

An investigation of the mitochondria as a potential source of racial differences in reactive oxygen species production and cellular respiration in peripheral blood mononuclear cells of healthy adults

by

Dulce Hiraci Gomez

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 7, 2022

Keywords: PBMCs, Racial Differences, Cell Respiration,
Mitochondrial Complexes, ROS production

Copyright 2022 by Dulce Hiraci Gomez

Approved by

Michael D. Brown, Professor, School of Kinesiology, Auburn University
Austin Robinson, Assistant Professor of Kinesiology, Auburn University
Andreas Kavazis, Professor of Kinesiology, Auburn University
Suzanne Oparil, Professor of Cell, Developmental & Integrative Physiology, University of
Alabama Birmingham
Jianzhong Shen, Professor, Harrison School of Pharmacy, Auburn University

Abstract

Background: Non-Hispanic Black (NHB) adults exhibit a higher prevalence of cardiovascular disease (CVD) compared to Non-Hispanic White (NHW) adults. Vascular oxidative stress may be one contributing factor to these racial disparities. Peripheral blood mononuclear cells (PBMCs) are primary contributors to systemic oxidative stress with documented racial differences in PBMC-derived reactive oxygen species (ROS) production. However, it is unclear whether the racial differences in PBMC-derived ROS are associated with alterations in mitochondrial function. Therefore, the purpose of this study was to investigate potential racial differences in protein expression for mitochondrial complexes, antioxidant defenses, superoxide-generating NADPH oxidase subunits, and cellular respiration in PBMCs isolated from NHB and NHW adults.

Methods: PBMCs were isolated as an ancillary subproject for the “Neighborhood Disadvantage, Sleep, and Vascular Health: Racial Disparities in Cardiometabolic Health and Blood Pressure” Study (NCT04576338) from healthy, young participants (17 NHB & 24 NHW). PBMCs were cultured 14-18 hrs prior to cell respiration and protein harvest. A Clark Electrode measured rate of basal, leak, maximal, and non-mitochondrial respiration. Formulas were also used to determine coupling efficiency, adenosine triphosphate (ATP) demand, spare respiratory capacity, and OCR metabolic potential. Western blotting was used to measure antioxidant modulators of oxidative stress (superoxide dismutase [SOD] isoforms 1 & 2; acetylated SOD2 [AcSOD2], inactivate form; NAD-dependent deacetylase Sirtuin-3 [SIRT3]), superoxide-generating enzyme NADPH oxidase subunits (p47phox and gp91phox), and mitochondrial complexes I – V. Non-adjusted differences between groups were analyzed with a student’s t-test or Man-Whitney U test, if they showed non-normal distributions. **Results:** There were no racial differences in the modulators of oxidative stress or superoxide-generating NADPH oxidase subunits. PBMCs isolated from NHBs adults

exhibited a significantly higher expression for Complex I ($p=0.014$). There were no sex differences in NADPH oxidase subunits or mitochondrial complexes. However, PBMCs isolated from females exhibited a greater expression for the antioxidant enzyme SIRT3 ($p=0.038$). PBMCs isolated from NHBs adults exhibited a significantly lower rates for basal respiration, leak respiration, and non-mitochondrial respiration ($p=0.017$, $p=0.009$, $p=0.014$ respectively). Finally, there were no sex differences in cell respiration. **Conclusion:** While speculative, the lower respiration rates (basal, leak, and non-mitochondrial derived) and higher protein expression for Complex I in PBMCs isolated from NHB adults may contribute to elevated ROS production. While no racial differences were found in the redox regulating proteins, additional information are needed on SOD activity, NADPH oxidase activity, mitochondrial damage, and changes in metabolic phenotype in activated PBMCs.

Acknowledgments

Pies, para que los quiero si tengo alas para volar – Frida Kahlo.

Estoy al fin de otro capítulo en mi carrera profesional, y es gracias a tu esfuerzo, dedicación, y amor que me ayudo atravesar momentos débiles en mi educación y en mi salud. ¡Estoy muy bendecida y orgullosa de tenerte a mi lado para ver nuestros logros! ¡Madre mía, gracias por ayudarme obtener mis alas!

Students must have initiative; they should not be mere imitators. They must learn to think and act for themselves – and be free – Cesar Chavez

My journey as a doctoral student was quite the whirlwind! As a first-generation college graduate, I learned to navigate academia and search for opportunities to grow as a scholar and scientist. I am extremely grateful to my mentors, Dr. Michael D. Brown and Dr. Austin T. Robinson, for their unconditional support and letters of recommendations ready at hand to submit to any and all fellowship/awards I came across. I am also grateful to my committee members: Dr. Andreas Kavazis, Dr. Suzanne Oparil, and Dr. Jianzhong Shen for their insight, feedback and patience as I transitioned projects.

A special thanks to my previous lab members, Dr. Adelola Adeyemo and Dr. Maitha Aldokhayyil, for helping me attain the skills I needed to be an independent researcher in the lab! For keeping in contact with me even after graduating to cheer me on as I reach the end of this journey too! To my partner, A. J. Kam, for holding me accountable when I was overwhelmed by my perceived lack in progress, as I completed my chapters, and then my presentation. Finally, I would like to thank my village/community of friends for their emotional support as I reached each and every milestones in my doctoral journey. I love you all so much!

Table of Contents

Abstract.....	2 – 3
Acknowledgments.....	4
List of Tables	7
List of Figures.....	8
List of Abbreviations	9 – 11
Chapter 1: Introduction.....	12 – 17
Chapter 2: Literature Review.....	18 – 42
2.1 Cardiovascular Disease (CVD).....	18 – 21
2.1.1 Overview of CVD.....	18 – 19
2.1.2 CVD Progression and Risk Factors	19 – 20
2.1.3 Vascular Dysfunction.....	20 – 21
2.2 Reactive Oxygen Species (ROS)	21 – 29
2.2.1 Overview of ROS and Molecular Targets	21 – 23
2.2.2 Sources of ROS.....	23 – 27
2.2.3 ROS Scavengers and Antioxidants	27 – 29
2.3 Mitochondria.....	29 – 35
2.3.1 Overview of the Mitochondria.....	29 – 30
2.3.2 Mitochondrial Complexes and Their Role.....	30 – 32
2.3.3 Intact Cell Respiration Measures	33 – 35
2.4 Peripheral Blood Mononuclear Cells (PBMCs)	35 – 40
2.4.1 Overview of PBMCs.....	35 – 36
2.4.2 Source of Oxidative Stress within PBMCs.....	36 – 38

2.4.3 Mitochondrial Dysfunction and its Role in Vascular Dysfunction.....	38 – 40
2.5 Racial Differences in Vascular Health.....	40 – 42
Chapter 3: Methods.....	43 – 48
3.1 Participant Recruitment	43
3.2 PBMCs Isolations	43 – 44
3.3 PBMCs Experimental Preparation	44 – 45
3.4 Cell Respiration	45 – 46
3.5 PBMCs Protein Isolation	46
3.6 Western Blotting.....	47 – 48
3.7 Statistical Analyses	48
Chapter 4: Results	49 – 66
4.1 Demographic Characteristics	49
4.2 Protein Expression of Antioxidant Modulators of Oxidative Stress.....	49
4.3 Protein Expression of Superoxide-Generating NADPH Oxidase Subunits.....	49 – 50
4.4 Protein Expression of Mitochondrial ETC Complexes	50
4.5 PBMC Respiration	50 – 51
Chapter 5: Discussion	67 – 73
References	74 – 88

\

List of Tables

Table 1. Participant Characteristics	52
Table 2. Participant Characteristics by Race	53
Table 3 Participant Characteristics by Sex	54

List of Figures

Figure 1. Vascular Dysfunction and NO Bioavailability	23
Figure 2. NADPH Oxidase and ROS Production	26
Figure 3. Mitochondria and ROS Production	27
Figure 4. Antioxidant Enzymes and Defenses	29
Figure 5. Formation of ATP During Oxidative Phosphorylation	32
Figure 6. Protein Expression of Antioxidant Modulators of Oxidative Stress in PBMCs isolated from NHW and NHB Individuals	55
Figure 7. Protein Expression of Antioxidant Modulators of Oxidative Stress in PBMCs isolated from Males and Females	56
Figure 8. Protein Expression of Superoxide-generating NADPH Oxidase Subunits in PBMCs isolated from NHW and NHB Individuals.....	57
Figure 9. Protein Expression of Superoxide-generating NADPH Oxidase Subunits in PBMCs isolated from Males and Females	58
Figure 10. Protein Expression of Mitochondrial Complexes in PBMCs isolated from NHW and NHB Individuals	59 – 60
Figure 11. Protein Expression of Mitochondrial Complexes in PBMCs isolated from Males and Females	61 – 62
Figure 12. Respiration Measures in PBMCs isolated from NHW and NHB Individuals...	63 – 64
Figure 13. Respiration Measures in PBMCs isolated from Males and Females	65 – 66

List of Abbreviations

NHB	Non-Hispanic Black
CVD	Cardiovascular Disease
NHW	Non-Hispanic White
PBMCs	Peripheral Blood Mononuclear Cells
ROS	Reactive Oxygen Species
ATP	Adenosine Triphosphate
SOD	Superoxide Dismutase
AcSOD2	Acetylated SOD2
SIRT3	Sirtuin-3
HTN	Hypertension
NO	Nitric Oxide
O ₂ ⁻	Superoxide
ONOO ⁻	Peroxynitrite
H ₂ O ₂	Hydrogen Peroxide
OH•	Hydroxyl
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
EC	Endothelial Cells
ETC	Electron Transport Chain
Complex I	NADH Dehydrogenase
Complex III	ubiquinone-cytochrome <i>c</i> reductase
O ₂	Oxygen
Complex II	Succinate Dehydrogenase

