

ABSTRACT

Title of Thesis: RACIAL DIFFERENCES IN VASCULAR
FUNCTION FOLLOWING INDUCED ACUTE
INFLAMMATION

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Kinesiology

African-Americans (AAs) have higher rates of cardiovascular disease (CVD), including hypertension and stroke, as compared to their Caucasian-American (CA) counterparts. High resting concentrations of systemic inflammatory biomarkers contribute to vascular dysfunction and are predictive of future cardiovascular events; differential resting levels of inflammatory markers between groups may reveal increased potential for CVD in at-risk groups. Additionally, impaired endothelial function and increased arterial stiffness, subclinical measures of CVD progression, have been reported in AA groups. The purpose of this study was to examine race differences between young, healthy AA and CA adults after a systemic inflammatory stimulus and subsequent endothelial responses to inflammation. Endothelial function, arterial stiffness, and hemodynamic variables were measured. The results suggest there were no race differences in vascular function or hemodynamic responses following an acute inflammatory stimulus.

**RACIAL DIFFERENCES IN VASCULAR FUNCTION
FOLLOWING INDUCED ACUTE INFLAMMATION**

by

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List of Abbreviations

AA – African American

AC – Arterial compliance

aDBP – Aortic diastolic blood pressure

AHA – American Heart Association

AIx – Augmentation index

AIx75 – Augmentation index standardized to heart rate of 75 beats per minute

ANCOVA – Analysis of variances

ANOVA – Analysis of covariances

AP – Augmentation pressure

aSBP – Aortic systolic blood pressure

ATP – Adenosine tri-phosphate

bDBP – Brachial diastolic blood pressure

BMI – Body mass index

bSBP – Brachial systolic blood pressure

CA – Caucasian American

cfPWV – Carotid-femoral pulse wave velocity

CRP – C-reactive protein

CVD – Cardiovascular disease

DBP – Diastolic blood pressure

ELISA – Enzyme-linked immunosorbent assay

eNOS – Endothelial nitric oxide synthase

FMD – Flow-mediated dilation

GTN – Glyceryl Trinitrate, also known as Nitroglycerin

IL-1/IL-6 – Interleukin 1/Interleukin-6

IRB – Institutional Review Board

L-NMMA – Nitric oxide synthase inhibitor NG-Monomethyl-L-Arginine

LPS – Lipopolysaccharide

MAP – Mean arterial pressure

NF- κ B – Nuclear factor κ B

NO – Nitric oxide

NSAID – Nonsteroidal anti-inflammatory drug

PWA – Pulse wave analysis

PWV – Pulse wave velocity

SBP – Systolic blood pressure

TNF- α – Tumor necrosis factor- α

VO₂ peak – Peak volume oxygen capacity/uptake

INTRODUCTION

Overview

The prevalence of cardiovascular disease (CVD) in the United States exhibits race-specific disparities. Research shows that cardiovascular health risks are increased in African American (AA) populations compared with their Caucasian American (CA) counterparts, with greater incidence of hypertension in AA groups. With vascular dysfunction contributing to future development of hypertension, measures of arterial stiffness, endothelial function, blood pressure and wave reflection may help in determining future CVD risk, even in young, healthy adults. Additionally, inflammation contributes significantly to the initiation and progression of CVD by impairment of vascular function. We sought to evaluate whether race differences were evident in inflammatory cytokine and vascular responses after administration of an acute inflammatory stimulus (Figure 1). The study aimed to examine whether AA and CA groups had different responses in concentrations of circulating inflammatory cytokines after induced acute inflammation. Additionally, the study sought to evaluate whether race differences were evident between young, healthy AA and CA groups in vascular responses after an induced bout of acute inflammation.

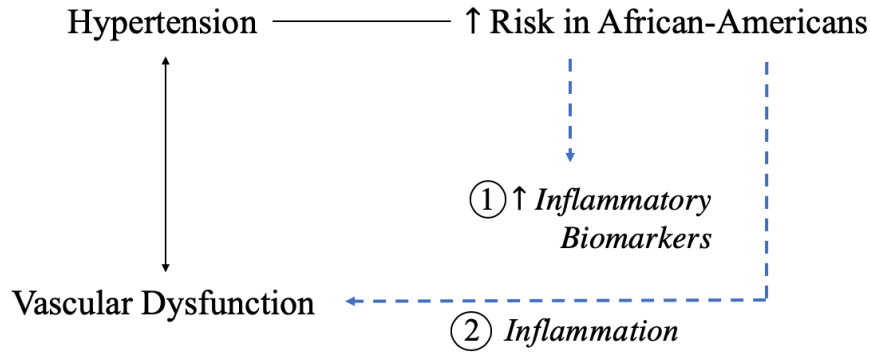


Figure 1. *Thematic diagram of specific aims.* **Aim 1** will examine the response of inflammatory cytokine concentration after influenza vaccine injection in AA and CA groups. **Aim 2** will investigate potential vascular dysfunction as a response to acute inflammation in AA vs. CA individuals.

Cardiovascular Disease and Hypertension

Cardiovascular disease continues to be the leading cause of death in the United States (Murphy et al., 2018). Nearly one in three American adults has hypertension – with less than half of those cases under control (Fryar et al., 2017). Hypertension is a condition of elevated blood pressure and a significant risk factor for CVD (Chobanian et al., 2003; Crim et al., 2013). Current American Heart Association (AHA) standards categorize hypertension by systolic and diastolic measurements; systolic blood pressure greater than or equal to 130 mmHg or diastolic blood pressure greater than or equal to 80 mmHg fall into the categories of hypertension (2017). The condition is considered to be controlled when lifestyle modification or medical treatment bring the systolic pressure to below 130 mmHg and diastolic pressure below 80 mmHg (Whelton et al., 2018; AHA, 2018).

Vascular Dysfunction

The endothelium is the innermost lining of the arteries and it plays a significant role in vascular tone. The endothelium has the ability to release vasoactive substances, especially in response to various stimuli, which affect the vasodilatory capacity of a blood vessel. Blood vessels may constrict, meaning their diameter has decreased from baseline, or dilate, meaning the diameter has increased from baseline. One example of an intrinsic stimulus is shear stress from blood flow, the dilatory response to which is endothelium-dependent by way of nitric oxide (NO) production. As illustrated in Figure 2, shear stress triggers a pathway that ultimately leads to the generation of NO from L-arginine via endothelial nitric oxide synthase (eNOS) (Chistiakov et al., 2017). NO produced by the endothelium acts upon the outer smooth muscle layer that lines larger conduit arteries. After diffusion into smooth muscle cells, NO activates guanylyl cyclase, leading to a G-protein cascade, ultimately causing vascular smooth muscle relaxation, observed as arterial dilation (Toblli et al., 2012). Higher dilatory capability of the endothelium represents healthier arterial function in normal, healthy populations.

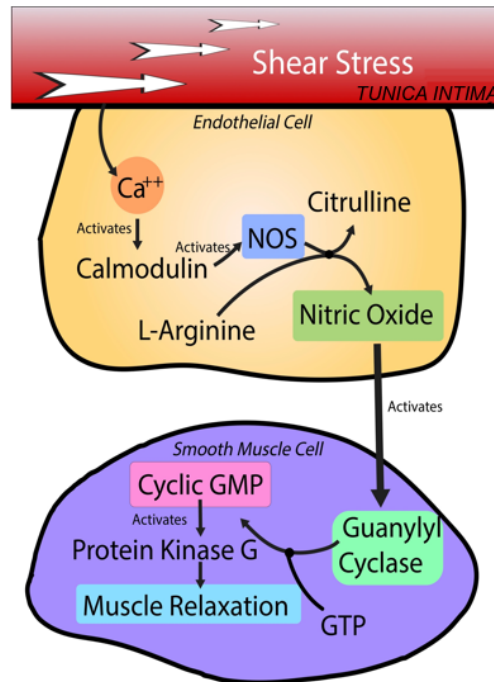


Figure 2. *Shear stress-induced endothelial dilation.* Increases in shear stress from blood flow trigger calcium release in the endothelial cells, thereby activating calmodulin. Calmodulin activation of nitric oxide synthase allows for the conversion of L-arginine into nitric oxide. Dispersion of endothelial-produced nitric oxide into the surrounding smooth muscle layer of the artery activates guanylyl cyclase. This activation causes a downstream protein cascade that culminates in smooth muscle relaxation, allowing for arterial dilation.

Measuring vascular function and arterial stiffness are common ways of evaluating future cardiovascular risk (Lerman & Zeiher, 2005). Vascular dysfunction is a pathological development that has been linked to impaired NO availability and metabolism, thus resulting in high levels of oxidative stress and augmented inflammation (Widmer & Lerman, 2014). Vascular dysfunction is evident in older adults as a result of aging (Celermajer et al., 1994), in populations with chronic illness or disease (Hadi et al., 2005; Rajendran et al., 2013), and is even seen in healthy individuals who have no cardiovascular risk factors except for family history of CVD

(Clarkson et al., 1997). Impaired vascular function has also been documented in patients with hypertension (Hadi et al., 2005; Konukoglu & Uzun, 2016).

Similar to endothelial function, arterial stiffness is an independent predictor of CVD and hypertension (Vlachopoulos et al., 2010b; Safar, 2017). While the inner and middle arterial layers are comprised of the endothelium and vascular smooth muscle, arterial walls also contain a matrix of collagen and elastin fibers. Collagen fibers provide tensile structure to the arteries, promoting rigidity, while the elastin fibers allow for expansion of vessel diameter and promote elasticity, which is essential in accommodation of pulsatile blood flow from cardiac contraction (Jacob, 2003). Stiffening of the arteries is seen in older and diseased populations, usually due to thickened walls and vascular remodeling. Vascular remodeling is a process in which the collagen-elastin ratio shifts toward increased collagen fibers. This collagen accumulation increases arterial stiffness, culminating in higher velocities of blood flow that place target end-organs at risk for damage (Tan et al., 2014). Silent coronary artery disease can be predicted by arterial wall thickening, thus forecasting future cardiovascular events (Lakatta & Levy, 2003). While arterial stiffness is used as a subclinical marker of CVD, there appears to be a positive feedback loop between the measure and hypertension; there is no clear answer to whether arterial stiffness is a cause or just a result of elevated blood pressure (Humphrey et al., 2016).

Flow-Mediated Dilation

Flow-mediated dilation (FMD) is widely used to evaluate vascular function. With the use of ultrasonography, the brachial artery is imaged at a resting baseline. For five minutes, a blood pressure cuff placed around the upper forearm is inflated to a

supra-systolic pressure to occlude blood flow. After cuff deflation, the post-ischemic hyperemic response stimulates dilation of the brachial artery, due to shear-stress mediated pathways of NO production (Corretti et al, 2002; Green et al., 2014). Administration of L-NMMA, a nitric oxide synthase inhibitor, markedly reduces the brachial dilation seen during FMD, confirming the importance of the contribution of NO to this response (Steyers & Miller, 2014). Compromised endothelial health is associated with increased cardiovascular risk, possibly due to atherosclerotic progression (Bonetti et al., 2002). In conjunction with the Framingham Risk Score, impaired FMD has been associated with future cardiovascular event risk, even in asymptomatic adults (Inaba et al., 2010; Yeboah et al., 2008; Zhong et al., 2018).

Pulse Wave Analysis

Brachial blood pressure can be easily evaluated in research settings to gauge cardiovascular health from the lens of hypertension. AHA clinical standards define categories of blood pressure that are used to stratify risk for hypertension, with elevated blood pressure beginning at 120/80 mmHg and Stage I Hypertension beginning at 130/80 mmHg (2017). Brachial pressure waves obtained from an automated oscillometric cuff on the upper arm can undergo a validated computer transfer function to reconstruct aortic waveforms; this is beneficial in that measures of central pulse pressures have been shown to be more reliable in predicting cardiovascular risk (Laurent et al., 2006). These aortic waveforms can be used to evaluate wave reflection, quantified as augmentation index (AIx), which serve as a marker of cardiovascular risk (van Trijp et al., 2004). When oxygenated blood is ejected from the heart during left ventricular contraction, it travels in a forward pressure wave toward the periphery

through the body's arterial tree. Forward pressure waves following ventricular contraction will initiate "backward," or reflected, pressure waves, mainly due to bifurcations and reduction of the diameter of branches in the arterial tree. Healthy individuals typically have more compliant arteries, in which elastin fibers of the arterial structure are able to absorb shock from pressure pulsatility. In these compliant arteries, reflected waves return at a time point later in the cardiac cycle, such as diastole, which is beneficial as return during diastole enhances coronary perfusion (Mitchell, 2008; Patvardhan et al., 2011). Older individuals with cardiovascular disease are typically observed to have stiffer, or less compliant arteries. Stiffer arteries that have an increased collagen to elastin ratio cause blood to travel faster through the arteries. Increased blood velocity causes reflected waves to return to the heart in a shorter wave transit time such that the cardiac cycle has not yet progressed from systolic contraction to diastolic relaxation (Klabunde, 2017; Patvardhan et al., 2011). In circumstances where the reflected wave returns during systole, there is an increased cardiac afterload (Patvardhan et al., 2011), and the heart needs to pump harder so that the pressure in the left ventricle is higher than the afterload in the aorta. When wave reflection arrives during systole, blood pressure waveforms generate two systolic peaks: the first systolic peak is generated by the initial left ventricular contraction and the second systolic peak is generated when the heart pumps harder to overcome increased afterload. AIx, the commonly used measure for wave reflection, is calculated as the difference in systolic peaks (second peak minus first peak) divided by pulse pressure (the systolic blood pressure minus diastolic blood pressure). When wave reflections return during the later diastole phase, AIx can become negative, commonly seen in healthy and young adults.

