolated and reverse transcribed. The resulting cDNA will be used to quantify ET-1 mRNA copy count by Droplet Digital polymerase chain reaction using TaqMan primer gene expression assays with human preproET-1 primers. • Insults during fetal life: Prenatal and at-birth medical records of each participant will be obtained from the UMMC and local hospitals database in paper copies and digital formats (*e.g.*, EPIC, DataArk). We will assess maternal comorbidities, such as over- or undernutrition, pre-eclampsia, diabetes, obesity, smoking, and alcohol consumption. Gestational age at birth will be assessed and classified as preterm (less than 37 weeks), early-term (37 weeks to 38 weeks 6 days), full-term (39 weeks to 40 weeks 6 days), late-term (41 weeks to 41 weeks 6 days), or post-term (over 42 weeks)[29]. Also, the birth weight will be assessed, and birth weight of fewer than 2500 grams will be a marker of impaired fetal growth[26]. • Adverse childhood experiences: The questionnaire ACE will measure the prevalence of 10 types of trauma over the first 18 years of life: emotional abuse, physical abuse, sexual abuse, emotional neglect, physical neglect, household substance abuse, domestic violence against mother (stepmother), household incarceration, household mental illness, and parental divorce[27]. • Perceived discrimination: The questionnaire SEE will measure the magnitude of perceived discrimination, ethnic identity, mainstream comfort, and social affiliation[28]. Summary of Research Procedures • Bosentan or Placebo: A single oral dose of Bosentan (125 mg; Tracleer, Actelion Pharmaceuticals US, San Francisco, CA) or Placebo will be administered in separate visits in a randomized, double-blind, crossover design. Bosentan is an FDA-approved medicine used for the treatment of cardiovascular diseases such as pulmonary hypertension. Pharmacists of the Investigational Drug Services at UMMC will prepare Bosentan and Placebo in liquid suspensions. • Arm blood vessel response: Participants will lie down on a bed with the arm abducted and elbow extended on a bedside table. A cuff will be placed around the forearm. After baseline arm blood flow measurements, the cuff will be rapidly inflated (< 1 s) and remain inflated for 5 min. Measurements will resume at the last 30 s of cuff inflation and continue for 3 min after rapid cuff deflation. • Leg blood vessel response: Participants will lie down on a bed with the legs extended and slightly rotated medially. A cuff will be placed around

	the calf. After baseline popliteal blood flow measurements, the cuff will be rapidly inflated (< 1 s) and remain inflated for 5 min. Measurements will resume at the last 30 s of cuff inflation and continue for 3 min after rapid cuff deflation. • Passive leg movement: Participants will lie down with their upper body and thighs on a bed, and the lower legs off the bed. One lower leg will be extended on a bedside table, and the other lower leg will hang downwards with the knee flexed at a 90° angle. After baseline femoral blood flow measurements, a researcher will move the participant's right lower leg (knee extension) through the range of motion of 90°–180°–90° for a maximum of 2 min, with continuous measurements of electrocardiogram, respiration, finger blood pressure, and leg blood flow. • Handgrip exercise: Participants will lie down on a bed with the arm abducted and elbow extended on a bedside table. Participants will perform rhythmic handgrip exercise by squeezing a handgrip dynamometer. Each handgrip exercise trial will last a maximum of 6 min or until fatigue. Participants will perform three to six handgrip exercise trials with continuous measurements of electrocardiogram, respiration, finger blood pressure, and arm blood flow. • Knee extension exercise: Participants will sit on a custom ergometer with the arms supported by side tables and one foot on a boot connected to the ergometer. Participants will perform rhythmic knee extension exercise by extending their lower leg through the range of motion of 90°–180°–90°. Each knee extension exercise trial will last a maximum of 6 min or until fatigue. Participants will perform three to six knee extension exercise trials with continuous measurements of electrocardiogram, respiration, finger blood pressure, and femoral blood flow. • Ischemic preconditioning: Participants will lie down on a bed with the arms and legs extended. A cuff will be placed around the upper arms or thighs. The cuffs will be rapidly inflated (< 1 s) and remain inflated for 5 min, then deflated for 5 min. This cycle will be repeated up to 4 times. • Ischemia–reperfusion: Participants will lie down on a bed with the arms and legs extended. A cuff will be placed around the upper arms or thighs. The cuffs will be rapidly inflated (< 1 s) and remain inflated for a maximum of 20 min continuously.
Inclusion Criteria	• Black (i.e. African American) or White (i.e. Caucasian American) individuals • 18 to 35 years of age • Born and raised in the United States • Biological parents of the same race (i.e. either Black or White)