Ang II	Angiotensin II
CHF	Congestive Heart Failure
HUVECs	Human Umbilical Vein ECs
NIH	National Institutes of Health
NHANES	National Health and Nutrition Examination Survey
AHA	American Heart Association
CV	Cardiovascular
VSMCs	Vascular Smooth Muscle Cells
NHA	Non-Hispanic Asian
BP	Blood Pressure
eNOS	Endothelial Nitric Oxide Synthase
BH ₄	Tetrahydrobiopterin
ET-1	Endothelin-1
RO ₂ •	Peroxyl Radical
RO•	Alkoxyl Radical
mtDNA	Mitochondrial DNA
K	Lysine residues
NADH	Nicotinamide Adenine Dinucleotide
FADH ₂	Flavin Adenine Dinucleotide
H ₂ O	Water
Complex IV	Cytochrome <i>c</i> Oxidase
Complex V	ATP synthase
ADP	Adenosine Diphosphate

FCCP	Carbonyl Cyanide <i>p</i> -trifluoromethoxyphenylhydrazone
OCR	oxygen consumption rate
NK	Natural-Killer Cells
IL-6	Interleukin-6
TNF- α	Tumor Necrosis Factor- α
CRP	C-Reactive Protein
SDoH	Social Determinants of Health
SES	Social Economic Status
NT	Normotensives
NDSVH	Neighborhood Disadvantage, Sleep, and Vascular Health
NHLBI	National Heart, Lung, and Blood Institute
LSM	Lymphocyte Separation Media
DPBS	Dulbecco's Phosphate-Buffered Saline
BCA	Bicinchoninic Acid
PVDF	Polyvinylidene Fluoride
NFDM	Non-Fat Dry Milk
TBST	Tris-Buffered Saline with Tween
HRP	Horseradish Peroxidase

Chapter 1: Introduction

Within the United States, cardiovascular disease (CVD) is the leading cause of death, with a marked racial disparity in prevalence, morbidity, and mortality.^{1,2} Non-Hispanic Black (NHB) adults are the most at-risk population with a higher prevalence for CVD risk factors (e.g., hypertension [HTN], obesity, diabetes) compared to Non-Hispanic White (NHW) adults or any other racial/ethnic group.^{1,3,4} The vast majority of research in CVD and racial disparities, including the majority of studies highlighted below, involves the comparison of NHB and NHW adults. Additionally, clinical data and cellular experiments have repeatedly shown NHB adults to exhibit higher systemic inflammation, an impaired vasodilatory response, heightened oxidative stress, and endothelial dysfunction that may contribute to the higher prevalence in CVD conditions compared to NHW adults.⁵⁻¹³ Vascular dysfunction, an antecedent to CVD progression, includes endothelial dysfunction which is characterized by a reduction in nitric oxide (NO)-dependent dilation.^{5,14-16}

Reactive oxygen species (ROS) are one way in which NO bioavailability and production can be hindered. The interaction of a superoxide (O_2^-) radical with NO generates peroxynitrite ($ONOO^-$) that further inhibits NO production and further generates ROS.^{17,18} ROS are highly reactive, oxygen bearing molecules (e.g., O_2^- ; hydrogen peroxide H_2O_2 ; hydroxyl radicals OH^\bullet) that inflict oxidative damage to DNA, proteins, and fatty acids which ultimately harm cell function and viability.^{19,20} The imbalance between the antioxidant defenses and ROS production within an organism leads to oxidative stress, that is associated with inflammation, hypertension, and CVDs.^{19,21}

There are various endogenous sources of ROS production which includes peroxisomes, lipoxygenases, cytochrome P450, xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the mitochondria.^{19,20,22,23} The main endogenous sources for ROS

production, known to impact NO bioavailability and initiate the process of vascular dysfunction are 1) NADPH oxidase 2) mitochondrial respiration, and 3) xanthine oxidase. NADPH oxidase is a multi-subunit cytosolic enzyme, known for generating ROS in response to growth factors, cytokines, or calcium signaling.^{20,22} NADPH oxidase enzymes are expressed in many cell types including immune cells (monocytes, T cells, macrophages), endothelial cells (ECs), and vascular smooth muscle cells. The mitochondria are also implicated as another major generator of ROS production during oxidative phosphorylation. The sites on the electron transport chain (ETC) recognized for the premature leakage of electrons are Complex I (NADH Dehydrogenase) and Complex III (ubiquinone-cytochrome *c* reductase) for reducing oxygen (O_2) into O_2^- .²³⁻²⁸ However, there are also other sites within the mitochondria recognized as producers of O_2^- and H_2O_2 that include 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, Succinate dehydrogenase (Complex II), and glycerol 3-phosphate dehydrogenase.²⁹

There are various enzymatic (e.g., SOD, catalase, glutathione peroxidases, and NADH peroxidase), and non-enzymatic (e.g., glutathione, vitamin C, vitamin E) antioxidant defense systems within cells that aid in combating excess ROS production.^{19,30} The following section will detail the role of superoxide dismutase (SOD) in modulating mitochondrial-derived ROS. SODs are a group of enzymes that scavenge ROS by catalyzing the conversion O_2^- into O_2 and H_2O_2 .^{31,32} In eukaryotes, there are three isoforms of this antioxidant enzyme: 1) SOD1 located throughout the cytoplasm, nucleus, and lumen between the outer and inner membranes of the mitochondria, 2) SOD2 located within mitochondrial matrix, and 3) SOD3 located extracellularly.³²⁻³⁴ Sirtuin-3 (SIRT3) is a mitochondrial deacetylase that plays a central role in the antioxidant defense system as a SOD2 activator.³²⁻³⁶ Mitochondrial dysfunction has gained attention as major contributor to the pathogenesis of various CVDs and is characterized by the dysregulation of mitochondrial-

derived ROS as a result of an increase in oxidant production, a decrease in antioxidant defenses, or both.^{37,38}

As primary contributors to systemic ROS, research is now focusing on further understanding the role of peripheral blood mononuclear cells (PBMCs) in vascular dysfunction, as they are in constant communication with the endothelium.²¹ PBMCs are identified as blood cells with a round nucleus (e.g., lymphocytes, monocytes, or dendrite cells) that provide selective responses to immune systems in order to fight infections and adapt to future intrusions.³⁹⁻⁴¹ Most PBMCs are in a naïve or resting state without effector functions due to the absence of an ongoing response.⁴⁰ Upon stimulation, activated PBMCs can produce inflammatory cytokines (e.g., Interleukin-6 or Tumor Necrosis Factor- α), upregulate activation markers, and initiate inflammatory pathways that can promote vascular dysfunction and CVD progression.^{21,42} Mice infused with angiotensin II (Ang II), a potent vasoconstrictor that causes HTN, showed a significant increase in their macrophage population compared to the sham-infused mice. Furthermore, the depletion of monocytes in a subset of mice that were then infused with Ang II has been shown to significantly reduce systemic O_2^- levels, ONOO⁻ formation, attenuate vascular dysfunction, and lower Ang II-induced increases in blood pressure compared to control mice.²¹

One mediator to the PBMC-derived contribution to systemic ROS is NADPH oxidase subunit gp91phox.²¹ PBMCs express the NOX2 isoform of NADPH oxidase that is characterized by gp91phox spanning across the cell membrane and generating large quantities of O_2^- that is released extracellularly to augment systemic ROS.²² PBMCs isolated from NHB males has shown a higher protein expression of NADPH oxidase subunits (gp91phox and p47phox) and greater intracellular O_2^- production compared to PBMCs isolated from NHW males.⁴³ Presumably the

greater ROS production in the PBMCs isolated from NHB adults could contribute to the racial differences in systemic oxidative stress.

Mitochondrial-derived ROS in PBMCs isolated from individuals with moderate to severe congestive heart failure (CHF) demonstrated the key role oxidative stress plays in the pathophysiology of CVDs. Specifically, PBMCs isolated from patients with CHF exhibited a marked reduction in mitochondrial transmembrane potential, an increase in intracellular ROS formation, impaired mitochondrial respiratory capacity, and reduced SOD activity when compared to PBMCs isolated from patients with mild CHF or a control group.⁴⁴⁻⁴⁶ Also decreased mitochondrial respiration in PBMCs isolated from patients with early-stage CHF was inversely related to circulating inflammatory cytokines (e.g., C-reactive protein, Interleukin-6, Tumor Necrosis- α) when compared to PBMCs isolated from a control group.⁴⁷ With oxidative stress, the mitochondria also become a target for ROS and can result in oxidative damage to the ETC complexes.^{48,49} These oxidative reactions not only serve to further amplify mitochondrial-derived ROS during respiration but can also result in the nitration of tyrosine residues on SOD2 and potentially inhibiting its enzymatic activity⁵⁰.