When wave reflections return during systole, systolic pressure peaks are amplified and AIx can increase, resulting in a positive value. Increased AIx can strongly predict future cardiovascular events and all-cause mortality (Vlachopoulos et al., 2010a). Due to the timing of reflected pressure waveforms, heart rate has been reported to have an inverse effect on AIx (Wilkinson et al., 2000). For this reason, AIx is commonly normalized to a heart rate of 75 beats per minute (AIx75).

Pulse Wave Velocity

Large artery stiffness assessed via applanation tonometry, is a measure to evaluate cardiovascular health. Applanation tonometry employs a high-fidelity tonometer at specific pulse sites to map out blood pressure waveforms. Carotid-femoral pulse wave velocity (cfPWV) is considered the gold standard measurement in assessment of central arterial stiffness (Laurent et al., 2006). Measurement of cfPWV requires acquiring blood pressure waveforms from the common carotid artery while an oscillometric cuff on the thigh obtains waveforms from the femoral artery. Distances from the carotid artery to the suprasternal notch and from the automated thigh cuff to the femoral pulse site are used to calculate the distance an aortic pressure waveform travels through a body's central cavity. Increased arterial stiffness causes pulse wave velocity to increase, meaning a higher cfPWV. Measures of aortic stiffness can be used to clinically determine one's risk of cardiovascular disease and mortality – increased arterial stiffness has been shown to strongly predict future cardiovascular events and all-cause mortality (Nelson et al., 2010; Laurent et al., 2001; Vlachopoulos et al., 2001 (b)).

Large Artery (Carotid) Stiffness

Ultrasonography can also be used as a non-invasive method to image the common carotid artery and these assessments can provide measures of carotid arterial beta-stiffness (β -stiffness), compliance and elasticity. As arteries stiffen with age or diseased conditions, pressure pulsatility through the microvasculature, especially the capillary beds, increases (Mitchell, 2008). Therefore, for high-perfusion organs, such as the brain and kidney, increased pressure pulsatility presents the possibility of end-organ damage. Aortic and large artery stiffness also contribute to increased systolic and pulse pressure (Hashimoto & Ito., 2009), all of which have been shown to be associated with markers of kidney damage (Cirillo et al., 2000; Safar et al., 2004; Vasan et al., 2019), cerebral damage and cognitive impairment (Mitchell, 2008; Mitchell et al., 2011). Ultrasonography measures of large artery stiffness at the carotid artery are useful because cardiac damage has been shown to associate with increased carotid artery stiffness (Bruno et al., 2017) and pulsatility, as measured by central hemodynamic pressure measures (Kaess et al., 2016). Additionally, changes in carotid stiffness have been found to be associated with higher incidence of cardiovascular events and mortality (Sedaghat et al., 2018; van Sloten et al., 2014).

Racial Disparities

The prevalence of CVD in the United States exhibits race-specific disparities. (Heron, 2019). While CVD incidence in CA males and females is 38% and 35%, respectively, AA exhibit higher rates with male and female prevalence at 46% and 48%, respectively (Benjamin et al., 2017). Hypertension is a significant contributor to the

early onset of cardiovascular disease seen in AA populations (Buie et al., 2019; Carnethon et al., 2017). Similar race disparities are observed in AA versus CA adults with regard to hypertension (Hertz et al., 2005). From 2015-2016, 41% of AA men and 40% of AA women had hypertension, higher than the recorded 30% of CA men and 26% of CA women (Fryar et al., 2017).

With the known contribution of vascular dysfunction to future cardiovascular events, it is critical to evaluate racial disparities in vascular function. Endothelial dysfunction is a known contributor to cardiovascular risk; impaired FMD at rest in individuals suggests future risk for cardiovascular events. Resting measures of vascular function can be studied to determine whether differences exist between individuals of different groups. Compared to CA counterparts, AA groups have been reported to exhibit lower endothelial function as measured by FMD at rest (Campia et al., 2002). Ranadive et al. reported lower baseline forearm blood flow and reduced reactive hyperemic responses in AA as compared to CA adults aged 18-35 years old (2016). AA also have greater arterial stiffness (Din-Dzietham et al., 2004) and lower arterial compliance (Zion et al., 2003). While CVD tends to target older adult populations, measures of vascular dysfunction have also been documented in young cohorts. Heffernan et al. found that young, healthy AA men had elevated central blood pressures and wave reflection as compared to young, healthy CA men (2008). Furthermore, AA children between the ages of nine and twelve years old had significantly higher pulse wave velocity as compared to CA children, which was maintained even after covarying for a number of potentially confounding variables (Lefferts et al., 2017).

Acute Inflammation

Inflammation is a major contributing factor to vascular dysfunction, which may ultimately increase cardiovascular risk. There are both acute and chronic facets to systemic inflammation. As a result of aging, older adults exhibit low-grade chronic inflammation (Ferrucci & Fabbri, 2018); circulating inflammatory marker concentrations are especially augmented in those with long-term illnesses or health conditions (Steyers & Miller, 2014). Patients with rheumatoid arthritis, a chronic inflammatory disease, were found to have a higher risk of myocardial infarction as compared to the general population (Levy et al., 2008). Interestingly, acute bouts of inflammation, like injury or infection, may carry similar cardiovascular implications (Smeeth et al., 2004). Therefore, in young and healthy cohorts, acute bouts of inflammation may provide possible insight into the future vascular responsiveness following a systemic stressor.

Inflammatory Cytokines

Several biomarkers, including C-reactive protein (CRP), interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF- α), determine the magnitude of an inflammatory response. Evaluation of circulating inflammatory cytokines can be useful in determining risk for future cardiovascular events (Cesari et al., 2003). CRP is often used as an assessment of future cardiovascular risk; high levels of CRP have been shown to be strong predictors of future coronary heart disease (Pai et al., 2004) and cardiovascular events (Ridker et al., 2002). Additionally, Ridker et al. found that, even in apparently healthy men, elevated resting concentrations of IL-6 were associated with increased future myocardial infarction risk (2000).

IL-6 is an important cytokine in acute inflammatory responses by the immune system (Nakahara et al., 2003). It is imperative to understand the process related to the systemic inflammation associated increase in cytokines. In brief, pathogen-associated molecular patterns (PAMPs) are cellular markers on foreign pathogens that are recognized by human immune cells (Kumar et al., 2011). PAMP recognition by immune cells initiates downstream immune signaling cascades, including that of the nuclear factor- κ B (NF- κ B) genomic pathway (Brasier, 2010; Tanaka et al., 2014). These pathways stimulate transcription of genes for production and secretion of immune cytokines like TNF- α , IL-1 β and IL-6 (Tanaka et al., 2014), which then enter the systemic circulation. Therefore, circulating IL-6 concentrations assist in quantifying the scale of inflammatory responses.

Racial disparities seem to exist in resting levels of inflammation; AA adults have been found to have higher levels of CRP than CA counterparts (Veerabhadrapa et al., 2011), as well as significantly higher concentrations of IL-6 (Paalani et al., 2011). However, these cohorts were middle-aged to older-adult populations in which there is an established positive relationship between age and inflammatory biomarkers (Milan-Mattos et al., 2019; Xia et al., 2016). Further research is needed to elucidate the involvement of race in inflammatory responses, specifically in young, healthy cohorts.

Vascular Response to Inflammation

In addition to potential roles in cardiovascular risk, inflammation has detrimental effects on vascular function. Studies have shown inflammation-mediated vascular dysfunction by mechanisms of reduced nitric oxide bioavailability and increased oxidative stress (Clapp et al., 2004). One of the proposed pathways by which

this occurs is the effect of TNF- α on the endothelium. TNF- α mediated inhibition of eNOS protein expression leads to attenuated NO production (Steyers & Miller, 2014). In addition to TNF- α , elevated concentrations of CRP have been found to associate with blunted vascular function (Fichtlscherer et al., 2000). Specifically, Hingorani et al. demonstrated impaired endothelium-dependent dilation after inducing an acute bout of systemic inflammation (2000). In addition to the deleterious effects exhibited on vascular function, circulating inflammatory markers have been found to associate with increased arterial stiffness (Jain et al., 2014).

Induced Systemic Inflammation

Various methods have been utilized as models of inducing acute systemic inflammation and studying vascular responses to the inflammation. Most notably, bacterial lipopolysaccharide (LPS), salmonella typhi vaccine, and the seasonal influenza vaccine have been successfully used to induce systemic inflammation. Bacterial inflammatory stimuli, such as administration of gram-positive and -negative bacteria, can impair endothelial function through several mechanisms. Gram-positive bacteria secrete toxins that can directly induce apoptosis of endothelial cells; gram-negative bacteria secrete toxins that can disrupt the structural integrity of endothelium, leading to vascular leakage and, in more severe cases, sepsis (Lubkin & Torres, 2017).

Bacterial endotoxin has been shown to induce inflammation in animal (Kuo, 2016) and human models (Kiers et al., 2017). In one study, Copeland et al. administered *Escherichia coli* type O113 endotoxin to both mice and humans (2005). Blood samples were collected at baseline before endotoxin administration, with follow-ups at two,

four, six, nine, and twenty-four hours after injection to quantify levels of IL-6, TNF- α , and IL-1 β . IL-6 and TNF- α increased in mice and human plasma soon after injection, returning to baseline by six hours post (Copeland et al., 2005).

Salmonella typhi vaccines have been successfully used to generate systemic inflammation. In response to *Salmonella typhi* vaccine, Vlachopoulos et al. demonstrated increased inflammatory marker concentrations in humans (2005). Significant increases of high sensitivity C-reactive protein (hsCRP) were observed at eight and thirty-two hours post injection, while high sensitivity interleukin-6 (hsIL-6) significantly increased eight hours post injection. In addition to augmented circulating concentrations of inflammatory biomarkers, the study reported increases in arterial stiffness, measured by PWV, at eight hours post vaccine and decreases in wave reflection, measured by AIx, at eight and thirty-two hours post-vaccine (Vlachopoulos et al., 2005).

Another study using typhoid vaccine measured an hourly time course of circulating inflammation levels for eight hours, with additional follow up thirty-two hours post *S. typhi* injection (Hingorani et al., 2000). Hingorani et al. reported no increases in TNF- α or IL-1 β , although a significant increase from baseline was found after 3 hours for IL-1ra. IL-1ra is released in response to IL-1 β , indicating that while IL-1 β may not have increased significantly systemically, there may have been increased production of this inflammatory cytokine at a local cellular level, leading to greater production of IL-1ra (Hingorani et al., 2000). Despite increased concentrations, IL-6 was not found to be significant after the inflammatory injection. In the same study, FMD significantly decreased eight hours post-acute inflammation injection (Hingorani

et al., 2000). Inflammation was shown to only have impacted endothelium-dependent dilation; nitroglycerin, a vascular smooth-muscle relaxer, continued to cause endothelium-independent vasodilation after induced inflammation (Hingorani et al., 2000). This inflammatory impact on endothelial function was later supported by Clapp et al., who found that typhoid vaccination generated an inflammatory response which led to endothelial dysfunction and attenuated NO bioavailability after eight hours (2004).