Exclusion Criteria	• Known chronic or ongoing disease, or on prescribed pharmacological treatment • History of liver disease • Pregnant, trying to become pregnant, or breastfeeding women • Use of hormonal contraceptives • Smoking or vaping in the last 6 months • Body mass index > 35 kg/m^2
Number of Participants (anticipated)	50 participants (i.e. 25 on each racial group). Based on data from Schreuder et al.[30], taking the improvement of blood flow response to rhythmic handgrip with Bosentan as the primary outcome, the estimated sample size is 42 participants (i.e. 21 per group). We anticipate having to recruit about 20% more individuals to account for possible loss of data (e.g. participants who cannot complete all visits, problems during analyses, etc.), therefore needing to enroll 50 participants.
Screening and Consent Process	Announcements will be made via UMMC e-mail listserv, UMMC intranet, Research Participant Recruitment app, flyers on campus, and word of mouth. Potential participants will contact the study personnel via e-mail or phone, and answer screening questions. If they meet the inclusion criteria, Visit 1 will be scheduled. Consenting will be conducted in Visit 1 at the research clinic by Dr. Barbosa or by a study person after supervision and adequate training with Dr. Barbosa. Following consenting, there will be assessments of medical history, and familiarization with the research measurements and procedures. After that, those who qualify for the study will be scheduled for Visit 2 and, subsequently, Visit 3.

Risks and benefits	Risks: • Electrocardiogram: Minor local skin irritation momentarily. • Blood pressure: Minor temporary discomfort, numbness, and/or tingling at the fingertips during cuff inflation, which will subside after cuff release. Bruising at the area where the cuff is inflated, which will disappear within a few days. • Finger blood pressure: Minor numbness, tingling, and bluish coloration at the fingertip. The finger cuff will be turned off between the research procedures, and these symptoms will subside. • Blood vessel response, ischemic preconditioning, and ischemia–reperfusion: Minor temporary discomfort, numbness, and/or tingling at the fingertips and toes during cuff inflation, which subside after cuff release. Bruising at the area where the cuff is inflated, which will disappear within a few days. • Handgrip and knee extension exercise: Minor fatigue and exhaustion momentarily. • Bosentan: We will administer a single dose of 125 mg of Bosentan orally. These dose and route are effective to improve blood flow response to exercise[30]. Furthermore, Bosentan is well tolerated up to 2400 mg[31]. Therefore, we do not expect any adverse events caused by participation in the study. Minor effects that may be expected are headache and swelling. Nevertheless, we will consider the following[32]: Chronic use of Bosentan has been associated with alterations in liver function. Additionally, studies in animals indicate that Bosentan may cause harm to an unborn fetus. Furthermore, there is a possibility of failure in contraception when Bosentan and hormonal contraceptives are co-administered. Therefore, out of an abundance of caution, we will exclude from participation in the study individuals with a history of liver disease, women who are pregnant, trying to become pregnant or breastfeeding, and women using hormonal contraceptives. Also, male participants will be informed about a possible risk of a decrease in sperm count due to using Bosentan. • Blood and endothelial cell sampling: Intravenous collection of blood and endothelial cells may include minor risks for local infection, local bruising, or occasional light-headedness. These risks are minimized by being performed under sterile conditions only by research personnel with the required training and experience with this technique. Blood drawn will not exceed 150 milliliters on any experimental testing day. To run the assays all at one time and avoid variability, samples will be stored in a –80° C freezer until analysis. After analysis, samples will be discarded into a container labeled with a biohazard sign and stored in secondary waste containers. All stored samples will be coded to protect confidentiality. The code-key will be kept in the Principal Investigator's

	office in a locked cabinet. Only the Principal Investigator and study personnel will have access to the files via key. No other students or faculty will have access. • ACE and SEE questionnaires: Given the sensitive nature of the topics assessed with these psychometric tests, participants will be informed that they may experience some distress or discomfort, may decline to answer any question, and may request to interrupt or end the assessment at any time. Our research personnel will observe the participants and may also interrupt or end the assessment if it seems necessary. In the event of an emergent psychiatric crisis, Dr. Barbosa and the research personnel will take appropriate steps including calling an emergency contact person indicated by the participant in the Medical History Form, and facilitating transportation to emergency health care. Furthermore, all participants will receive referral information and telephone numbers for local and national emotional support services, such as the Student Counseling and Wellness Center, the Center for Integrative Health, the Behavioral Health Specialty Clinic, Crisis and Suicide Prevention lines. Benefits: • There will be no direct benefit from participating in this research study. We hope to learn information that may help others in the future.
Randomization and blinding	We will use a Randomization Model tool in the REDCap system to randomly select between Bosentan and Placebo to be administered on Visit 2 for each individual, aiming at achieving a balanced distribution between Black and White individuals, and between men and women. Once the random drug selection is made for Visit 2, selection for Visit 3 will automatically be the alternative drug. Therefore, participants who complete Visits 2 and 3 will have received both Bosentan and Placebo. The participants and study personnel will be blind to the administration of Bosentan or Placebo on any visit. Pharmacists of the Investigational Drug Services at UMMC will retain the information about the drug selection per visit and will reveal it to the study personnel after completing data collection and analyses for that study participant or in case of emergency health care.