With mitochondrial dysfunction, a decrease in the antioxidant defense mechanisms is observed. Genetically modified mouse models demonstrated the role mitochondrial SOD2 plays in vascular biology. Specifically, SOD2 silencing in apolipoprotein-deficient mice resulted in endothelial dysfunction in carotid arteries.³⁴ Furthermore, even a heterozygous mouse for SOD2 (SOD2^{+/-}) exhibited mitochondrial dysfunction, diminished SOD2 activity, higher susceptibility to oxidative stress, and myocardial ischemia.⁵¹⁻⁵³ SIRT3 knockout mouse models also demonstrated an increase in SOD2 acetylation, elevated mitochondrial-derived O₂⁻ levels, and diminished endothelial derived NO production promoting the pathogenesis of HTN.³⁶ In static conditions,

human umbilical vein ECs (HUVECs) isolated from NHB individuals has been shown to have significantly higher protein expression for NADPH oxidase subunits (p47phox and gp91phox), lower protein expression for SOD2, lower SOD1 activity, and total SOD activity compared to HUVECs isolated from NHW individuals.^{8,54}

Lymphocytes and monocytes are key players in the progression of atherosclerosis by modulating the inflammatory and immune response.⁵⁵ Lymphocytes (e.g., helper T cells, B cells, and natural-killer cells) are essential mediators of immune cell homeostasis but can also contribute to systemic inflammation.^{40,41} Whereas, macrophages and monocytes with a proinflammatory profile are essential for driving both inflammatory and oxidative signaling pathways involved in vascular dysfunction.²¹ At the site of inflammation, monocytes transmigrate from the blood, initiate adhesion to the endothelium, differentiate into macrophages, and initiate the formation of an atherosclerotic plaque.⁵⁶

Mitochondrial dysfunction in PBMCs is suggested to play a key role in endothelial dysfunction, atherosclerosis, HTN, and CVD; yet the specific mechanism(s) are still unclear. Whether superoxide generating NADPH oxidase expression is elevated or antioxidant defenses/activator (SOD1, SOD2, SIRT3) expression is diminished in PBMCs isolated from NHB adults is unknown. Also, alterations in mitochondrial function (cell respiration and ETC complexes) could be one mechanism contributing to the elevations in oxidative stress observed in PBMCs isolated from NHB adults. Limited work has been performed to investigate this area. Therefore, it is imperative for research to continue exploring the role of the mitochondria as a potential player in the racial differences in vascular function and the pathophysiology of CVDs.

The aims of the proposed study are:

Aim 1: To investigate possible racial differences in the protein expression of enzymes regulating ROS production and ETC complexes in PBMCs. We hypothesize that PBMCs isolated from NHB individuals would have a lower expression in antioxidant modulators of oxidative stress (e.g., SOD1, SOD2, SIRT3), higher expression in acetylated SOD2 (AcSOD2), higher expression in superoxide-generating NADPH Oxidase subunits (e.g., gp91phox, p47phox), and/or higher expression of mitochondrial ETC complexes that promote vascular dysfunction.

Aim 2: To examine possible racial differences in PBMC respiration. We hypothesize that PBMC isolated from NHB individuals would have lower cell respiration that would promote mitochondrial and vascular dysfunction.

Aim 3: To explore potential sex differences in regulators of ROS production and respiration in PBMCs; given the National Institutes of Health (NIH) stance on incorporating sex as a biological variable that should be factored into basic and biomedical research. Human studies on mitochondrial biology demonstrated a lack in consistent sex differences for ETC complexes protein expression in PBMCs and skeletal muscle, greater antioxidant capacity in PBMCs isolated from females, and comparable maximal respiration rates between sexes.⁵⁷⁻⁶⁰ With this in mind, we hypothesize that PBMCs isolated from females would either have a greater protein expression in antioxidant modulators of oxidative stress or lower expression in superoxide-generating NADPH oxidase subunits, with no differences in mitochondrial complex expression. We also hypothesize that there would be no sex differences in cell respiration.

Chapter 2: Literature Review

2.1 Cardiovascular Disease (CVD)

2.1.1 Overview of CVD

Cardiovascular disease (CVD) has and continues to be the leading cause of death in the United States, with mortality slightly higher than 870,000 from CVD alone.^{2,3,61} The CVD-related mortality and morbidity also takes an economic toll on the healthcare system, with the 2016-2017 Medical Expenditure Panel Survey declaring over \$363.4 billion spent annually in direct and indirect health care costs (e.g., physician visits, hospital services, medications, procedures), as well as \$147.4 billion lost in future productivity due to time spent away from the workforce.² According to data from the 2015 to 2018 National Health and Nutrition Examination Survey (NHANES), the prevalence of CVD among US adults was 49.2% affecting 126.9 million US adults in 2018.² By 2035, the US population is projected to have over 130 million adults with some form of CVD, in addition to a total of \$1.1 trillion in direct and indirect costs.⁴

CVD is an umbrella term that incorporates various heart conditions (e.g., arrhythmias, heart valve problems), heart diseases (e.g., coronary artery disease, peripheral artery disease, high blood pressure), and stroke. If left untreated, these diseases/conditions could result in heart failure, heart attack, or even death. When breaking down the percentage of deaths attributed to CVD in the US, the leading cause is coronary heart disease (42.1%), followed by stroke (17.0%), high blood pressure (11.0%), heart failure (9.6%), diseases of arteries (2.9%), and other CVD causes (17.4%).² The American Heart Association (AHA) emphasizes “Life’s Simple 7” as the risk factors known to increase the risk of CVD progression, which includes core health behaviors (e.g., physical activity, diet, smoking status) and health factors (cholesterol, body weight, blood glucose, and blood pressure).² It is important to point out that there are also social/environmental risk factors

(e.g., family income, education level, race/ethnicity, single-living status) related to cardiovascular (CV) health outcomes.^{2,62-66} Moreover, it is imperative to understand the progression of CVD at the molecular level, develop pharmacological and non-pharmacological interventions, and focus on assisting at-risk populations to prevent, or at least, manage the symptoms; with the ultimate goal of reducing the health and economic burdens within the United States, as well as globally.

2.1.2 CVD Progression and Risk Factors

CVD develops as a result of low-grade inflammation, vascular dysfunction, and atherosclerosis that allows for plaque development and potential ischemic complications.⁶⁷ Atherosclerosis is the buildup of plaque within the artery walls that leads to the hardening/narrowing of the blood vessel and reduction in blood flow that ultimately limits the O₂ and nutrients supplied to the affected organs and tissues throughout the body. Plaque buildup is initiated by the adhesion of blood leukocytes to an activated endothelium which stimulates the migration of leukocytes in between the endothelium and vascular smooth muscle cells (VSMCs) where monocytes mature into macrophages.⁶⁸ Once matured, these macrophages can take up cholesterol and other lipids forming foam cells that reduce vascular elasticity and flexibility.

As previously described, risk factors that contribute to the development of atherosclerosis and increase risk of CVD include health behaviors (e.g., smoking, sedentary lifestyle, poor diet) and health factors (e.g., obesity, hypertension, diabetes mellitus, high cholesterol, and genetic predispositions).^{2,66} Interestingly, prevalence of these risk factors is not uniform between sexes and racial/ethnic groups. Within the United States, Non-Hispanic Black (NHB) adults have the highest prevalence for high blood pressure (Males 58.3%, Females 57.6%) compared to Non-Hispanic White (NHW; Males 51.0%, Females 40.5%), Non-Hispanic Asian (NHA; Males 51.0%, Females 42.1%), and Hispanic adults (Males 50.6%, Females 40.8%). Another risk factor in which

NHB adults have a higher prevalence of obesity (Males 38.2%, Females 55.2%) compared to Hispanic (Males 44.0%, Females 46.2%), NHW (Males 40.7%, Females 38.7%), and NHA adults (Males 13.5%, Females 15.9%). As for the prevalence for Type 2 Diabetes, Hispanic adults have the highest prevalence (Males 15.1%, Females 14.1%), followed by NHB (Males 14.7%, Females 13.4%), NHA (Males 12.8%, Females 9.9%), and NHW adults (Males 9.4% vs. Females 7.3%).²

These racial disparities across risk factors also contribute to the differences in prevalence, morbidity, and mortality rates for CVD.^{1,2} Within the US, the most at-risk population for CVD prevalence is NHB adults (Males 60.1% vs. Females 58.8%) compared to NHW (Males 53.6% vs. Females 42.1%), Hispanic (Males 52.3% vs. Females 42.7%), and NHA adults (Males 52.0% vs. Females 42.5%).²

Hypertension (HTN) is defined as high blood pressure and considered a major risk factor for CVD. In 2017, the American College of Cardiology and AHA redefined the blood pressure (BP) guidelines for HTN, with the ultimate goal of providing earlier treatment and management opportunities for patients.³ The 2017 guidelines lowered the BP threshold for HTN categorization from $\geq 140/90$ mmHg to $\geq 130/80$ mmHg, which raised the percentage of individuals categorized for HTN from 30.7% to 45.7% respectively. Not only do NHB individuals have a higher prevalence for HTN but are also more likely to develop HTN earlier in life, exhibit an expedited progression of the disease, and develop more CV risk factors (e.g., diabetes, obesity) compared to NHW individuals.^{14,69}

2.1.3 Vascular Dysfunction

The endothelium is a monolayer of endothelial cells (ECs) that lines the interior surface of blood vessels and plays a critical role in vascular homeostasis by sensing and integrating hemodynamic and hormonal stimuli to generate various messenger molecules that influence the

physiology of the surrounding tissues.^{16,70} In healthy endothelium, ECs have vasomotor control within conduit and resistance vessels by balancing the release of vasodilator and vasoconstrictor substances.^{11,14,71,72} Throughout most of the vasculature the endothelium's most potent vasodilator is nitric oxide (NO), the production of which is catalyzed by the enzyme, endothelial NO synthase (eNOS) from L-arginine.^{5,73} NO is a powerful antithrombotic, anti-atherogenic, and anti-inflammatory agent that plays a role in platelet aggregation, adhesion of leukocytes to ECs, VSMCs proliferation, and alterations in lipoprotein metabolism to sustain the blood vessel's elasticity and flexibility.^{16,70}