Seasonal influenza vaccines have also been used as a model for inducing acute bouts of systemic inflammation in both young and older adults (Liuba et al., 2007; Jae et al., 2009; Ranadive et al., 2014; Schroeder et al., 2018). Induced inflammation via influenza vaccine administration have also been shown to impair vascular function in immuno-compromised individuals (Vlachopoulos et al., 2011), as well as increase arterial stiffness in healthy adults. Lane-Cordova et al. demonstrated significant increases in CRP twenty-four and forty-eight hours after administration of influenza vaccine in both young and older adults, with a concurrent increase in IL-6 after twenty-four hours only in young adults (2016). FMD was reduced twenty-four and forty-eight hours after influenza vaccination in only the young adult cohort, however it should be noted that older adults had higher baseline levels of inflammatory markers than their young adult counterparts (Lane-Cordova et al., 2016). Furthermore, Schroeder et al. examined young and older adults, with respect to low or high cardiorespiratory fitness in response to seasonal influenza vaccine (2018). Systemic inflammation was induced, yet no changes were observed in any groups for measures of wave reflection or arterial stiffness; however, at 24 hours post influenza injection, FMD in fit, older adults and all

young adults decreased, while FMD in low-fit older adults was reported to have no change (Schroeder et al., 2018).

Jae et al. investigated the effect of influenza vaccines on arterial stiffness in healthy older adults (aged 57-75 years) in South Korea who were administered either influenza vaccine or a sham vaccine (2013). Compared to baseline measurements, CRP and IL-6 were significantly increased at twenty-four and forty-eight hours post influenza vaccine administration, but not sham injection (Jae et al., 2013). While measures of blood pressure and augmentation index did not change following induced inflammation, cfPWV increased twenty-four hours after the inflammatory influenza vaccine (Jae et al., 2013). This finding aligned with previous literature that linked inflammation with increases in arterial stiffness (Vlachopoulos et al., 2005) after *Salmonella typhi* injection, suggesting the inflammation induced by both injections resulted in similar augmented arterial stiffness indices. Vaccine models are clearly capable of inducing acute bouts of systemic inflammation, which then affect vascular function. By studying vascular responses to stressors such as inflammatory states, we may be able to elucidate race differences between groups that may indicate future cardiovascular risk not seen at resting baseline states in young, healthy adults.

SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1: To assess quantities of circulating inflammatory cytokine IL-6 at baseline compared to 24 hours and 48 hours after influenza vaccine administration in AA and CA participants.

Hypothesis 1: AA participants will exhibit significantly greater concentrations of circulating inflammatory marker IL-6 compared with their CA counterparts.

Specific Aim 2: To assess endothelial function following acute induced inflammation between AA and CA participants.

Hypothesis 2: AA will exhibit attenuated FMD measures as compared to their CA counterparts.

Exploratory Aims

If baseline differences for variables were detected between race groups, linear regressions were run with the variable as a covariate to determine any correlations that may exist. Additionally, statistical tests run on changes from baseline (for example: value of FMD at 24 hours minus value of FMD at baseline) typically accounts for baseline differences, which is explained in more detail in the methods section later. Exploratory aims examined racial differences in the hemodynamic (brachial and central pressures) and arterial stiffness responses (central pulse wave velocity, arterial compliance and β -stiffness) to the induced inflammation. Additionally, in response to inflammation, vascular function seems to be preserved in fit older adults, mimicking

vascular responses to inflammatory stressors as seen in young adults. Therefore, cardiorespiratory fitness was measured by VO_2 peak as an independent factor that may affect the vascular responses to inflammation.

METHODS AND PROCEDURES

IRB Approval

This study was approved by the Institutional Review Board of the University of Maryland, College Park. All recruited participants provided written informed consent after thorough explanation of the study protocol and testing measures.

Experimental Design

Recruitment and Eligibility

Recruitment for the study comprised of AA and CA human participants. Study recruitment flyers with email contact information were posted throughout the University of Maryland's School of Public Health building. Once participants contacted the email, an IRB-approved digital questionnaire was sent out with preliminarily screening criteria questions. The email questionnaire screened participants for study eligibility with the following questions:

- 1) Are you between the ages of 18-39?
- 2) Do you weigh less than 110 pounds (49.9 kgs)
- 3) Do you have a BMI greater than 30?
- 4) Do you have a heart disease?
- 5) Do you have high blood pressure (i.e. greater than 140/90)?
- 6) Do you have a lung disease?
- 7) Do you have diabetes or another metabolic disease?
- 8) Are you currently pregnant?
- 9) Have you been sick in the past two months?
- 10) Do you currently smoke (cigarettes, vaping and juuling) or use any tobacco products?
- 11) Are you taking any prescription medications (other than oral contraceptives)?

Email recipients were also asked to self-identify their race as African-American, Caucasian-American, biracial, multiracial, or other. Potential participants who met

eligibility criteria were then scheduled for their first visit to the laboratory. Eligible participants were between the ages of 18-39 years and healthy. During their first visit, all recruited participants provided signed written consent into the study with forms approved by the University of Maryland IRB. After written consent was provided, participants filled out a printed eligibility screening questionnaire. Any participants that identified as bi- or multi-racial, or did not self-identify exclusively as AA or CA, were excluded. Additional eligibility criteria required participants to be nonsmokers, not currently pregnant, with a weight greater than 110 pounds, and body mass index (BMI) less than 30 kg/m².

After completing the eligibility screening questionnaire, participants filled out a health history questionnaire. This form screened participants for cardiometabolic conditions, diagnosed medical conditions, prescription medication use, family history of cardiovascular disease, smoking/tobacco product habits, and menstrual cycle history. Exclusionary criteria included being diagnosed with or taking medications for: cardiovascular disease (uncontrolled or medication-controlled hypertension, stroke, coronary artery disease, cardiac arrhythmia), metabolic disease (diabetes mellitus), and inflammatory diseases (rheumatoid arthritis, systemic lupus erythematosus). Participants were excluded if they had contracted the common cold, influenza, bacterial or viral infections, or an upper respiratory tract infection within the previous two months, had already received the influenza vaccine for the specified season, had a bleeding disorder, were undergoing anticoagulant therapy, or did not meet the Center for Disease Control's guidelines for vaccination (CDC, 2019). If the resting blood pressure, taken during the first visit, was equal to or greater than 140/90 mmHg, the

potential participant was then excluded. These criteria ensured recruitment of healthy participants, which would limit confounding factors that may have an effect on an inflammatory response. With each completed visit, participants were eligible for \$15 compensation. If all seven visits are completed in full, a participant could be financially compensated up to a total of \$105 in cash.

Laboratory Visits

Prior to each visit, participants were instructed to refrain from food, drinks, alcohol, caffeine and exercise for 12 hours. They were also instructed to refrain from over-the-counter pain medications (NSAIDs including aspirin, ibuprofen, naproxen) for 24 hours and recreational drug use for 48 hours before each visit. These fasting restrictions were intended to limit any effect of medication or drug use on the inflammatory responses observed in the study.

The study employed a randomized, double-blind, sham shot-controlled crossover design. Participants came to the laboratory for seven total visits. During Visit 1, participants were consented in to the study, filled out eligibility screening and health history questionnaires, had their resting blood pressure taken, and underwent a maximal exercise test on a cycle ergometer. The remaining six visits were scheduled into two groups of three days in a row: a baseline visit (Visits 2 and 5), a 24-hours post shot follow-up (Visits 3 and 6), and a 48-hours post shot follow-up (Visits 4 and 7). Males were tested with a minimum 10-day period between Visits 4 and 5 to allow for a wash-out period of any effects from the first administered shot. Females were scheduled during the first seven days of their menstrual cycle for both sets of 3-day visits. This was intended to serve as a control for hormone concentration fluctuation: testing

occurred during the low hormone portion of the follicular phase in naturally menstruating females, or during the placebo pill week in oral contraceptive users. This gap of approximately four weeks allowed ample wash-out time for any shot effects between Visits 4 and 5. The protocol timeline is summarized in Figure 3.

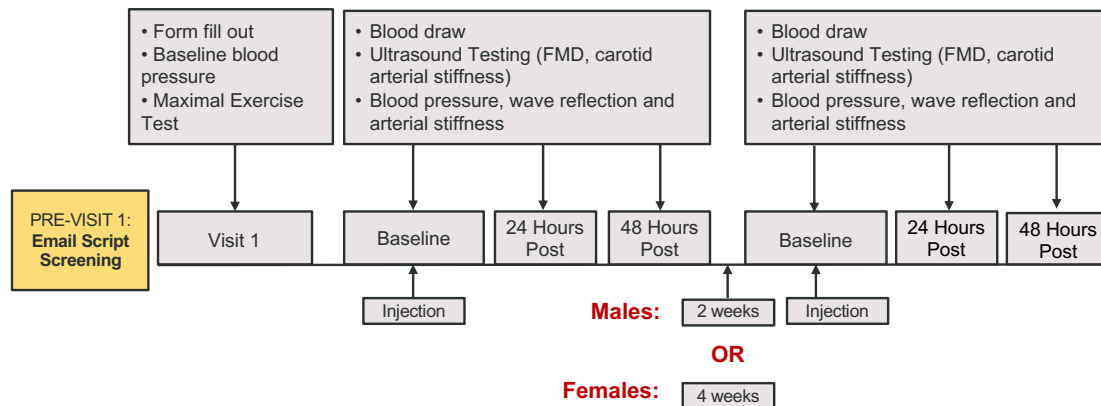


Figure 3. Protocol timeline. The middle row of seven gray boxes represent individual visits. Visits 2 through 7 have identical testing, with 2 and 5 designated as injection days. On injection days, participants are walked over to the University of Maryland Health Center for either Shot A or Shot B after completion of testing. Males typically have a two-week wash-out period between Visits 4 and 5, while females typically have a four-week wash-out.

Visits 2-7 had identical testing protocols that last approximately 45 minutes per visit, with the exception of the days when participants were walked to the University of Maryland Health Center for either a sham saline shot or the seasonal flu vaccine. At the completion of baseline testing on Visit 2, participants were designated as Shot A or Shot B recipients and were scheduled to receive the remaining shot at Visit 5. The sham and vaccine shots were coded to blind the participant and data collectors to prevent data interpretation bias. Only the Health Center nurse and Principal Investigator, Dr. Ranadive, knew the code translation as to which shot was the saline or vaccine injections.

In each testing session, measurements were taken in a dimly lit room after ten minutes of supine rest. Procedures included a venous blood draw, non-invasive blood pressure and heart rate monitoring, flow-mediated dilation, carotid ultrasonography, brachial blood pressure, and pulse wave velocity. All testing is described in further detail below. After each blood draw, samples were frozen in a -80° C freezer for future analysis. To examine **Specific Aim 1**, enzyme-linked immunosorbent assays (ELISA) were used to quantify circulating inflammatory markers after recruitment and data collection completion.

Procedures

VO₂ Peak Test

A maximal exercise test was administered on a cycle ergometer to measure VO₂ peak. Participants were fitted with a Bluetooth Polar heart rate monitor and brachial blood pressure cuff. An appropriately sized face mask that covers the nose and mouth was put on, ensuring an airtight seal to prevent air leakage. The face mask was connected to a system which analyzed exhaled gas composition to determine volume of oxygen exhaled in relation to room air. After a few minutes of cycling warm up, the test began at a cycling resistance of 50 Watts and the participant was instructed to maintain a cycling cadence of 80 revolutions per minute (rpm) throughout the test. Every two minutes, resistance intensity increased by 25 Watts. At the end of each two-minute stage, heart rate was recorded, brachial blood pressure was manually measured, and the participant was asked to indicate their perceived level of exertion on a Borg scale of 6 to 20, with 20 being maximal perceived exertion. The test continued until the

cadence of 80 rpm is no longer maintainable, or volitional exhaustion. Data are collected and binned in 15-second intervals to obtain relative VO₂ peak measurement.

Flow-Mediated Dilation (FMD)

The brachial artery was imaged using ultrasonography (Aloka, Hitachi, Tokyo, Japan) with a high frequency linear array probe (5-13 MHz). An automated blood pressure cuff was placed around the forearm of the same arm, just below the elbow. One minute of baseline measurements was recorded, followed by five minutes of ischemia due to supra-systolic cuff inflation (220 mmHg). After occlusion, the cuff was automatically deflated, and 150 seconds of hyperemic response were recorded. The FMD was recorded via computer analysis software, CV Suite, where edge detection software calculated brachial artery diameter by measuring distances between the two edges of the arterial lining. FMD can be expressed as an absolute measure, in millimeters to represent change from peak diameter to mean baseline diameter, or expressed as a relative percentage, to represent the change in diameter with respect to the mean baseline diameter. Below are the equations used to calculate measures of FMD.

$$\textbf{Absolute FMD (mm)} = [\text{peak diameter (mm)} - \text{baseline diameter (mm)}]$$

$$\textbf{Relative FMD (\%)} = \frac{(\text{peak diameter (mm)} - \text{baseline diameter (mm)})}{\text{baseline diameter (mm)}} \times 100$$

The edge-detection software that automatically calculates FMD values, CV Suite, also allows for each FMD data file to be exported; these exported data files provide second-to-second measurements of arterial diameter from each test. *FMD binning* refers to the manual calculation of FMD by averaging small time (i.e. 3 second) intervals of the arterial diameter to generate average baseline and peak diameters. This analysis is completed to account for small fluctuations in diameter due to the pulsatile contractions of the artery to accommodate blood flow. Evaluation of baseline diameter, shear rate and blood pressure are commonplace in FMD analysis in order to account for their contributions to this measurement. To account for these variables, Absolute FMD can be divided by the secondary measure (i.e. FMD/baseline diameter).