Outcome Measures	The primary measurements obtained by duplex Doppler ultrasound are: 1) brachial artery flow-mediated dilation (i.e., the increase in arterial diameter in response to the increased shear stress following forearm cuff release); 2) forearm reactive hyperemia (i.e., the increase in blood velocity following forearm cuff release); 3) blood flow responses to passive leg movement; 4) blood flow responses to low, moderate, and high-intensity rhythmic handgrip exercise; 5) blood flow responses to low, moderate, and high-intensity rhythmic knee extension exercise. Other measurements are beat-to-beat heart rate and beat-to-beat blood pressure, which can be used for further analyses of heart rate variability, blood pressure variability, and baroreflex sensitivity.
Study Endpoint	To determine whether there is an augmented contribution of ET-1 to the basal vascular tone in Black individuals compared with White individuals. We anticipate that the antagonism of ET-1 receptors will improve vasodilator responses in Black individuals but not in White individuals.
Data Safety Monitoring Plan	Dr. Gailen D. Marshall Jr., Professor of the Department of Medicine, will serve as an independent Data Safety Monitor for this study. The study personnel will conduct semi-annual meetings with the Monitor to present the research progress and discussion of observed events upon drug administration. In case of a participant's emergency health care that could be associated with the study, the study personnel will notify the Monitor within 24 h.
Protected Health Information (PHI)	The information collected in this study includes name, telephone, home address, e-mail address, date of birth, age, race and ethnicity, medical history, and pictures. Each study participant will be given a study ID and the data will be associated with this ID. All data will be accessed via HIPAA compliant methods and stored using REDCap.
Statistical Methodology	Flow-mediated dilation and reactive hyperemia will be compared between groups (Black vs. White) and between drugs (Bosentan vs. Placebo) using a mixed model ANOVA (two-way). Responses to rhythmic handgrip exercise will be compared between groups (Black vs. White), between drugs (Bosentan vs. Placebo), and between intensities (low, moderate, high) using a mixed model ANOVA (three-way).

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Data Collection Sheet	The following data collection instruments were created using REDCap, and PDF copies were included in this proposal: • Medical History Form • Randomization Form • Protocol Sheet Visit 1 • Protocol Sheet Visits 2 and 3 • Questionnaire Adverse Childhood Experiences • Questionnaire Scale of Ethnic Experience

Funding Sources	NIH (P20GM104357) Department of Physiology & Biophysics

INCLUSION OF INDIVIDUALS ACROSS THE LIFESPAN

Healthy Black and White men and women will be recruited between the ages of 18 to 40 years. The focus of our research is to examine differences in vascular function associated with race. Therefore, to avoid individuals with impaired vascular function due to aging and comorbidities, we will recruit a relatively young cohort of participants.

INCLUSION OF WOMEN AND MINORITIES

This research proposal is designed to elucidate deleterious alterations in vascular function specifically in young Black individuals. Accordingly, Black men and women will be examined and, as traditionally done in previous studies in this field, results will be compared with those from White men and women. Subjects will qualify for the study if they identify their race and the race of their biological parents as being Non-Hispanic Black (i.e., African American) or Non-Hispanic White (i.e., Caucasian American). Individuals whose parents are mixed Black and White, or of other races (Hispanic or Latino, Asian, Native American, etc.) will not qualify for the study. To avoid confounding factors associated with lifestyle, only individuals born and raised in the U.S. will be included. We anticipate to include a total of 60 individuals, with a distribution of 15 Black men, 15 Black women, 15 White men, and 15 White women. While men and women will be primarily grouped according to race, this large and balanced planned sample size will allow for sub-analyses comparing the effects of race on vascular responses separately in men and women.