Vascular dysfunction is a key factor in the initiation and progression of atherosclerosis, HTN, and CVD development.¹⁴⁻¹⁶ Vascular dysfunction is characterized by the stiffening of large arteries and endothelial dysfunction that results in a reduction in NO-dependent dilation, increased expression of proinflammatory adhesion molecules, and the dysfunction of messenger molecules.^{5,15,16,74} The deficiency in NO can occur at multiple levels: 1) absence of ECs or high EC apoptosis due to a toxic stimuli (e.g., reactive oxygen species, proinflammatory cytokines), 2) alterations in eNOS activity (e.g., posttranslational modifications), 3) a decrease in substrate (L-arginine) or eNOS cofactor (e.g., tetrahydrobiopterin [BH₄]) availability, 4) inhibition of NO production (e.g., endothelin-1, ET-1), or 5) interception of NO generation by reactive oxygen species (ROS).^{6,16,75}

2.2 Reactive Oxygen Species (ROS)

2.2.1 Overview of ROS and Molecular Targets

ROS was originally coined to explain a class of endogenous, highly reactive, oxygen-bearing molecules, also known as free radicals, that were implicated in oxidative damage inflicted on DNA, proteins, fatty acids, and other cellular components.^{19,20} There are various types of ROS

which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^\bullet) radicals, peroxy (RO_2^\bullet), and alkoxy (RO^\bullet).^{19,22} The reactivity of ROS molecules determines its selectivity in choosing biological targets. There are specific ROS molecules (e.g., H_2O_2 or O_2^-) that are very selective with its molecular targets.^{19,20} Whereas other ROS molecules (e.g., OH^\bullet) that are not selective, have an extremely short lifetime, and a broad range of nonspecific targets.¹⁹ Oxidative stress is an excess in ROS as a result of an imbalance in ROS production and antioxidant abundance/activity that is associated with inflammation, aging, HTN, CVD, and neurodegenerative diseases.^{19,21}

As previously discussed, ROS is one way in which NO bioavailability and production can be hindered. Excess O_2^- rapidly interacts with NO to produce peroxynitrite ($ONOO^-$), another highly reactive oxidant, that can induce cell cytotoxicity, apoptosis, and increase the generation of ROS (e.g., O_2^- and H_2O_2).^{17,18,76} $ONOO^-$ also diminishes NO production by 1) releasing zinc from the zinc-thiolate cluster of eNOS and 2) oxidizing the BH_4 cofactor, both result in eNOS uncoupling and increase O_2^- production, ultimately causing endothelial dysfunction.⁷⁷⁻⁷⁹ Vascular dysfunction and NO bioavailability is depicted in Figure 1. This highly reactive oxidant can also disrupt mitochondrial function by inhibiting the electron transport chain (ETC) complexes and inhibiting the antioxidant defenses (e.g., superoxide dismutase 2, or SOD 2), which further fuels the formation of mitochondrial-derived ROS.⁷⁶

ROS can also react with different biomolecules that lead to molecular damage.²⁰ Molecular targets of ROS include proteins via protein oxidation, where following the attack, conformational changes can occur that lead to alterations in protein structure and/or protein activity.¹⁹ For example, ROS can oxidize cysteine residues, which are important targets in molecular pathways, and ultimately alter protein phosphorylation capacity.¹⁹ A second molecular target for ROS is

nuclear and mitochondrial DNA (mtDNA) via single- and double-strand breaks, abasic sites, and base damages.^{19,80,81} Finally, a third molecular target for ROS are lipids whereby free radicals (e.g., OH•) react with fatty acids to generate lipid carbon radicals that trap O₂ and form lipid peroxy radicals. This lipid peroxy radical formation alters the fatty acyl chains from *cis* to a *trans* configuration, reduces membrane fluidity, and produces secondary cytotoxic products involved in cell apoptosis and atherosclerosis.¹⁹

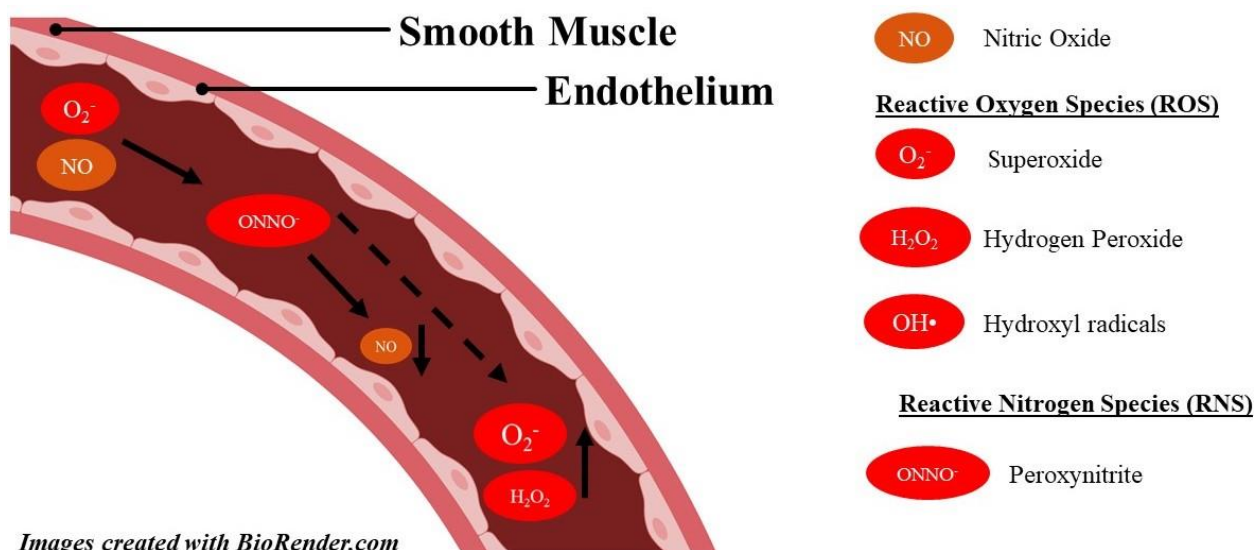


Figure 1. Vascular Dysfunction and NO Bioavailability

ROS is one way in which NO bioavailability and production can be hindered. O₂⁻ immediately react with NO to form ONOO⁻, a highly reactive oxidant that further inhibits NO production and activates cell signaling cascades that further increase the formation of ROS production.

2.2.2 Sources of ROS

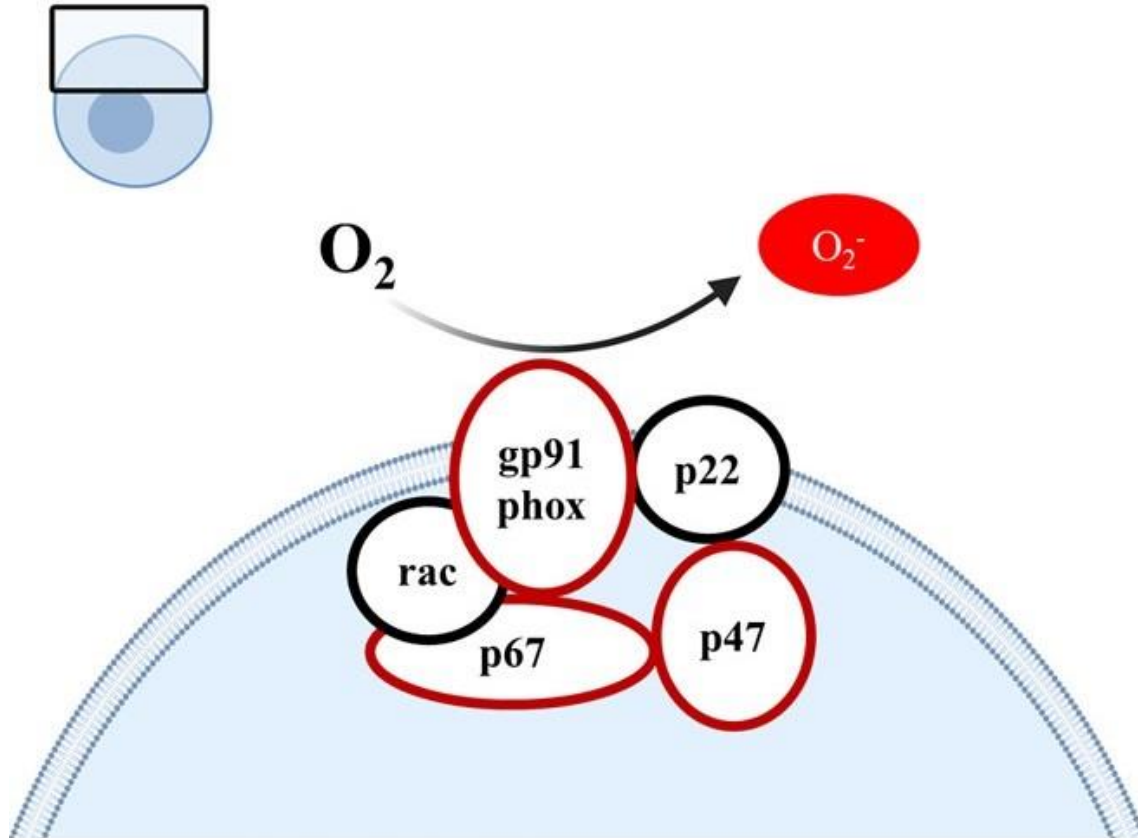
Briefly, there are exogenous sources of ROS production that arise from environmental sources (e.g., ultraviolet light, ionizing radiation, or pollutants), environmental agents (e.g., carcinogens), and chemotherapeutic cancer drugs.¹⁹ Yet the following section will discuss the various endogenous sources of ROS production and detail the role of two main sources contributing to ROS production, oxidative stress, and vascular dysfunction.