Non-invasive Blood Pressure

Non-invasive beat-to-beat blood pressure was monitored via finger plethysmography (AD Instruments) throughout the FMD test. A four-lead ECG concurrently measured heart rate. Data were collected by Labchart computer software for later analysis of mean and beat-to-beat blood pressure analyses.

Carotid Ultrasonography

Ultrasonography was used to image the right common carotid artery, proximal of the carotid bulb, with a high frequency linear array probe. Automated wall detection software was used to determine measures of carotid arterial stiffness, including arterial compliance, beta stiffness, and wave intensity. A three-lead ECG measured heart rate during carotid imaging. In the equations below for β -stiffness and arterial compliance, SBP and DBP respectively refer to systolic and diastolic blood pressures measured by

ultrasonography at the right common carotid artery (CCA). D_s and D_d refer to the CCA diameter during systole (maximum) and diastole (minimum), respectively.

$$\beta \text{ stiffness (AU)} = \frac{\ln\left(\frac{SBP}{DBP}\right)}{\left[\frac{D_s - D_d}{D_d}\right]}$$

$$\text{Arterial Compliance} \left(\frac{mm^2}{kPa}\right) = \frac{\pi(D_s^2 - D_d^2)}{4(SBP * DBP)}$$

Pulse Wave Analysis (PWA)

Pulse wave analysis was used to evaluate measures of blood pressure and wave reflection. Participants rested in a supine position while brachial artery blood pressure waveforms were obtained from an oscillometric cuff (Sphygmocor, AtCor Medical, Sydney, Australia). A validated, computerized transfer function reconstructed aortic blood pressure waveforms. Augmentation Index (AIx) was used to measure wave reflection. Left ventricular contraction of the heart generates a forward traveling pressure wave toward the periphery called an early systolic pressure peak; upon reaching bifurcations or changes in vasomotor tone, reflected pressure waves return toward the heart and generate a late systolic peak. If wave reflection occurs during the systolic phase, then the heart must contract harder to overcome this increased afterload pressure and the difference of late – early systolic peaks (or augmentation pressure (AP)) is positive. If the wave reflection occurs during the diastolic phase, then the ventricle is closed off to this afterload, and the AP is negative. AIx was calculated as the ratio of the difference between early and late systolic pressure peaks ($P_2 - P_1$) over pulse pressure ($SBP - DBP$). Due to the effect of heart rate on AIx, this measure was

also normalized to a heart rate of 75 beats per minute (AIx75) by pulse wave analysis software, Sphygmocor.

$$\mathbf{AIx} (\%) = \frac{(P_2 - P_1)}{(SBP - DBP)} \times 100$$

Pulse Wave Velocity (PWV)

PWV is obtained through carotid applanation tonometry (Sphygmocor, AtCor Medical, Sydney, Australia). A high-fidelity tonometer is used to obtain pressure waveforms from the right common carotid artery while femoral pressure waveforms are measured by a blood pressure cuff secured around the right upper thigh. A standard tape measure is used to measure three distances: from the right common carotid artery pulse site to sternal notch, from the sternal notch to the top of the thigh cuff, and from the top of the thigh cuff to the femoral artery pulse site. PWV is measured as the time a blood pressure waveform takes to travel from the aorta to the femoral artery. Thus, these distance measurements are used to calculate a central path distance to gauge central arterial stiffness.

$$\mathbf{PWV} \text{ (m/s)} = \frac{\Delta \text{ distance}}{\Delta \text{ time}}$$

Induced Inflammation via Influenza Vaccine

Participants were screened for seasonal influenza vaccine eligibility according to CDC and University of Maryland Health Center guidelines. After all baseline vascular testing was completed during Visits 2 and 5, participants were given a notecard labeled “Shot A” or “Shot B” to present to the nurse who administered the injections. Participants were walked by researchers to the University of Maryland

Health Center, where the nurse additionally screened each participant for vaccine eligibility on both visits and administered the assigned shot.

Blood Collection, Storage, and Analysis

Blood was collected from an antecubital vein using University of Maryland Health Center-certified aseptic techniques. Plasma samples were spun immediately at room temperature and at 2,000 x g for 20 minutes. Serum samples sat for 45 minutes at room temperature before being spun at 1,500 x g for 15 minutes at -4°C. Immediately after centrifugation, serum and plasma were pipetted into 50 μ L aliquots and stored in a -80° C freezer until future analysis.

Interleukin-6 was selected as the primary systemic inflammatory marker for analysis. Quantification of circulating serum IL-6 was measured using commercially available high sensitivity enzyme-linked immunosorbent assay kits ((ELISA) HS600C; R&D Systems, Biotechne, Minneapolis, MN). ELISA kits are a common laboratory technique consisting of a 96-well plate that is used to quantify concentrations of antigen, antibodies proteins or hormones in blood samples (Engvall & Perlmann, 1971). To quantify IL-6 concentrations, a sandwich ELISA is used. The 96-well plate contains embedded antibodies that will attach to the IL-6 in serum or plasma, then an antibody specific to binding with IL-6 is added after an incubation and washing period. Solutions are added to provide a colorimetric effect: presence of the substance being quantified will be indicated by color in the plate wells (Engvall & Perlmann, 1971). Using a micro-plate reader, absorbances of the color can be plugged into an equation to determine well-specific concentrations.

In young, healthy adults, circulating serum IL-6 have been found in concentrations less than 5 pg/mL (Schroeder et al., 2018). Due to the low ranges of circulating IL-6, high-sensitivity ELISA kits are to be used for inflammatory marker quantification in this project. R&D Systems' high sensitivity IL-6 ELISA has an assay range of 0.2 – 10 pg/mL with a sensitivity of 0.09 pg/mL, as compared to the normal sensitivity kit that has an assay range of 3.1 – 300 pg/mL with a sensitivity of 0.7 pg/mL. Due to serum IL-6 being on the lower end of this detection range, high-sensitivity is deemed the more accurate option in accurate biomarker quantification.

Statistical Analysis

This study included two groups, defined by race. For both the aims, the analyses will be performed using repeated measures analysis of variance (ANOVA) with race as the between-subject condition, and inflammation stimulus as the within-subject effects. Normality and distributional assumptions were assessed and non-parametric methods were used when appropriate.

For **Specific Aim 1**, inflammatory cytokine concentrations were measured under two conditions: inflammation (influenza vaccine) and control (saline shot). The dependent variable was change in levels of proinflammatory cytokine (IL-6) concentration. The ANOVA examined effect of race and time in responses to each condition, with race categorized as AA or CA and time defined as change in IL-6 concentration from baseline to the 24 hour and 48 hour follow up visits, 24-BL and 48-BL.

For **Specific Aim 2**, flow-mediated dilation (FMD) in AA and CA was assessed before and after administration of the inflammatory stimulus and control. The dependent variable was change in FMD. The ANOVA examined effect of race and time in responses to each condition, with race categorized as AA or CA and time defined as change in IL-6 concentration from baseline to the 24 hour and 48 hour follow up visits, 24-BL and 48-BL.

Post-hoc ANOVAs were run for the vascular measures listed in the exploratory aims (A1x, A1x75, brachial and aortic blood pressures, carotid artery β -stiffness and arterial compliance, carotid-femoral PWV). In addition, body mass index (BMI) and VO₂ peak values were compared using Student's t tests to determine similarity in these measures for participants from both race groups. Since BMI over 30 kg m⁻² (classification of Obesity; National Heart, Lung and Blood Institute, 2015) was considered exclusionary criteria, all eligible participants should be underneath this categorization, thus were free of the potentially confounding impact of obesity on vascular measures. Since participants were not screened based on physical activity or fitness levels, there may be variation in cardiorespiratory fitness between the two cohorts. If Student's t-tests were to reveal differences in BMI or VO₂ peak between AA and CA groups, then that respective variable were to be examined as a covariate in an analysis of covariance (ANCOVA). ANCOVAs would determine whether VO₂ peak or BMI values influenced or interacted with the vascular or inflammatory responses in AA and CA groups using linear regression analysis.

Sample size/statistical power considerations: For repeated measures ANOVA type models, conventions of magnitude of the effect size are 0.1 for small effect, 0.25 for

moderate effect, and 0.4 for large effect size, according to Cohen's d. A power analysis was completed based on previously published literature that examined FMD in induced-inflammation studies. In order to observe a large effect size, a minimum per group sample size of 10 was needed to achieve a power of 95% at significance level of 0.05 for detecting within subject and between subject interactions for FMD. Statistically significant effects were calculated to be capable of detection with 10 participants per group, at an α -level of $p = 0.05$. With at least 10 AA participants and 10 CA participants, we were looking to recruit approximately 20 participants in total.

RESULTS

Participant Characteristics

A total of 31 participants consented into the study. Due to dropouts, scheduling issues, or inability to obtain vascular measures or blood draws, only participants with full data sets of 24-BL and 48-BL measures were included in analyses for each experimental or control condition. For the two primary variables, IL-6 data included 9 AA and 15 CA for the influenza vaccine (inflammation condition) and 11 AA and 14 CA for the saline injection (control condition); FMD data included 10 AA and 17 CA for the inflammation condition and 11 AA and 16 CA for the control condition. All remaining measures of blood pressure, wave reflection and arterial stiffness were comprised of 11 AA and 15 CA for inflammation condition and 12 AA and 15 CA for control condition data. Characteristics for all included participants are listed in *Table 1*. There were no differences between AA and CA participants for age or BMI. Aerobic capacity, as measured by VO_2 peak, was significantly higher in the CA group as compared to their AA counterparts ($41.1 \pm 8.0 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ vs. $34.3 \pm 7.1 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, $p<0.05$).

Table 1. Participant characteristics

	African Americans (n=13)	Caucasian Americans (n=17)
Females/Males	6/7	9/8
Age (years)	21 ± 2	21 ± 3
BMI ($\text{kg}\cdot\text{m}^{-2}$)	24.1 ± 3.5	23.0 ± 2.5
VO_2 peak ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	34.3 ± 7.1	$41.1 \pm 8.0^*$

Data are expressed as means $\pm$ SD. BMI, body mass index. * $p<0.05$ statistically significantly different from AA group.

Table 2 shows IL-6 and vascular data for participants at baseline visits for both inflammation and control conditions. Student's t tests confirmed no statistically significant differences between race groups or condition baseline visits for any of the data ($p>0.05$). In evaluation of the hemodynamic measures, all participants had clinically normal baseline brachial blood pressures.

Table 2. Participant data at baseline visits for each condition

Variables	Inflammation Condition		Control Condition	
	AA	CA	AA	CA
[IL-6] pg/mL	1.42 $\pm$ 2.1	1.48 $\pm$ 2.4	1.18 $\pm$ 0.8	1.42 $\pm$ 1.7
FMD (%)	7.94 $\pm$ 5.8	6.22 $\pm$ 2.9	9.32 $\pm$ 4.1	8.23 $\pm$ 4.1
Brachial SBP (mmHg)	118 $\pm$ 9	120 $\pm$ 9	121 $\pm$ 8	119 $\pm$ 9
Brachial DBP (mmHg)	71 $\pm$ 5	68 $\pm$ 7	70 $\pm$ 5	69 $\pm$ 5
Aortic SBP (mmHg)	103 $\pm$ 7	104 $\pm$ 7	106 $\pm$ 6	104 $\pm$ 7
Aortic DBP (mmHg)	71 $\pm$ 5	69 $\pm$ 7	71 $\pm$ 4	70 $\pm$ 5
MAP (mmHg)	83 $\pm$ 6	82 $\pm$ 7	83 $\pm$ 4	82 $\pm$ 5
AIx (%)	7 $\pm$ 6	4 $\pm$ 7	6 $\pm$ 7	3 $\pm$ 11
AIx75 (%)	1 $\pm$ 7	-5 $\pm$ 9	-2 $\pm$ 7	-5 $\pm$ 13
cfPWV (m/s)	5.6 $\pm$ 0.5	5.5 $\pm$ 0.5	5.6 $\pm$ 0.4	5.8 $\pm$ 0.6
β -Stiffness (a.u.)	4.9 $\pm$ 1.1	5.5 $\pm$ 1.0	5.1 $\pm$ 1.3	5.4 $\pm$ 1.7
AC (mm ² kPa ⁻¹)	1.1 $\pm$ 0.2	0.9 $\pm$ 0.2	1.01 $\pm$ 0.15	1 $\pm$ 0.3
Elastic Modulus (a.u.)	60 $\pm$ 16	68 $\pm$ 13	63 $\pm$ 15	67 $\pm$ 23

Data are expressed as means $\pm$ SD. AA, African-American; CA, Caucasian-American; IL-6, interleukin-6; FMD, flow-mediated dilation; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure, AIx, augmentation index; cfPWV, carotid-femoral pulse wave velocity; AC, arterial compliance.