RECRUITMENT AND RETENTION PLAN

Individuals will be recruited via UMMC e-mail listserv, UMMC intranet, flyers on campus, and word of mouth. Potential participants will contact the study personnel via e-mail or phone, and answer screening questions. If they meet the inclusion criteria, a consenting and familiarization visit will be scheduled. Consenting will be conducted at the research laboratory by a study person. After signing the Informed Consent Form, participants will answer a medical history questionnaire, and be familiarized with the research measurements and procedures.

Study visits will be scheduled in pairs according to each specific aim of the project. In other words, each intervention and its respective placebo will be scheduled for consecutive visits. Participants will receive gift cards after completing each pair of visits. Furthermore, participants will learn more about the development of physiological studies in humans. Particularly for UMMC students, this experience may contribute for deciding to pursue a career in human physiology research.

	Fall 2020	Spring 2021	Fall 2021	Spring 2022	Fall 2022	Spring 2023	Fall 2023
Studies – Specific Aim 1a							
Studies – Specific Aim 1b							
Studies – Specific Aim 1c							
Studies – Specific Aim 2							
Abstract submissions							
Manuscript submissions							
IRB – Initial submission							
IRB – Amendments and new protocols							
Funding – COBRE Pilot Grant Program submission							
Funding – AHA Career Development Award submissions							
Funding – NIH Research Project Grant (R01) submissions							

PROTECTION OF HUMAN SUBJECTS

- Risks to Human Subjects
  a. Human Subjects Involvement, Characteristics, and Design

Cardiovascular diseases (CVD) are disproportionately more prevalent in Black (i.e., African American) men and women than in other racial groups in the U.S., which has been associated with deleterious alterations in vascular function starting early in life in Black individuals. Our research is designed to examine the impairments in vascular function in young Black individuals compared with White individuals, understand the contributions of nitric oxide (NO; Aim 1) and endothelin-1 (ET-1; Aim 2) driving these racial differences, generate evidence on potential interventions to improve vascular function and support future clinical practices to prevent CVD in the Black population.

Healthy Black and White men and women of age between 18 and 40 years will be recruited via UMMC e-mail listserv, UMMC intranet, flyers on campus, and word of mouth. Subjects will qualify for the study if they identify their race and the race of their biological parents as being only Black (i.e., African American) or only White (i.e., Caucasian American). Individuals whose parents are mixed Black and White, or of other races (Hispanic or Latino, Asian, Native American, etc.) will not qualify for the study. To avoid confounding factors associated with lifestyle, only individuals born and raised in the U.S. will be included. Also, to avoid comorbidities, we will not include individuals with any chronic or ongoing disease, on prescribed pharmacological treatment, smoking or using tobacco, and with obesity (BMI > 30 kg / m^2). We anticipate to include a total of 60 individuals.

In each visit, we will examine cardiovascular measurements during various stimuli commonly used in research (i.e., flow-mediated dilation, passive leg movement, rhythmic handgrip exercise, and knee extension exercise). Prior to, or at the beginning of each visit, participants will undergo Placebo-controlled interventions [single oral administration of nitrate-rich (~12.8 mmol) beetroot juice; 7-day oral administration of L-citrulline (6 g daily); single oral administration of Sildenafil (100 mg); single oral administration of Bosentan (125 mg)] to acutely modulate NO and ET-1 signaling.

  b. Sources of Materials

The following data will be collected from the subjects: Peripheral arterial blood flow (duplex Doppler ultrasound); beat-to-beat mean arterial pressure (finger photoplethysmography); heart rate (electrocardiogram); plasma concentrations of nitrate, nitrite, L-arginine, L-citrulline, and ET-1. All data will be coded, i.e., each study participant will be given a study ID and the data will be associated with this ID. Only study personnel will have access to the ID coding system, which will be accessed only if needed. All data will be assessed via HIPAA compliant methods, directly collected in or transferred to digital format, and stored using REDCap or other UMMC-provided cloud system. Plasma samples will be disposed of after conclusion of the study analyses.

  c. Potential Risks

There are no psychological, financial or legal risks associated with participating in this research.

Physical risks are only minor and brief discomforts associated with the normal operation of the research measurements and procedures, such as local skin irritation during electrocardiogram; numbness and tingling during blood pressure measurements and procedures with cuff inflations; muscle fatigue after handgrip and knee extension exercise, and minor risks for infection, bruising or occasional light-headedness due to venous blood draws.

Oral administration of beetroot juice, L-citrulline and Sildenafil implicate no known risks. Prolonged use of Bosentan has been associated with alterations in liver function, potential harm to an unborn fetus, and a possibility of failure in contraception when co-administered with hormonal contraceptives.

There are no alternative interventions in the study. If a subject chooses not to undergo an intervention, he or she will not be included in that portion of the study.