There are different endogenous sources of ROS production as a by-product which includes peroxisomes, lipoxygenases, and cytochrome P450.^{19,22} Peroxisomes are cytoplasmic organelles that catalyze the breakdown of long fatty acid chains via beta-oxidation and generates H₂O₂. lipoxygenases are enzymes that catalyze the peroxidation of arachidonic acid to generate prostaglandins or thromboxane, along with ROS production.⁸² Finally, Cytochrome P450 are enzymes whose function is to oxidize steroids, fatty acids, and xenobiotics, generating ROS through the course of their reaction cycles.⁸³

One cytosolic enzyme system known for generating ROS in mammalian cells is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, also known as a superoxide-generating system.²⁰ NADPH oxidase is a multi-subunit enzyme with seven isoforms in this transmembrane family, whose function is to generate ROS in a regulated manner in response to growth factors, cytokines, or calcium signaling.^{19,20,22} NADPH oxidase is known as a primary source of ROS production in the CV system and is expressed in many cell types including immune cells (e.g., monocytes, T cells, macrophages), ECs, and VSMCs.^{21,84} Figure 2 depicts the subunits of NADPH Oxidase, which consists of a catalytic membranous subunit (gp91phox), along with various regulatory subunits that are either found in the cytosol (e.g., p47phox, p67phox, and p40phox) or membrane bound (p22phox).²⁰ NADPH oxidase forms a stabilizing complex with subunits gp91phox and p22phox that remains inactive until the cell is exposed to microorganisms, inflammatory mediators, or specific agonists (e.g., angiotensin II, Ang II) that initiate the translocation and assembly of the cytosolic subunits to create an active enzyme.²⁰ This multi-subunit assembly allows the cytosolic domain to transfer an electron from NADPH to the FAD cofactor, then transfer to the heme group, and finally donate the electron to O₂ to generate O₂⁻ on the extracellular side.^{19,22} It is important to note that the NADPH oxidase isoform plays an

important role in its contribution to systemic oxidative stress. For example, ECs predominantly express the NOX4 isoform that has the gp91phox subunit located on the intracellular organelles instead of the cell membrane, thereby limiting ECs capacity to produce and release O_2^- extracellularly. However, peripheral blood mononuclear cells (PBMCs) express the NOX2 isoform with a gp91phox that spans across the cell membrane, has the capacity to generate large quantities of O_2^- to release extracellularly, and increase systemic oxidative stress.²²

The mitochondria are also implicated as another major generator of ROS during oxidative phosphorylation. Figure 3 depicts the sites on the electron transport chain (ETC) recognized for the premature leakage of electrons, Complex I (NADH Dehydrogenase) and Complex III (ubiquinone-cytochrome *c* reductase) for reducing O_2 into O_2^- .²³⁻²⁸ However, there are also other sites within the mitochondria recognized as producers of O_2^- and H_2O_2 that include 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase, Succinate dehydrogenase (Complex II), and glycerol 3-phosphate dehydrogenase.²⁹ *In vitro* studies on isolated mitochondria have shown these ETC complexes convert up to 5% of O_2 molecules into O_2^- .³³ Furthermore, experiments on isolated mitochondria from the gastrocnemius and soleus muscle in mice demonstrated that Complex I-derived ROS was exclusively released into the mitochondrial matrix, whereas Complex III-derived ROS was released into the mitochondrial matrix and intermembrane space.^{19,27,31} The O_2^- generated from Complex III can transverse into the cytosol via voltage-dependent anion channels, however most of this O_2^- is immediately converted into H_2O_2 via the cytosolic SOD1 enzyme.^{31,85}

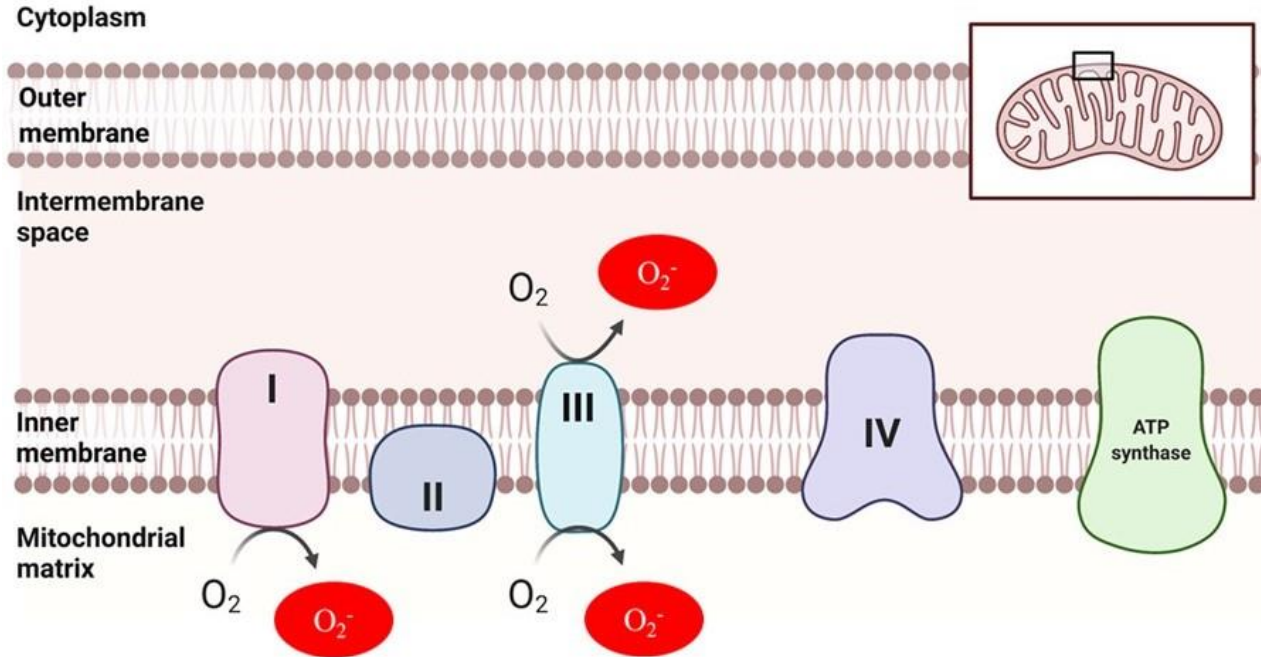


Images created with BioRender.com

Figure 2. NADPH Oxidase and ROS Production

NADPH oxidase is one cytosolic enzyme known for generating ROS. This multi-subunit contains a catalytic membranous subunit (gp91phox) and requires various regulatory subunits found in the cytosol (p47phox, gp91phox, and p40phox) or membrane bound (p22phox). When a cell is exposed to inflammatory mediators or agonists, it initiates the assembly of the cytosolic subunits to create an active enzyme. In PBMCs, the catalytic gp91phox subunit spans the cell membrane and has the capacity to generate large quantities of O_2^- extracellularly. However, regulatory subunits (p47phox and p67phox) are also involved in NADPH oxidase activity and ROS formation.

(p22, p22phox. p47, p47phox. p67, p67phox. O_2 , oxygen. O_2^- , superoxide)



Images created with BioRender.com

Figure 3. Mitochondria and ROS Production

The mitochondria are another major generator of ROS during oxidative phosphorylation. Complex I and Complex III are the two main sites recognized for the premature leakage of electrons and reducing O_2 into O_2^- . Complex I-derived ROS is exclusively released into the mitochondrial matrix, while Complex III-derived ROS can be released into the mitochondrial matrix or the intermembrane space.

(O_2 , oxygen. O_2^- , superoxide)

2.2.3 ROS Scavengers and Antioxidants

There are various enzymatic (e.g., SOD, catalase, glutathione peroxidases, and NADH peroxidase), and non-enzymatic (e.g., glutathione, vitamin C, vitamin E) antioxidant defense systems within cells that aid in combating excess ROS production.^{19,30} However, the following section will focus on two antioxidant defenses that play a role in modulating mitochondrial-derived ROS.