Induced Inflammation

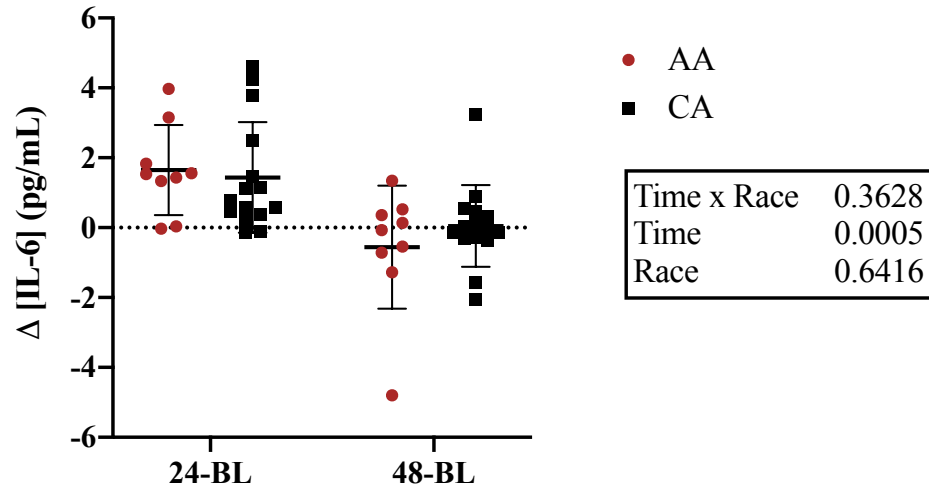
To assess the magnitude of a systemic inflammatory response, serum concentration of IL-6 was measured as differences, or deltas (Δ), of follow-up visit concentrations as compared to baseline concentrations (24-BL vs. 48-BL). Previous studies that have used this vaccine model of induced inflammation have evaluated inflammatory and vascular responses as changes from baseline, which typically accounts for any baseline differences between groups. However, no baseline differences were found for any measures in this study between race groups. *Figure 4* illustrates the change in serum IL-6 concentration in AA and CA groups, in panels separated by experimental conditions of influenza vaccine and sham saline injections. After administration of the influenza vaccine, two-way ANOVA revealed significance for an effect of time on the changes in IL-6 concentration ($p=0.0005$), but not race, as was hypothesized ($p>0.05$). While inflammation was induced in all participants as a result of the influenza vaccine, AA and CA adults seem to respond similarly to this stimulus. After administration of the saline injection, no significance was found for changes in IL-6 concentration for time, race or interaction effect, indicating no inflammation was present as a result of the saline control condition.

This study was not appropriately powered for an ANOVA that would examine race, time, and experimental condition factors in one analysis. In order to further examine between experimental conditions within each race group, follow-up ANOVAs were run to compare serum IL-6 concentrations in response to influenza vaccine and saline in AA and CA groups separately, as shown in *Figure 5*. In the AA group, two-way ANOVA confirmed an interaction effect for time and experimental condition

($p=0.0384$). In CA, significance was found for effects of time ($p=0.0019$), experimental condition ($p=0.0051$), and interaction ($p=0.0096$). These data suggest that AA and CA groups had differential responses between experimental conditions, which further confirms the influenza vaccine was successful as an acute systemic inflammatory stimulus and the saline injection as a control counterpart.

A)

[IL-6] Responses to Influenza Vaccine



B)

[IL-6] Responses to Saline

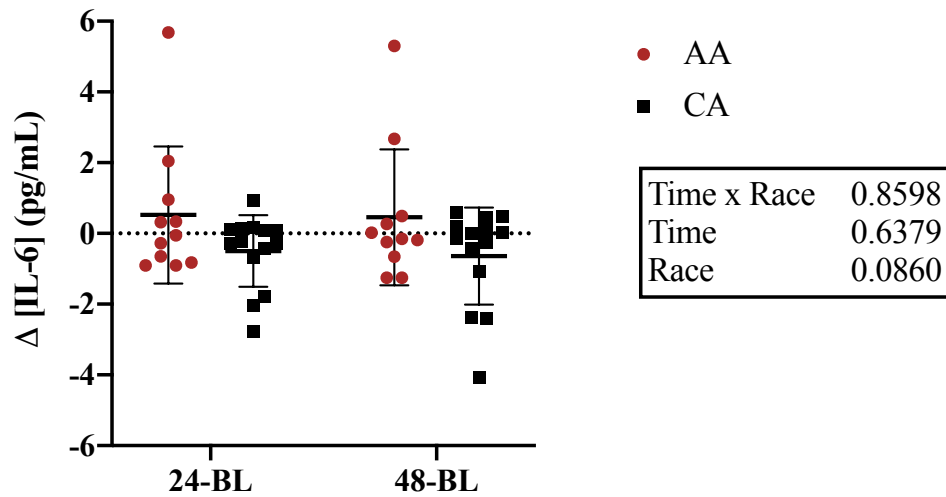
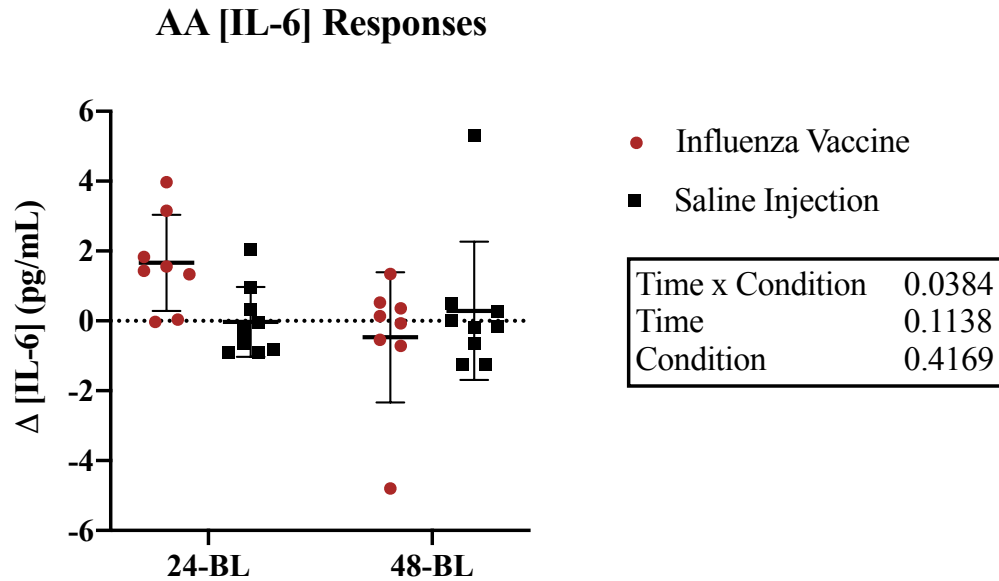


Figure 4. Changes in IL-6 concentration after influenza and saline injections. Values are expressed as means $\pm$ SD. Two-way ANOVA *p* values are included in each panel for factors of race, time, and interaction effect.

A)



B)

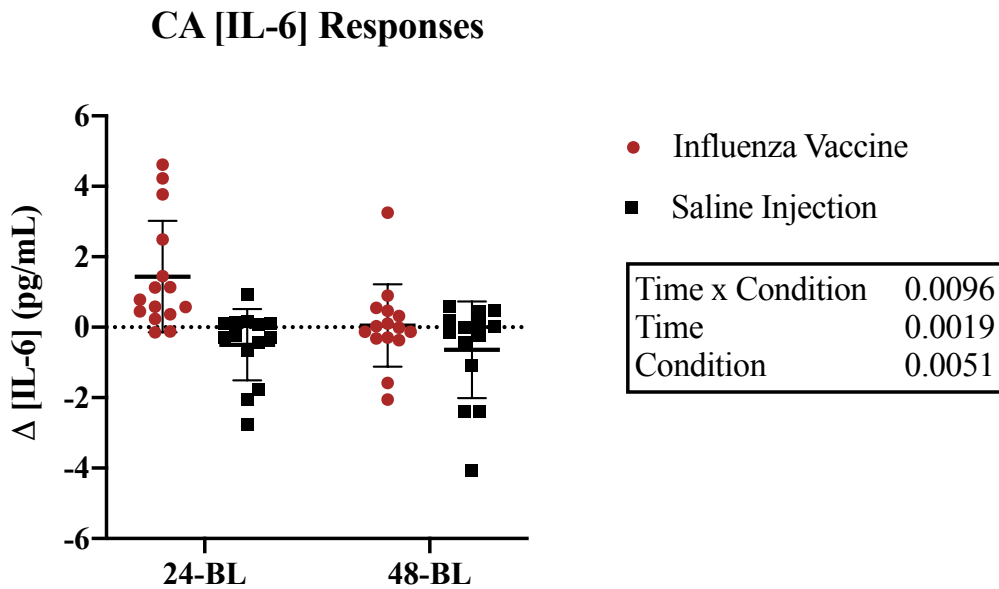
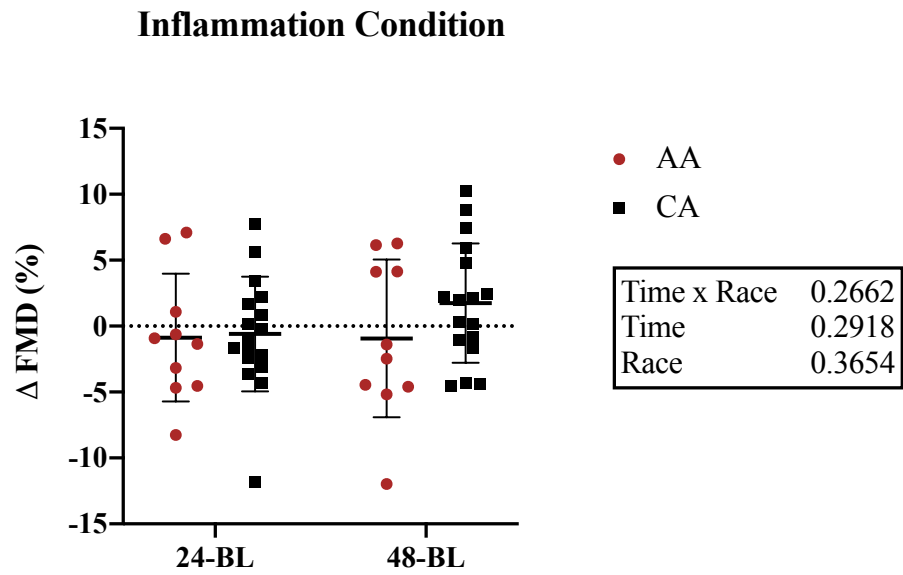


Figure 5. *Changes in serum IL-6 concentration by race in A) AA and B) CA participants. Values are expressed as means $\pm$ SD. Two-way ANOVA p values are included in each panel for factors of race, time, and interaction effect.*

Vascular Function

All reported FMD values were calculated from 3-second binned averages, mentioned earlier. Two-way ANOVA revealed no significant effects of time, race or interaction on FMD responses in AA and CA after administration of the influenza vaccine, as shown in *Figure 6* ($p>0.05$). Interestingly, two-way ANOVA for FMD responses during the control condition revealed a significant effect for time ($p=0.0490$) and interaction of time and race ($p=0.0483$), despite lack of inflammation. Student's *t* tests were run to analyze these data and no baseline differences in FMD were found between race groups or experimental conditions (*Table 1*). Student's *t* tests were then run to compare each baseline FMD of AA and CA groups to their respective 24 hour and 48 hour follow up values within each experimental condition, reported in *Table 3*. The AA follow up visit FMD values were not statistically significantly different from baseline for either condition. For the inflammation condition, CA follow up visit FMD values were not statistically significantly different from baseline. However, during the control condition, CA 24 hour follow up FMD was statistically significantly lower than baseline ($8.41 \pm 4.1\%$ at baseline vs. $5.86 \pm 2.0\%$ at 24 hours, $p<0.05$), but the 48 hour follow up was not statistically significantly different. The drop in FMD during control condition was not expected, however, daily fluctuations are possible and the values returned to baseline at 48 hours after saline injection administration.