- Adequacy of Protection Against Risks
  a. Recruitment and Informed Consent

Announcements will be made via UMMC e-mail listserv, UMMC intranet, flyers on campus, and word of mouth. Potential participants will contact the study personnel via e-mail or phone, and answer screening questions. If they meet the inclusion criteria, a consenting and familiarization visit will be scheduled. Consenting will be conducted at the research laboratory by a study person. After signing the

Informed Consent Form, participants will answer a medical history questionnaire, and be familiarized with the research measurements and procedures.

b. Protections Against Risk

Research personnel will only get involved in this study after approval by the IRB, which will require completion of institutional and/or web-based training (e.g., CITI Program) in protection of human subjects. Furthermore, all personnel will be extensively trained in the specific research techniques. Study visits will be conducted in the Clinical Research and Trials Unit at UMMC, where medical doctors and clinical researchers will provide oversight, technical assistance, safety monitoring, and explanation of incidental findings (e.g., high blood pressure) to the subjects. The Investigational Drug Services at UMMC will assist in the preparation, storage, and dispensing of drugs, supplements, and respective placebo. A portion of the procedures and interventions proposed in this study has already been approved by the IRB of the Human Research Office at UMMC, and amendments will be submitted as this study progresses.

Although the physical risks in this study are only minor and brief, the researchers will continuously interact with the subjects in the study visits to monitor their level of discomfort. The techniques using cuff inflation and arterial occlusion (i.e., blood pressure measurement and flow-mediate dilation) will be discontinued at the subject's request if deemed too uncomfortable. Similarly, exercise trials will be discontinued if not tolerated. Also, the risks associated with blood draws will be minimized by being performed under sterile conditions only by research personnel with required training and experience with this technique.

Oral administration of beetroot juice, L-citrulline and Sildenafil implicate no known risks. The use of Bosentan is well tolerated up to 2400 mg in health individuals and, accordingly, we do not expect any adverse events caused by Bosentan in this study. Nevertheless, out of an abundance of caution, we will exclude from participation in the study individuals with a history of liver disease; women who are pregnant, trying to become pregnant or breastfeeding; and women using hormonal contraceptives, since these conditions may be more vulnerable to adverse events with Bosentan.

- Potential Benefits of the Proposed Research to Human Subjects and Others

  The benefit for the participants in this study is to learn more about the development of research in humans. Particularly for UMMC students, this experience may contribute for deciding to pursue a career in human physiology research. As discussed above, the risks to participants are minimal, thus it is our opinion that the benefits derived from the proposed studies largely outweigh the risks.

- Importance of the Knowledge to be Gained

  Healthy young Black individuals must cope with subclinical impairments in vascular regulation that place continuous stress on the cardiovascular system and predispose to vascular remodeling and dysfunction, which are predictors of CVD. With this investigation, we strive to provide evidence on the roles of NO and ET-1 underlying the racial differences in vascular function and generate preliminary data on tailored novel interventions to improve vascular regulation in Black individuals, ultimately contributing to reduce mortality and improve cardiovascular health in this underrepresented group of the American population.

DATA SAFETY MONITORING PLAN

As approved by the IRB of the Human Research Office at UMMC, a physician and senior faculty of the Clinical Research and Trials Unit will serve as an independent Data Safety Monitor for this study. The Principal Investigator and study personnel will conduct semi-annual meetings with the Monitor to present the research progress and discussion of adverse events observed, if any. In case of a participant's emergency health care that could be associated with the study, the study personnel will notify the Monitor within 24 h.

STATISTICAL DESIGN AND POWER

Sample size estimation (statulator.com) is 11 individuals per group, based on the femoral artery blood flow response to knee extension exercise from our preliminary data, using Student's t-test for independent samples, and considering a two-tailed alpha of 0.05, and power of 0.8 as parameters. This estimated sample size is adequate relative to our previous investigations. Due to potential influence of sex and race on vascular function, we will duplicate the estimated sample size to recruit an equal number of men and women. To account for incompletion and exclusion of data during analyses, additional participants will be recruited, leading to an estimate of 30 individuals enrolled in each racial group. We will use a mixed-model ANOVA to compare vascular responses according to the effects of drug administration (Placebo vs. Drug), group (Black vs. White), and interaction. Subjects' characteristics will be compared using Student's t-test for independent samples and, in case of significant differences, such characteristics will be used as covariates in the ANOVA. Additionally, while men and women will be primarily grouped according to race, the large and balanced sample size will allow for sub-analyses comparing the effects of race on vascular responses separately in men and women.