SODs are a group of enzymes that scavenge ROS by catalyzing the conversion of O_2^- into O_2 and H_2O_2 to minimize the imbalance of oxidative stress.^{31,32} In eukaryotes, there are three isoforms of this antioxidant enzyme: 1) CuZn-SOD (SOD1) located throughout the cytoplasm,

nucleus, and intermembrane space of the mitochondria, 2) MnSOD (SOD2) located within mitochondrial matrix, and 3) CuZn-SOD (SOD3) located extracellularly.³²⁻³⁴ Genetically modified mouse models demonstrated the role of mitochondrial SOD2 in vascular biology, specifically in relation to CVD, with SOD2 silencing resulting in apolipoprotein-deficient mice exhibiting endothelial dysfunction in carotid arteries.³⁴ Even a heterozygous mouse for SOD2 (SOD^{+/-}) exhibited mitochondrial dysfunction, diminished SOD2 activity, greater susceptibility to oxidative stress, and myocardial ischemia.⁵¹⁻⁵³ Furthermore, SOD2 knockout rodents demonstrated vascular remodeling, arterial stiffening, and heightened O₂⁻ levels, which are all characteristics of endothelial and vascular dysfunction.⁸⁶

Sirtuin-3 (SIRT3) is a mitochondrial deacetylase that plays a central role in the antioxidant defense system by regulating SOD2 activity.³⁵ Acetylation is a major post-translational modification that inhibits SOD2 (AcSOD2) activity and as a result increases circulating ROS levels.⁸⁷ Antioxidant enzymes and defenses within cells are depicted in Figure 4. SIRT3 functions to deacetylate lysine (i.e., K) residues K68 and K122 to activate SOD2 and scavenge cellular ROS.^{35,36} SIRT3 is NAD⁺-dependent and acts as a metabolic sensor to regulate mitochondrial function to match nutrient supplies.⁸⁸ SIRT3 knockout-mouse models exhibit an increase in SOD2 acetylation, elevated mitochondrial-derived O₂⁻, and diminished endothelial NO, all of which contribute to the pathogenesis of HTN.³⁶ Moreover, reduced SIRT3 activity can be caused by Ang II (potent vasoconstrictor) and inflammation.⁸⁹ In older populations, SIRT3 protein expression declines with aging, in parallel with increased incidence of HTN.^{90,91} In summary, SIRT3 protein expression promotes vascular inflammation, endothelial dysfunction, vascular hypertrophy, and end-organ damage.⁹²

Superoxide Dismutase (SOD)

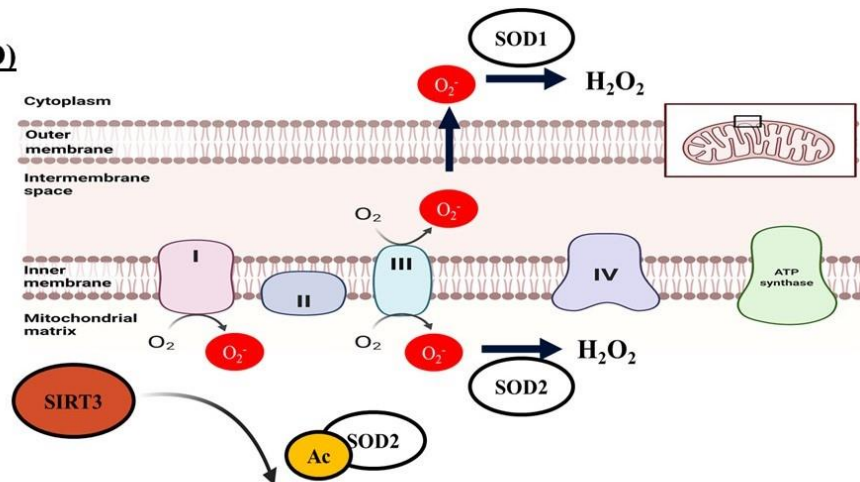
SOD1 – Cytoplasm

SOD2 – Matrix

AcSOD2 – Acetylated
SOD2

Sirtuin-3 (SIRT3)

SIRT3 – Mitochondrial
deacetylase



Images created with BioRender.com

Figure 4. Antioxidant Enzymes and Defenses

SODs are a group of enzymes localized to specific compartment to combat ROS production. SOD2 can be inactivated with the addition of an acetyl group (AcSOD2) which reduces the enzyme's scavenging activity. SIRT3 is a mitochondrial deacetylase and an important regulator of SOD2 activity by removing the acetylation on SOD2 and allowing SOD2 to continue converting O_2^- into H_2O_2 .

(O_2 , oxygen. O_2^- , superoxide. Ac, acetyl group)

2.3 Mitochondria

2.3.1 Overview of the Mitochondria

The modern eukaryotic cell is thought to have originated as a result of the engulfment of an α -proteobacteria that evolved into the mitochondria known today.^{93,94} The most striking evidence for this association between the mitochondria and α -proteobacteria is the similarities in the respiratory chain complexes, the double membrane characteristics, and adenosine triphosphate (ATP) production.^{93,94} With the evolution of the mitochondria, most of the genetic material from the progenitor was lost or transferred to the nuclear genome.^{94,95} The human mitochondria have a circular genome of ~16 kilobases that only code for 13 mitochondrial proteins known as the core constituents for the hydrophobic subunits in the respiratory chain complexes I-IV or of the ATP synthase which are imbedded on the inner mitochondrial membrane.^{93,94} The rest of the

mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm by ribosomes, and then imported into the mitochondria.⁹⁶

The mitochondria are cytoplasmic membrane bound organelles found across various cells (e.g., ECs, VSMCs, PBMCs) and are predominantly known for their role in ATP production via oxidative phosphorylation.^{28,94,97} The mitochondria also function in the: 1) generation and removal of ROS, 2) cell apoptosis pathways, 3) calcium regulation within the cytoplasm and mitochondrial matrix, 4) immune function, 5) cell growth, 6) stem cell function, and 7) synthesis and catabolism of metabolites that are then transported to the appropriate locations within the cell.^{97,98} The mitochondria are comprised of an inner and outer mitochondrial membrane to create two aqueous regions known as the intermembrane space and the matrix.²⁸ The outer membrane is porous and as a result allows ions and small uncharged molecules to cross freely via voltage dependent anion channels; while larger molecules (e.g., proteins) require translocases for their relocation.^{94,99} The inner membrane on the other hand, has a very controlled diffusion barrier to all ions and molecules that require membrane transport proteins selective to the individual molecule or ion. This allows for the electrochemical membrane potential to build up across the inner mitochondrial membrane, where oxidative phosphorylation takes place.⁹⁹

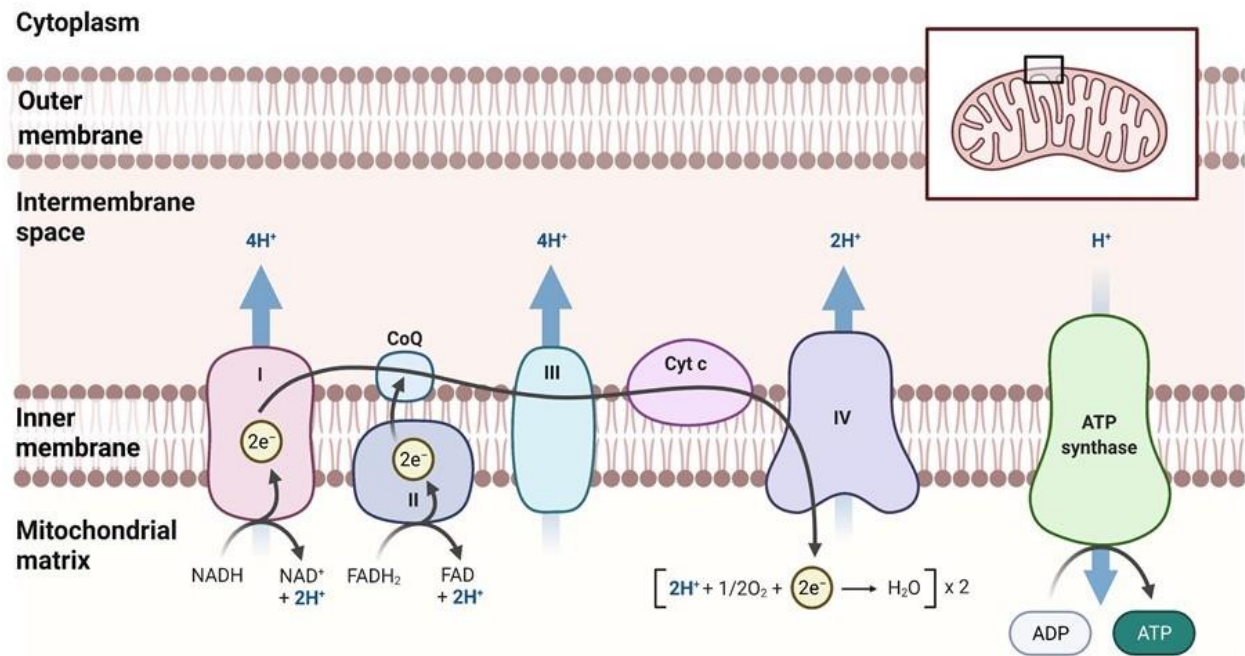
2.3.2 Mitochondrial Complexes and Their Role

The mitochondria house important enzymatic systems to finalize the oxidation of sugars, fats, and proteins with the ultimate goal of producing usable energy as ATP.¹⁰⁰ Each substrate is essentially broken down into acetyl-CoA and transferred to the mitochondrial matrix to enter the Krebs cycle, which is a series of seven enzymatic steps that pass electrons to cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂).²⁸ The electrons removed from NADH and FADH₂ in the electron transport chain power the pumping of

protons from the matrix into the intermembrane space that allows for a membrane potential difference to power the synthesis of ATP during the final step of oxidative phosphorylation.