A)



B)

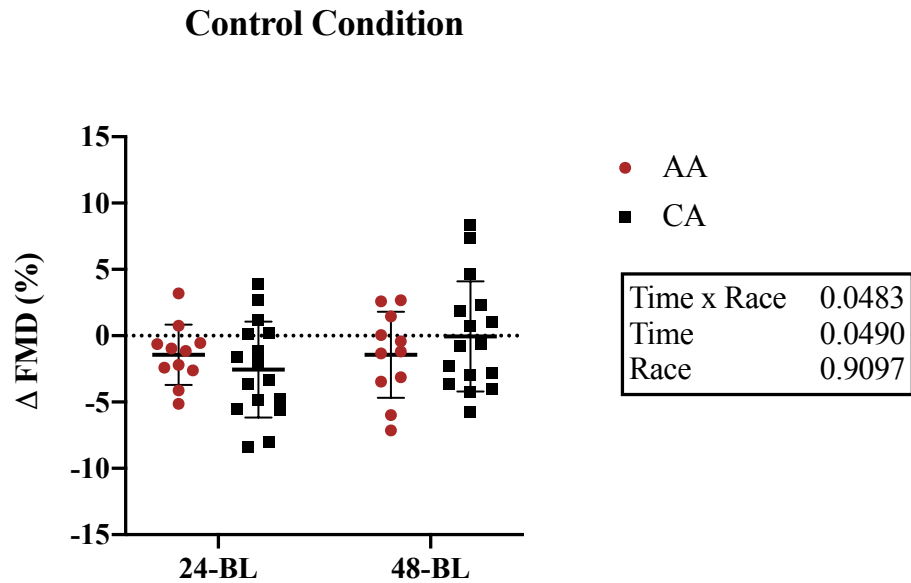


Figure 6. Changes in FMD in response to influenza vaccine and sham saline injections. Two-way ANOVAs revealed no significant differences for effects of time, race, or interaction after administration of A) influenza vaccine ($p > 0.05$). After administration of the B) sham saline, two-way ANOVA revealed significant effects of time ($p = 0.0490$) and interaction of time and race ($p = 0.0490$). Values are expressed as means $\pm$ SD.

Table 3. FMD values at each visit.

	Inflammation Condition			Control Condition		
	BL	24 post	48 post	BL	24 post	48 post
AA	8.42±5.9	7.54±4.3	7.48±5.4	9.85±3.8	8.41±3.6	8.40 ±4.5
CA	6.22±2.9	5.62±2.8	7.97±4.1	8.41±4.1	5.86±2.0*	8.35±3.2

Data are expressed as means $\pm$ SD. * represents statistically significantly different from baseline of the same condition, $p<0.05$.

Hemodynamics

Hemodynamic variables included brachial systolic and diastolic blood pressures (bSBP, bDBP), aortic systolic and diastolic blood pressures (aSBP, aDBP), and mean arterial pressure (MAP). Two-way ANOVA revealed no significant effects of time, race, or interaction on any of the brachial blood pressure responses in either inflammation or control conditions ($p>0.05$), as illustrated in *Figure 7*. *Figure 8* shows responses of aortic hemodynamics. Two-way ANOVA revealed no significant effects of time, race, or interaction for either inflammation or control conditions on responses of aortic systolic, diastolic or mean arterial pressures ($p>0.05$). These results suggest inflammation had no impact of brachial or aortic hemodynamics and that AA and CA groups respond similarly to the inflammatory stimulus.

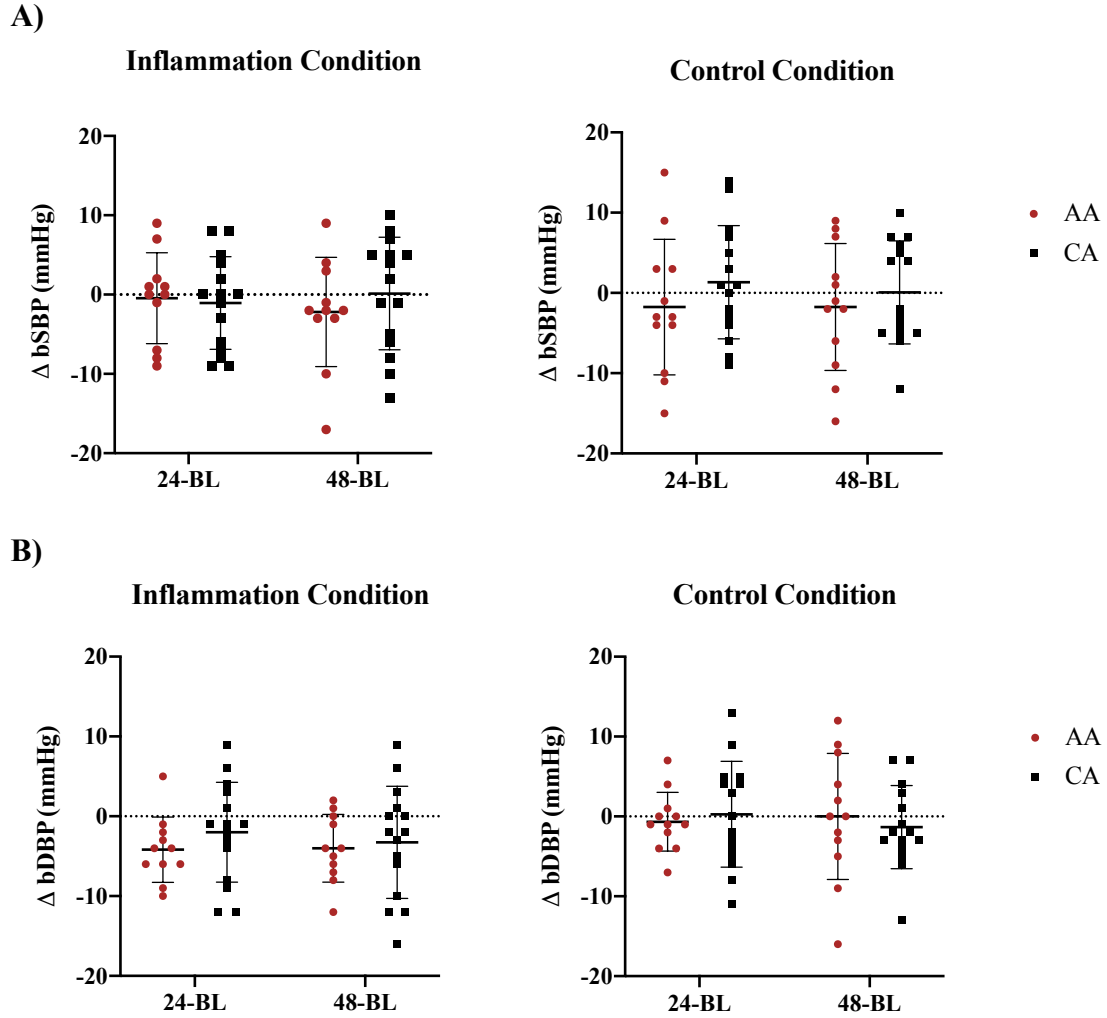


Figure 7. Changes in brachial blood pressures after administration of influenza vaccine and sham saline injection. Two-way ANOVAs revealed no significant effects of time, race, or interaction for A) brachial systolic blood pressure or B) brachial diastolic blood pressure in either experimental condition ($p > 0.05$). Values are expressed as means $\pm$ SD.

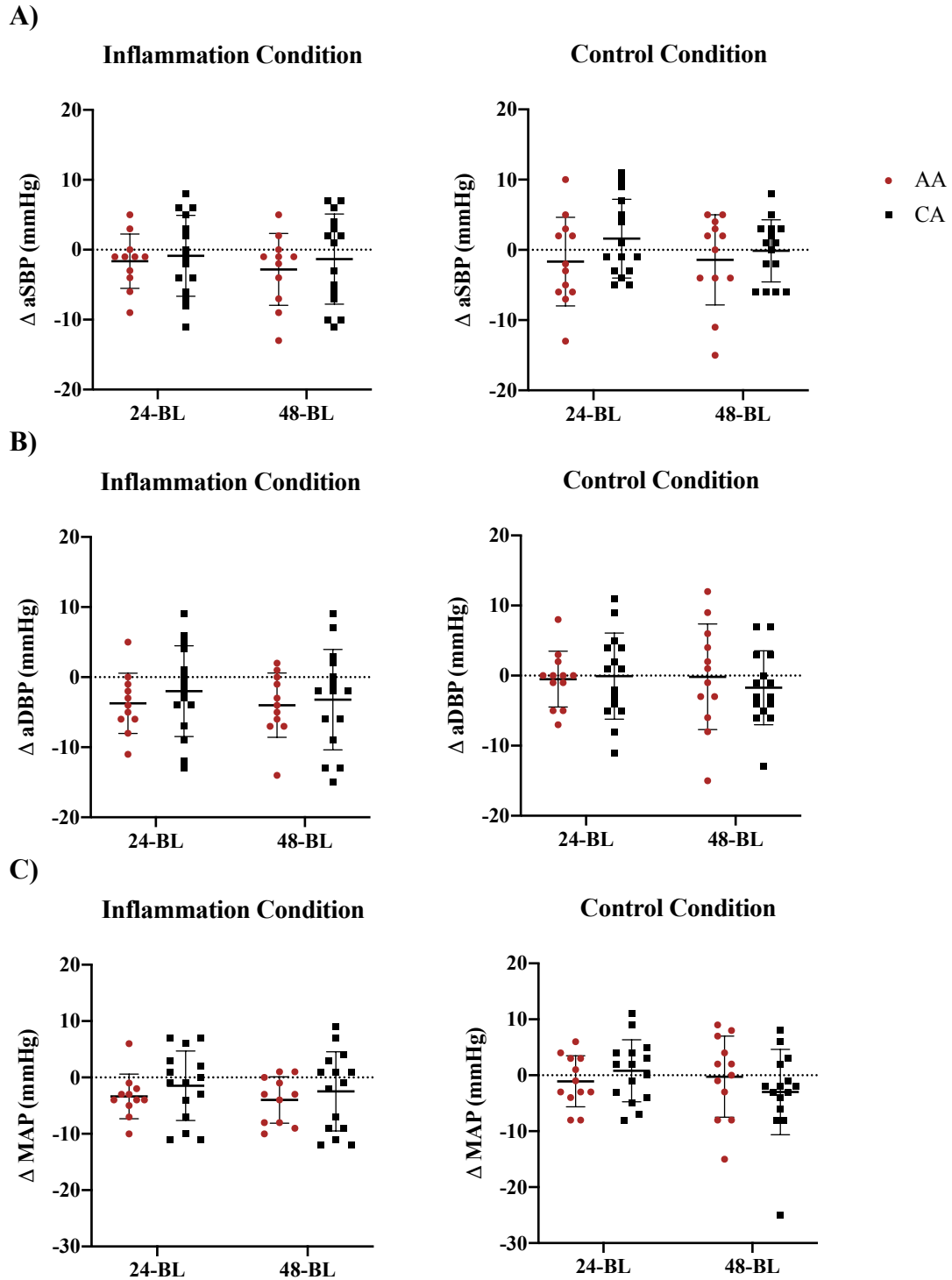


Figure 8. Changes in aortic blood pressures after administration of influenza vaccine and sham saline injection. Two-way ANOVAs revealed no significant effects of time, race, or interaction for A) aortic systolic blood pressure, B) aortic diastolic blood pressure, and C) mean arterial pressure ($p > 0.05$). Values are expressed as means $\pm$ SD.

Wave Reflection and Arterial Stiffness

Two-way ANOVA revealed no significant effects of time, race, or interaction on responses of wave reflection, as measured by AIx and AIx75, in either inflammation or control conditions (*Figure 9*). Additionally, no significant effects were detected for measures of central arterial stiffness, as measured by cfPWV (*Figure 10*), or ultrasound indices of carotid artery stiffness, as measured arterial compliance (AC), β -stiffness, and elastic modulus (Ep) (*Figure 11*) for either experimental condition. As no race differences were detected in wave reflection or arterial stiffness measures in response to inflammation, these data suggest young, health AA adults respond similarly to such a stressor as do young, healthy CA adults.