The ETC is a series of multi-subunit complexes within the inner mitochondrial membrane. Complex I is the largest of the ETC complexes with a total of 45 subunits and a mass of 1 MDa. This Complex is an L-shaped enzyme with a hydrophobic domain within the inner mitochondrial membrane and a hydrophilic arm protruding into the mitochondrial matrix that contains an NADH binding site.²⁸ NADH is oxidized at Complex I, this results in two electrons donated to Complex I and allows for the translocation of four protons from the matrix into the intermembrane space. Succinate dehydrogenase (Complex II), involved in the catalyzation of FAD to FADH₂ in the Krebs cycle, has a mass of 123 kDa and contains an FAD in the enzyme to assist with the donation of electrons to coenzyme Q.^{28,101} Complex II is also the only complex among complexes I - IV that does not pump protons from the mitochondrial matrix. Coenzyme Q can be reduced by Complex I or II and freely diffuse through the inner mitochondrial membrane to reduce to Complex III. As the smallest of the ETC complexes, with a mass of 13kDa, Complex III obtains two electrons from the oxidation of coenzyme Q and translocates two protons from the mitochondrial matrix.¹⁰² The electrons transferred across the ETC aid in the conversion of O₂ to water (H₂O) at cytochrome *c* oxidase (Complex IV). Four molecules of Complex IV must have an electron bound at its active site in order to catalyze two H₂O molecules from O₂.²⁸ Simultaneously, Complex IV translocates four protons from the mitochondrial matrix into the intermembrane space.¹⁰³ ATP synthase (Complex V) contains two domains (F₀ and F₁) for the enzyme to mimic a rotary motor that is necessary to accomplish the final step of oxidative phosphorylation, the phosphorylation of adenosine diphosphate (ADP).^{28,99,104} The protons deposited into the intermembrane space by the ETC complexes move down their electrochemical gradient at Complex V via the F₀ domain which

contains a transmembrane proton channel. This initiates the rotor turn in the head of the F₁ domain in Complex V, which allows the F₁ domain to pass the three binding sites for ADP and phosphate and induces the conformational change to produce three ATP molecules for every turn of the rotor.²⁸ The formation of ATP during oxidative phosphorylation is depicted in Figure 5.



Images created with BioRender.com

Figure 5. Formation of ATP During Oxidative Phosphorylation

The mitochondria are predominantly known for their role in ATP production during oxidative phosphorylation. Electrons from cofactors (NADH and FADH₂) are accepted at Complex I and II and continue to be passed down to Complexes III and IV, all while pumping protons from the matrix into the intermembrane space. This creates the electrochemical gradient needed for the generation of ATP at Complex IV (ATP Synthase). Oxygen is the final electron acceptor and becomes water at Complex IV.

(NADH, nicotinamide adenine dinucleotide. FADH₂, flavin adenine dinucleotide. H⁺, proton. e⁻, electron. H₂O, Water. O₂, oxygen. ADP, adenosine diphosphate. ATP, adenosine triphosphate)

2.3.3 Intact Cell Respiration Measures

The mitochondrial proton circuit is essential for its physiological functions and consists of Complexes I, III, and IV to pump protons across the mitochondrial inner membrane.⁹⁸ Mitochondrial dysfunction can be assessed in isolated mitochondria, in intact cells, or *in vivo* with various methods in place to measure mitochondrial function.⁹⁸ One advantage to using intact cells for quantifying mitochondrial function and cellular bioenergetics is the removal of artefacts associated with mitochondrial isolation that could offer greater physiological relevance. However, this model also lacks the *in vivo* context, and the outcomes of the experiments are influenced by the experimenter's decision on substrates, serum, pH, and oxygen concentrations used.⁹⁸ Measurement of oxygen utilization in intact cells can offer a bioenergetic analysis of the proton circuit similar to that in isolated mitochondria.^{98,105–107}

The Clark-type oxygen electrode and chamber is one of the most cited methods for measuring respiration in intact cells.¹⁰⁸ For intact cells, several measures of intracellular mitochondrial function can be derived by the sequential injection of mitochondrial inhibitors.^{98,109–111} This report includes the rate of basal respiration, leak respiration, maximal respiration, and non-mitochondrial respiration. Basal respiration is the absence of mitochondrial inhibitors and represents the net sum of all the processes within the cell capable of consuming oxygen (e.g., mitochondrial and non-mitochondrial).^{98,110,111} The assumption being that basal respiration represents the demand on the proton motive force to produce ATP or move ions across the inner membrane.¹¹¹ Leak respiration is determined with the addition of oligomycin, an inhibitor of Complex V, which inhibits the proton flux through this enzyme and the remaining respiration rate depicts the proton leak in to the mitochondrial matrix.^{98,110,111} An increase in proton leak could result from alterations in mitochondrial morphology (e.g., damage to the inner mitochondrial

membrane and/or ETC complexes); also oxidative stress has been shown to increase proton leak¹¹⁰⁻¹¹². In isolated rat brain mitochondria, sequential peroxynitrite stimulation decreased the mitochondrial membrane potential and stimulated state 4 respiration, suggesting an increase in the proton leak.^{113 110,111} Maximal respiration in cells is estimated with the addition of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), a mitochondrial uncoupler, that collapses the inner membrane gradient and is no longer controlled by the proton gradient which increases oxygen consumption due to the inner membrane being permeable to protons.^{110,111,114,115} Lastly, non-mitochondrial respiration is measured with the addition of rotenone, Complex I inhibitor, and antimycin, Complex III inhibitor, to abolish all mitochondrial-associated respiration. Non-mitochondrial respiration determines the attribution of non-mitochondrial O₂-consuming processes (e.g., cyclo-oxygenases, lipoxygenases, NADPH oxidases, endoplasmic reticulum, xanthine oxidase), which are typically associated with inflammation, and serve as a negative indicator of bioenergetic health.^{98,110,111,114,115} The activity of NADPH oxidase also contributes to cellular oxygen uptake, and is specifically important within PBMCs (e.g., macrophages) in which NADPH oxidase activity may dominate O₂ uptake.⁹⁸ Non-mitochondrial respiration has been shown to increase in the presence of stressors (e.g., ROS).^{110,111,116}

The use of specific formulas allows for additional measurements on ATP demand, coupling efficiency, spare respiratory capacity, and oxygen consumption rate (OCR) metabolic potential. ATP demand is an estimate of the rate of oxygen required for ATP synthesis and is calculated by subtracting the rate in oxygen consumption with the inhibitor oligomycin from basal respiration.^{98,109} A decrease in ATP demand would indicate a lack in substrate availability or severe damage to oxidative phosphorylation that can impede flow of electrons and result in lower respiration rates.¹¹⁰ Coupling efficiency is the fraction of basal respiration used for ATP demand;

with high coupling efficiency representing a cell attaining high ATP production with relatively low energy consumption.⁹⁸ Spare respiratory capacity is the differences between basal and maximal respiration and represents the cell's ability to respond to increases in ATP demand, with low spare respiratory capacity indicating mitochondrial dysfunction.^{98,110,115,117,118} It has been shown that spare respiratory capacity is depleted in conditions of severe stress, specifically under conditions of oxidative stress.^{116,119–124}

2.4 Peripheral blood mononuclear cells (PBMCs)

2.4.1 Overview of PBMCs

In addition to delivering oxygen throughout the body, blood ensures is a vehicle for circulating immune cells that ward off potential infections. Blood is composed of erythrocytes (transports oxygen/carbon dioxide), granulocytes (involved in immune response), plasma (contains proteins, sugars, and lipid particles), platelets (assists in blood clotting), and PBMCs (major cells in the human body immunity) that are in constant communication with ECs.^{39–41} PBMCs are identified as any blood cell with a round nucleus (e.g., lymphocytes, monocytes, or dendritic cells) that provide a selective response to immune systems in order to fight infections and adapt to future intrusions.^{39–41} The percentage breakdown of PBMCs vary across individuals but typically include 70-90% lymphocytes, 10-20% monocytes, and 1-2% dendritic cells.⁴⁰ The two types of white blood cells found within PBMCs are 1) monocytes, a type of leukocyte that develops into a macrophage when it leaves the blood and transmigrates into blood vessels, and 2) lymphocytes that includes helper T cells, B cells, and natural-killer (NK) cells.^{40,41} Within lymphocytes, the percentage breakdown range is at 70-85% T cells, 5-10% B cells, and 5-20% NK cells.⁴⁰

In the absence of an ongoing immune response, most PBMCs are in a naïve or resting state without effector functions. For example, naïve B cells lack antigens bound to their surface-anchored antigen receptor and naïve T cells express low levels of their cognate antigen/activation markers due to its lack of encountering a foreign antigen (e.g., CD25, CD44, CD69).⁴⁰ When B cells are activated, this initiates the differentiation into highly specific antibody producing plasma cells.^{40,41} When T cells are activated, this initiates a differentiation program for the development of effector functions and the opportunity to further differentiate into memory T cells to secrete a faster/stronger immune response at future antigen encounters.^{40,41}

While T cells are critical players in immune cell homeostasis and host defense, they also contribute to immune and inflammatory diseases.⁴² Monocytes and macrophages have also been important immune cells involved in inflammatory pathways in CVD.⁵⁶ Upon stimulation, monocytes exhibit a pro-atherogenic profile that alters their potential for migration and adhesion to the endothelium and facilitates their maturation into macrophages.¹²⁵ Macrophages with an inflammatory profile continue producing higher levels of inflammatory mediators/cytokines (e.g., e.g., interleukin-6 [IL-6]; tumor necrosis factor- α [TNF- α]) and play a critical role in the development of atherosclerotic plaque.^{56,126}