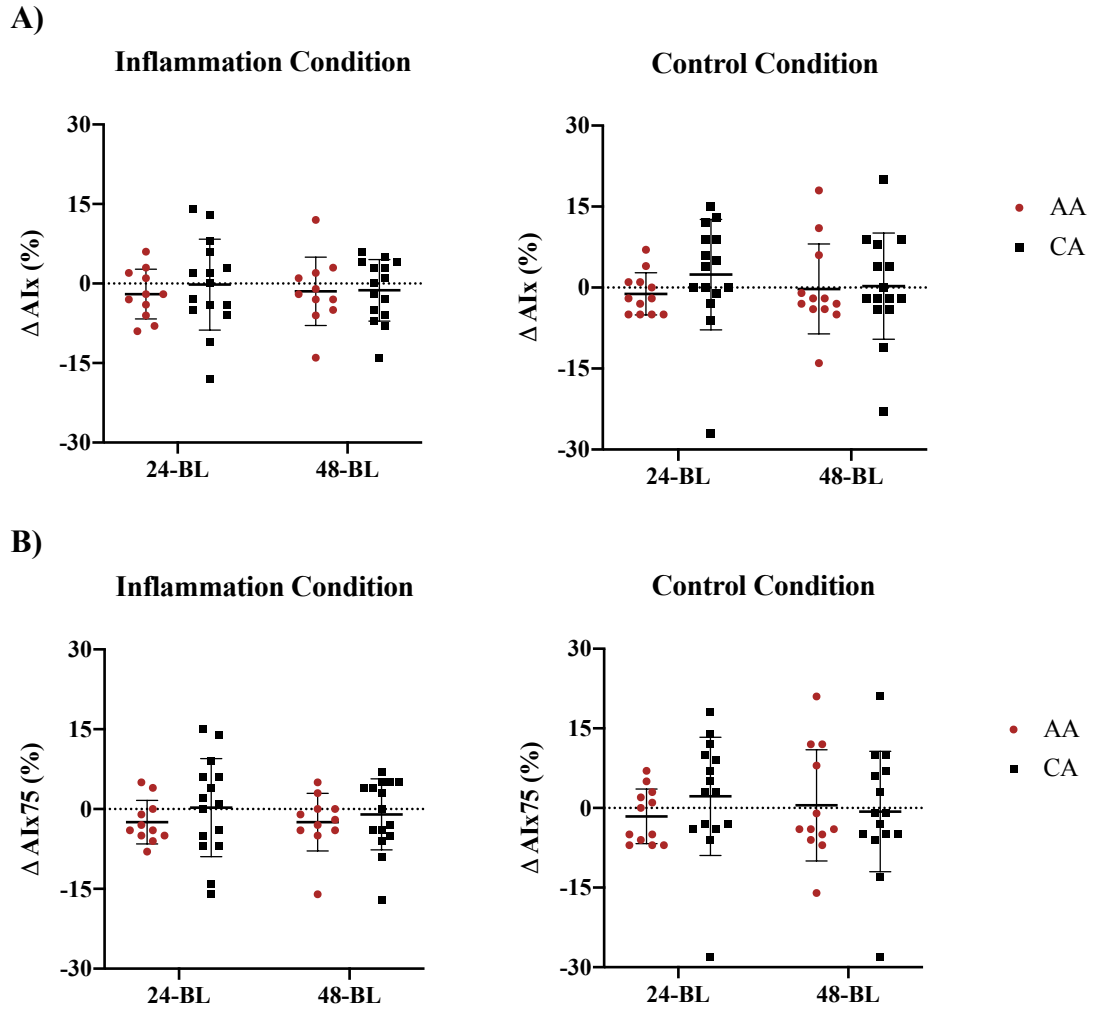


Figure 9. *Changes in augmentation index after administration of the influenza vaccine and sham saline injection. Two-way ANOVAs revealed no significant effects of time, race, or interaction for responses of A) augmentation index and B) augmentation index standardized to heart rate of 75 beats per minute ($p > 0.05$). Values are expressed as means $\pm$ SD.*

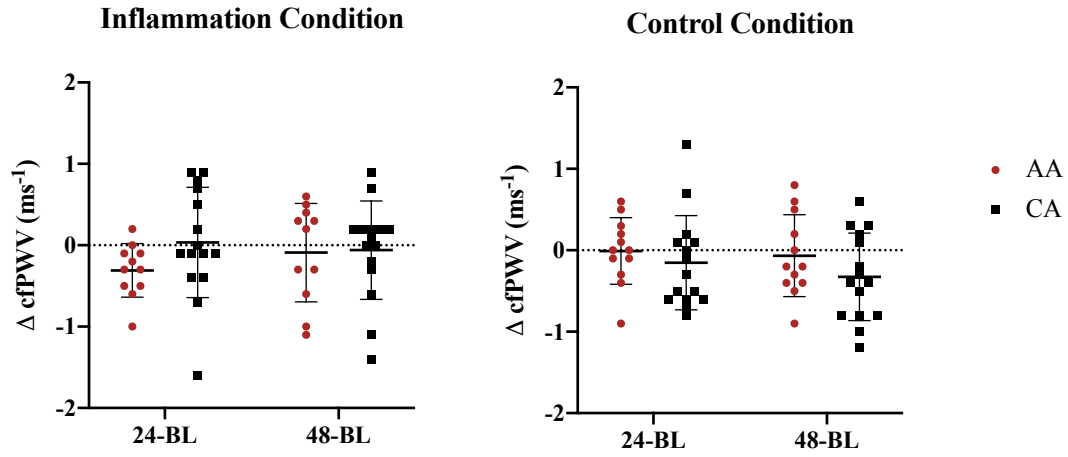


Figure 10. Changes in cfPWV after administration of the influenza vaccine and sham saline injection. Two-way ANOVAs revealed no significant effects of time, race, or administration of the influenza vaccine ($p > 0.05$). Values are expressed as means $\pm$ SD.

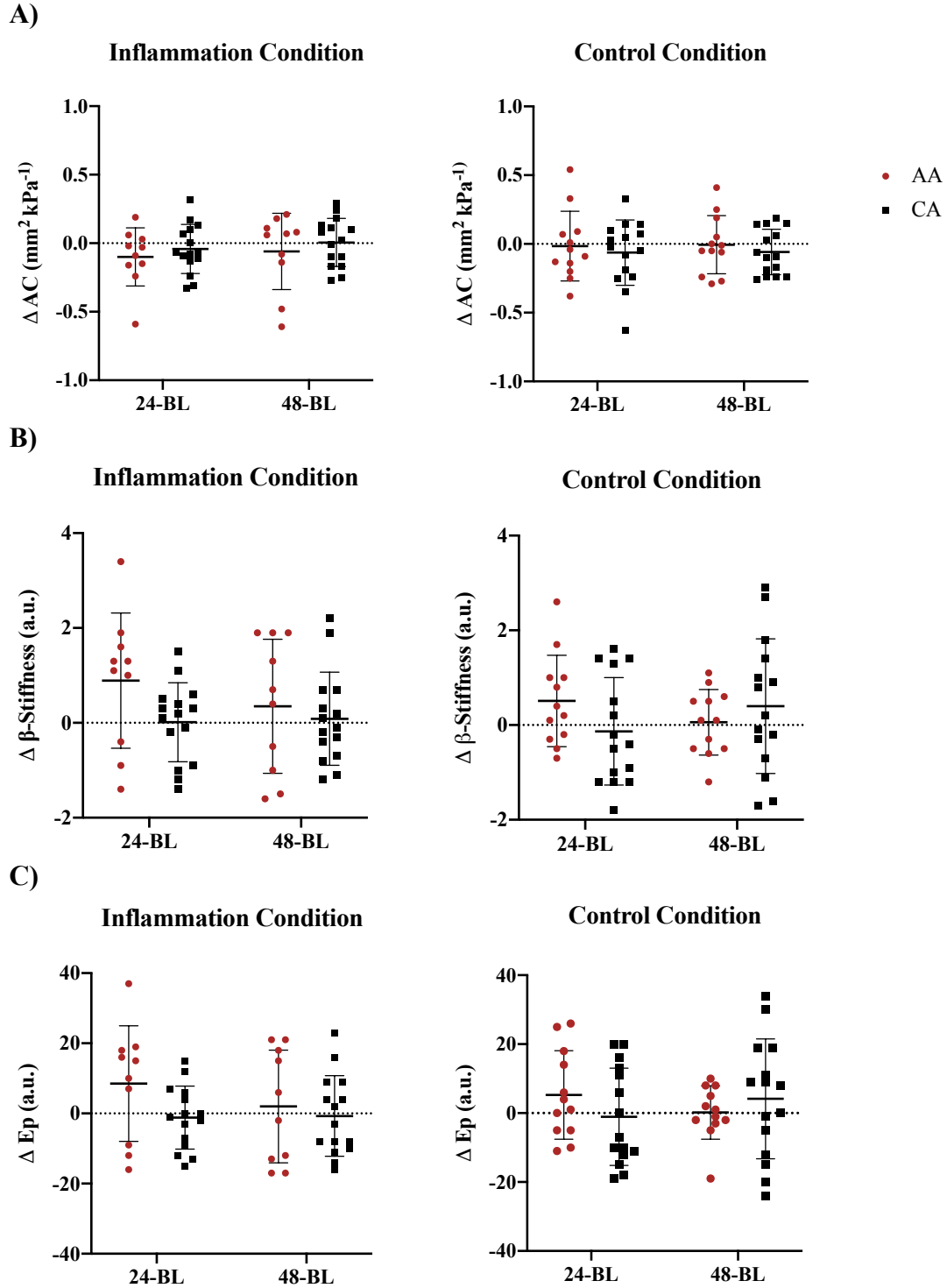


Figure 11. Changes in ultrasound measures of carotid artery stiffness after administration of the influenza vaccine and sham saline injection. Two-way ANOVAs revealed no significant effects of time, race, or interaction for delta measures of A) arterial compliance, B) β -stiffness, and C) elastic modulus ($p > 0.05$). Values are expressed as means $\pm$ SD.

DISCUSSION

The study sought to determine whether young, healthy AA adults would exhibit higher concentrations of inflammatory cytokines in response to the seasonal influenza vaccine compared with their CA counterparts and whether systemic inflammation would result in a larger detriment to vascular function in the AA participants compared with the CA participants. Seasonal influenza vaccines have previously been used to induce systemic inflammation (Liuba et al., 2007; Jae et al., 2009; Ranadive et al., 2014). The influenza vaccine model of inducing inflammation was validated in this study, as shown by the significant increase in IL-6 seen 24 hours after vaccine administration – an increase not demonstrated after sham saline injection (*Figure 4*). Despite the increase in IL-6 observed in both AA and CA groups separately (*Figure 5*), there were no race differences in the IL-6 response to induced inflammation.

Induced Inflammation

Circulating levels of IL-6 are commonly used to measure inflammatory responses. In both AA and CA groups, serum IL-6 increased significantly 24 hours after influenza vaccine administration, confirming that systemic inflammation was induced with our model. The inflammatory response returned to baseline levels of IL-6 concentrations at 48 hours. With administration of the sham saline injection, serum IL-6 levels did not change significantly after 24 or 48 hours, confirming no inflammatory response was present during this control condition.

Previous literature has reported race differences in resting inflammatory cytokine concentrations, with older AA adults exhibiting augmented inflammation as

compared to CA counterparts; however, there are limited studies of this evidenced in young, non-diseased cohorts. In 2011, Paalani et al. conducted a study where participants aged 35-102 years old self-reported as either “black” or “white”; the black cohort was found to have higher circulating levels of CRP (mean 0.12 mg/mL in black vs. 0.09 mg/mL in white, $p=0.010$) and IL-6 (mean 3.37 pg/mL in black vs. 2.34 pg/mL in white, $p=0.000$) compared with their white counterparts. Approximately 29% of these participants reported they were diagnosed with cardiometabolic or inflammatory conditions; when these diagnoses were covaried for, IL-6 maintained its significant association with race, but CRP levels were no longer significantly different between blacks and whites (Paalani et al., 2011). Other studies with middle-aged and older adult AA populations have also shown higher levels of CRP (Khera et al., 2005; Slopen et al., 2010; Veerabhadrapa et al., 2011) and IL-6 (Carroll et al., 2009), yet these population-based cohorts included participants with cardiometabolic disease. Chronic disease and age are known contributors to increased chronic inflammation, thus elucidation of race differences in resting levels of inflammation needs to be evaluated in a population free of the potentially confounding factors of age and disease, such as the young, healthy cohort of this current study.

In order to examine *in vitro* versus *in vivo* oxidative stress and inflammatory responses, one group studied AA and CA human umbilical vein endothelial cells (HUVECs) in comparison to plasma from AA and CA adults aged 18-25 years old, (Feairheller et al., 2011). AA HUVECs had higher IL-6 concentrations than the CA HUVECs, yet this difference was not found in the young adult plasma samples. Augmented inflammatory profiles have been considered to contribute to the higher

incidence of hypertension and CVD in AA populations, yet this distinction is not entirely evident in young and healthy AA adults. To our knowledge, there is limited literature that has examined resting IL-6 concentrations between young, healthy AA and CA adults, which would control for the possibly confounding role that age plays in inflammatory biomarker concentration. Our participant cohort was comprised of healthy participants aged below 30 years old, eliminating the potential roles of age or disease on inflammatory responses. This study did not find any differences in baseline serum IL-6 concentrations between AA and CA groups, suggesting that perhaps the augmented inflammatory cytokine profiles seen in older AA adults becomes evident at a later age. Additionally, it is plausible that racial differences that exist in resting levels of inflammatory markers in young and healthy adults may be revealed with an inflammatory stimulus stronger than the influenza vaccine utilized in this study.

Vascular Function

Contrary to our hypothesis, inflammation had no effect on the FMD responses in AA and CA groups. These results are also in contrast to existing literature which have shown inflammation, induced by influenza vaccine, to impair endothelial function. FMD is largely attributable to endothelium dependent NO. Of note, a meta-analysis determined that the NO contribution to FMD ranges between 47-72%, depending on technique specifications and the population cohort studied (Green et al., 2014). Impaired endothelium-dependent dilation via reduced NO bioavailability during inflammation has been attributed to increased production of reactive oxygen species, which scavenge free NO (Clapp et al., 2004), as well as the inhibition of eNOS

expression by the inflammatory TNF- α – leading to less NO substrate availability (Steyers & Miller, 2014). We hypothesized that higher levels of inflammation after administration of the influenza vaccine would attenuate FMD due to this decrease in endothelium-dependent NO. While our control condition was confirmed by lack of inflammation, the results found a decrease in CA FMD at 24 hour follow up to the sham saline injection. As this was the control condition, no changes were expected in vascular measures after administration of the saline injection, however, daily fluctuations in FMD may occur due to outside factors such as stress or inadequate sleep. The drop in FMD was not unanimous throughout both groups, as AA FMD at follow up visits were similar to baseline for the control condition.

Clapp et al. demonstrated impaired endothelial function in conjunction with increases in IL-6 8 hours following *Salmonella typhi* vaccination (2004); additionally, Hingorani et al. reported elevated IL-6 concentrations and depressed FMD 8 hours after *S. typhi* vaccination (2000). Specific to the influenza vaccine, one study found elevated CRP 2 days after vaccine administration, concomitant with decreased FMD only in males (Liuba et al., 2007). Additionally, attenuated FMD was observed with increased IL-6 and CRP levels at 24 hour and 48 hour follow up visits to administration of influenza vaccine (Lane-Cordova et al., 2016). Interestingly, the same vaccine model did not result in changes in FMD in older adults, despite generation of systemic inflammation evidenced with the same biomarkers (Lane-Cordova et al., 2016; Ranadive et al., 2014). Our participants were young and healthy; due to the discrepancy in responses previously seen between young and older adults, it was hypothesized that FMD would decrease in response to inflammation in our young sample groups and that

this decrease would be augmented in the AA participants. However, with approximately half the dilation of FMD being attributed to NO (Green et al., 2014), it is possible that in the presence of inflammatory ROS, dilatory integrity was maintained due to upregulation of other vasoactive substances, such as adenosine triphosphate (ATP), prostaglandins, or endothelium-derived hyperpolarizing factor (Vanhoutte & Mombouli, 1996; Green et al., 2014).

Exploratory Measures

In comparing changes from baseline to 24- and 48-hours post-induced inflammation, the lack of significant effects of race, time or interaction for aortic and brachial blood pressures suggest that young, healthy adults maintain a cardiovascular adaptability in response to inflammation. Previous studies that used induced inflammation models have contradicting results for blood pressure responses. In 2005, Hingorani et al. found no significant changes in central or peripheral systolic and pulse pressures after *S. typhi* vaccination, despite reporting increases in arterial stiffness. In a separate study, young adults had significantly reduced MAP and DBP 24- and 48-hours post-induced inflammation (Lane-Cordova et al., 2016). Some differences in vascular responses have been postulated to be due to stimulus severity; vaccines that result in a more potent inflammatory response are thought to have differential effects on vascular reactions. However, this may be more evident in the magnitude of response of inflammatory cytokine concentration; while some studies utilizing vaccine models of inducing inflammation report differences in hemodynamic responses, others report differences in measures of arterial stiffness.

In our study, no significant effects of race, time or interaction were detected for changes in cfPWV or carotid artery ultrasound measures of AC, β -stiffness, and Ep at resting baseline visits or in response to inflammation; this is in contrast with literature that have found AA populations to have higher arterial stiffness than CA counterparts, in both young adults (Zion et al., 2003) and middle-aged and older adults (Din-Dzietham et al., 2004). Previous studies have also found augmented arterial stiffness in response to induced systemic inflammation in young adults (Vlachopoulos et al., 2005) and older adults (Jae et al., 2013). Studying acute responses of vascular measures like arterial stiffness in response to a physiological stressor like inflammation is valuable because acute responses may predict how long-term exposure to such stressors would affect vascular function, and in turn, cardiovascular risk. High resting levels of CRP have been shown to have direct correlations with increased aortic and brachial PWV (Yasmin et al., 2004). Increased arterial stiffness can augment wave reflection measures of AIx and AIx75 due to reduced wave reflection transit time, however this is not a concrete relationship. Vlachopoulos et al. reported increased arterial stiffness in response to induced inflammation, however, AIx had significantly decreased (2005). This may be attributed to medium- to small-branch arteriole dilation that slowed down wave reflections later in the arterial tree periphery; this suggested that AIx was controlled by downstream vasculature (Vlachopoulos et al., 2005). In our study, we found no changes in arterial stiffness or wave reflection in response to inflammation induced by the influenza vaccine, nor race differences between AA and CA groups' responses. Macrovascular function largely seemed unaffected by this acute inflammatory response. It is plausible that vasculature in young, healthy adults is able

to counteract the detrimental effects of inflammation through microvascular responses; this downstream mitigation could explain why arterial stiffness and wave reflection, as measured in our study, were unaffected during an inflammatory response. Measuring microvascular function was beyond the scope of this study, but worth investigating as a future direction.

Fitness and regular physical activity have long been recognized in combating CVD risk factors and all-cause mortality (Paffenbarger et al., 1989; Lee et al., 2011). Regular exercise, specifically aerobic training, has been shown to decrease resting levels of inflammatory cytokine concentrations in older adults (El-Kader & Al-Shreef, 2018; Monteiro-Junior et al., 2018). Furthermore, exercise training has been reported to normalize vascular dysfunction seen in obese adolescents (Watts et al., 2004). At rest, adults with higher levels of physical activity have been reported to exhibit decreased concentrations of inflammatory cytokines (Geffken et al., 2001; Colbert et al., 2004; Yu et al., 2009). Cardiorespiratory fitness may play an important role in vascular reactivity during systemic inflammation. In addition to the inverse association of cardiorespiratory fitness with CRP concentrations in young adults (Williams et al., 2005), aerobic fitness has also been shown to mitigate vascular dysfunction seen in response to induced inflammation in older adults. The increased arterial stiffness reported in older adults during inflammation is attenuated in older adults with higher aerobic fitness (Jae et al., 2013). Our study found the AA group had significantly lower VO_2 peak than the CA group; literature has shown that age-matched groups of AA populations typically exhibit lower VO_2 measures than their CA counterparts (Hunter et al., 2001; Wang et al., 2011). Covarying for VO_2 peak yielded no significant

associations between VO₂ peak and vascular responses to inflammation in our young, healthy cohort. In a previous study, FMD responses to inflammation were reported to be similar between young low-fit and moderately fit adults (Schroeder et al., 2018). It is possible that the benefits of aerobic fitness on vascular responses to inflammatory stressors are specific to older adult populations. Endothelial function is typically blunted in older adults when compared to their young counterparts; after induced inflammation, vascular function in the moderately fit, older adult group mimicked the responses seen in young adults (Schroeder et al., 2018). Thus, while aerobic fitness may not affect vascular integrity in young adults undergoing acute inflammation, enhancing fitness and increasing levels of habitual physical activity may preserve vascular integrity in diseased or older adult groups.

Limitations

One of the limitations of the current study is that IL-6 was the only inflammatory cytokine quantified. IL-6 was chosen due to its consistent expression in systemic inflammatory responses and its established use in detecting inflammation in vaccine model studies prior. Other biomarkers, such as CRP or TNF- α , have been previously used to validate generation of an inflammatory response and could have potentially exposed differential race responses that IL-6 could not identify. Additionally, this particular study did not specifically examine sex differences in inflammatory or vascular responses to the induced inflammation. Other factors that could contribute to stress within a human participant, like exams or sleep cycle

disturbances during perceived stressful weeks, could have potentially affected the results.

Conclusions

In conclusion, young, healthy AA and CA individuals had similar baseline levels of serum IL-6. After administration of the seasonal influenza vaccine, inflammation was induced in both groups, however, there were no differences in the way AA and CA groups responded in inflammatory cytokine concentrations. Baseline vascular function was similar between race groups, and acute systemic inflammation did not affect endothelial responses in these young, healthy AA and CA adults. AA individuals had similar hemodynamic, wave reflection, and arterial stiffness responses as compared to CA individuals following an acute systemic inflammation bout. Even when differences in cardiorespiratory fitness were covaried for, the lack of differential responses between race groups remained.

LITERATURE REVIEW

Cardiorespiratory Fitness and Inflammation

Cardiorespiratory fitness has been investigated as a potential mediator of vascular function in response to systemic inflammation. Paradoxically, an acute bout of exercise may serve as a physiological stressor on the human body. As regular exercise and physical activity have been established to decrease risk for CVD and all-cause mortality (Lee et al., 2011), it has also been investigated as a way to restore endothelium-dependent dilation in older males to look like the dilatory responses seen in young adult males (DeSouza et al., 2000). The short-term effects of single exercise bouts on vascular responses to inflammation have also been studied in order to elucidate a possible relation to the long-term benefits of cardiorespiratory fitness and regular exercise.

In young adults, exercise training has been shown to improve measures of vascular function and mitigate inflammatory responses. Cardiorespiratory fitness has been shown inversely associated with CRP levels in young adults, regardless of obesity, blood pressure, smoking or contraceptive use (Williams et al., 2005). One study found that in males, muscular fitness – independent of cardiorespiratory fitness – was inversely associated with CRP and IL-6 concentrations (Vaara et al., 2014). Obese adolescents underwent an 8-week circuit training program that benefited measures of functional capacity and strength, as well as improving FMD (Watts et al., 2004), which indicates that cardiovascular benefits do not only come from solely aerobic training, but can be seen from different forms of exercise training. To further this point, in a dual human-animal study where subjects were placed in a control,

aerobic training (AT), resistance training or high-intensity interval training (HIIT) group, PWV significantly decreased in both rats and humans that underwent either AT and HIIT programs (Hasegawa et al., 2018).

Numerous studies have shown that regular physical activity results in cardiovascular benefits for older adults. Regular physical activity, regardless of aerobic fitness level or race group, is established in decreasing concentrations of circulating inflammatory cytokines like CRP, IL-6, and TNF- α (Geffken et al., 2001; Colbert et al., 2004; Yu et al., 2009). In older adults, lower inflammatory marker levels have also been associated with improved physical fitness and performance (Taaffe et al., 2000). In response to induced inflammation, older adults with high cardiorespiratory fitness have displayed attenuated increases in arterial stiffness compared to low-fit older adults (Jae et al., 2013). In contrast, after influenza vaccine-induced inflammation, Schroeder et al. did not find any changes in arterial stiffness; however, they demonstrated preserved vascular reactivity in moderately-fit older adults as compared to their low-fit, age-matched counterparts (2018). While the low-fit older adults experienced no change in FMD, fit older adults exhibited decreased FMD after 24 hours, mimicking those of the young adults (Schroeder et al., 2018). As a possible explanation, it has been speculated that physically active older adults do not experience the same degree of NO bioavailability attenuation observed in non-fit older adults (Taddei et al., 2000). Additionally, Ranadive et al. suggested the concept of a basement effect for their cohort, wherein older adults may not elicit a typical impaired vascular response to inflammation due to already existing vascular dysfunction attributable to ageing, ROS, chronic low-grade inflammation, or decreased NO bioavailability (2014).

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