2.4.2 Source of Oxidative Stress within PBMCs

Research is focusing on further understanding the role of PBMCs in vascular dysfunction, as they are in constant communication with ECs and are primary contributors to systemic ROS.²¹ Macrophages and monocytes with a proinflammatory profile are essential for driving both inflammatory and oxidative signaling pathways involved in vascular dysfunction.²¹ Mice infused with Ang II, a potent vasoconstrictor that causes HTN, showed a significant increase in their macrophage population compared to the sham-infused mice. Furthermore, the depletion of

monocytes in a subset of mice that were then infused with Ang II has been shown to significantly reduce systemic O_2^- levels, ONOO⁻ formation, attenuate vascular dysfunction, and lower Ang II-induced increases in blood pressure compared to control mice.²¹ Along with a decrease in ROS production in the monocyte depleted mice, there was also decrease in the protein expression of the superoxide-generating enzyme NADPH oxidase subunit, gp91phox.²¹ PBMC-derived ROS not only increase ROS production and the secretion of proinflammatory cytokines, but further increase systemic oxidative stress that compromise mitochondrial respiration and impair immune cell function.^{55,127–131}

The implications of mitochondrial function and mitochondrial-derived ROS in circulating PBMCs have been researched in patients with CVD. Mitochondrial-derived ROS in PBMCs isolated from patients with moderate to severe congestive heart failure (CHF) have demonstrated the key role oxidative stress plays in the pathophysiology of CVDs. Specifically, PBMCs isolated from patients with CHF exhibited a marked reduction in mitochondrial transmembrane potential, an increase in intracellular ROS formation, impaired mitochondrial respiratory capacity, reduced SOD activity, and structural derangements (reduction in mitochondrial area with intact cristae) when compared to PBMCs isolated from patients with mild CHF or a control group.^{44–46} With oxidative stress, the mitochondria also become a target of ROS and can result in oxidative damage to the respiratory complexes.^{48,49} These oxidative reactions not only serve to further amplify mitochondrial-derived ROS during respiration but can also result in the nitration of tyrosine residues on SOD2 and potentially inhibit its enzymatic activity⁵⁰.

Furthermore, PBMCs isolated from patients with early-stage CHF have been shown to exhibit decreased mitochondrial oxygen consumption at Complex I and II compared to PBMCs isolated from a control group.⁴⁷ These impairments in the respiratory chain complexes were

inversely related to inflammatory cytokine (e.g., IL-6, TNF- α) and acute phase protein (e.g., C-reactive protein [CRP]) levels.⁴⁷ Reduced mitochondrial transmembrane potential has also been exhibited in PBMCs (e.g., leukocytes, monocytes, lymphocytes) isolated from CHF patients and was associated with increased ROS formation and inflammation.⁴⁶ However, reduced polarity may not be a major indicator of mitochondrial health as mononuclear cells isolated from type 2 diabetics have exhibited hyperpolarization of the mitochondrial membrane, reduced mitochondrial mass, and greater ROS production compared to mononuclear cells isolated from a control group.^{132–134} Interestingly, disease severity seems to play a role, as PBMCs isolated from patients with moderate to severe CHF also exhibited a reduction in mitochondrial respiration that is associated with higher mitochondrial-derived ROS production when compared to PBMCs isolated from patients with mild CHF.⁴⁵

2.4.3 Mitochondrial Dysfunction and its Role in Vascular Dysfunction

Mitochondrial density is considerably lower in vascular tissue when compared to skeletal muscle, liver, or heart.^{135,136} In skeletal muscle and cardiomyocytes, where cells rely heavily on oxidative phosphorylation, mitochondrial density accounts for 15% and 35% of the cell volume.^{137,138} On the other hand, ECs rely heavily on anaerobic glycolysis to meet energy demands and only have a mitochondrial density that accounts for 2-5% of the cell volume.^{139,140} Evidence suggests that the mitochondria in vascular tissue play a more critical role in maintaining cellular and tissue homeostasis by regulating calcium homeostasis, apoptosis, as well as responding to immune and inflammatory pathways.^{37,97}

The bioenergetic profiles in PBMCs (monocytes and leukocytes) have been characterized.¹⁴¹ For monocytes isolated from eight healthy donors, this percentage breakdown in oxygen consumption was 17% non-mitochondrial respiration, 39% spare respiratory capacity, 9%

leak respiration, and 35% is ATP demand. For lymphocytes isolated from eight healthy donors, the percentage breakdown in oxygen consumption was 19% non-mitochondrial respiration, 34% spare respiratory capacity, 15% leak respiration, and 32% is ATP demand.^{141 141141} It appears that activated lymphocytes will switch their metabolic phenotype to increase both their glycolytic function and mitochondrial oxygen consumption.¹⁴² Quiescent T cells show a primary reliance on oxidative phosphorylation to meet their metabolic needs, yet when these T cells are activated, they undergo a metabolic shift from oxidative phosphorylation to glycolysis.¹⁴³⁻¹⁴⁵ This metabolic switch is also seen in activated monocyte-macrophage cells as they shift from oxidative phosphorylation to glycolysis by inhibiting oxidative phosphorylation and in doing so, increases ROS production.^{146,147}

The dysregulation in mitochondrial-derived ROS leads to oxidative stress, as a result of an increase in oxidant production, a decrease in antioxidant defenses, or both that leads to mitochondrial dysfunction.^{37,38} If left unregulated, the oxidative stress within a cell causes mtDNA damage that will ultimately compromise mitochondrial function.¹⁹ For example, H₂O₂-induced DNA damage was more extensive in mtDNA than in nuclear DNA in fibroblasts, and resulted in mitochondrial dysfunction which persisted even with protracted treatment.^{81 81} Oxidative mtDNA damage has been linked to aging, CVD, and neuronal degeneration.⁸¹

Mitochondria are essential for maintaining cell viability and survival, and therefore changes in biogenesis, morphology, and function are all factors that may contribute to mitochondrial dysfunction and the development of CVDs.¹¹² Mitochondrial dysfunction is hypothesized to contribute to endothelial dysfunction, atherosclerosis, HTN, and increased risk of CVD.^{31,148-154} As previously stated, there is a racial disparity in the prevalence, morbidity, and mortality for CVD; with NHB individuals considered an at-risk population compared to NHW

individuals.¹⁻⁴ Cellular experiments provide further insight to potential pathways and avenues that can contribute to these racial differences in CVD progression.

2.5 Racial differences in Vascular Health

Social determinants of health (SDoH) encompass the economic, social, environmental, and psychosocial factors that influence the progression of CVD risk factors.¹⁵⁵ SDoH bring attention to the social position of vulnerable/disadvantaged groups, chronic exposure to stress, impact of health factors, and the disparities observed in CVD outcomes.¹⁵⁶ In the United States, an individual's lived experience includes race/ethnicity, discrimination, economic stability, housing stability, neighborhood violence, food security, sleep quality, education access, healthcare access, and community relationships.^{155,157}

Neighborhood socioeconomic environment is calculated with data on housing, income, education level, and occupation information, with a lower social economic status (SES) associated with increased incidence of coronary heart disease and heart failure.^{158,159} Individuals with a lower SES category also have greater exposure to compounding health behaviors (e.g., smoking, alcohol consumption, physical inactivity, and diet) and less access to medical support, all of which increase their risk of CVD.^{163,164} SES is one prominent variable that partially explains the racial disparities for CVD risk factors (e.g., HTN, diabetes, obesity), with a disproportionate percentage of NHB individuals in the lower SES category compared to NHW individuals.¹⁶⁰⁻¹⁶² Furthermore, there is a growing interest in targeting translational and basic research to unveil mechanisms by which SDoH influence the biological pathways.

NHB Americans are an at-risk population, this is largely attributed to the early onset of diminished endothelial function (e.g., heightened oxidative stress, inflammatory markers) and the elevated CVD risk factors with the African American/Black race.^{8,165} NHB Americans not only

exhibit a higher prevalence for HTN and CVD, but a higher prevalence for endothelial dysfunction, elevated levels of oxidative stress, and greater systemic low-grade inflammation (e.g., IL-6, CRP) compared to NHW individuals in epidemiological and clinical studies.^{6,8–10,69,166} Even among those with normotensive (NT) blood pressure, NHB individuals have greater vasoconstrictor responses with sympathetic stimulation, an impaired vasodilation responses, and a smaller lumen to media ratio compared to NHW individuals.¹⁶⁶ Across NT and HTN patients, NHB individuals also exhibit a four- to eight- fold higher concentration for plasma ET-1 levels, potent vasoconstrictor and inhibitor of NO production, compared to NHW individuals.^{167,168} These racial differences can even be observed during submaximal testing, with NHB individuals expressing a higher oxidative stress response compared to NHW individuals.¹⁶⁵ Along with an increase in oxidative stress, higher levels of circulating inflammatory cytokines (e.g., IL-6, TNF), and acute phase proteins (e.g., CRP) found in NHB individuals compared to NHW individuals contributes to the decrease in eNOS expression, NO bioavailability, and endothelial dysfunction.⁸ Prehypertensive BP values have been shown to significantly correlate with higher levels of circulating inflammatory biomarkers (TNF- α ; IL-6, CRP) that aid in creating a proinflammatory environment which contributes to endothelial damage and dysfunction.^{6,11,169} These racial differences may contribute impairment to both endothelium – independent and – dependent (e.g., decreased NO bioavailability and production) responses.

In basal conditions, human umbilical vein ECs (HUVECs) isolated from NHB adults have been shown to have significantly lower NO bioavailability accompanied by an increase in ROS production (O_2^- and $ONOO^-$), higher production of O_2^- from NADPH oxidase-stimulation, and higher protein expression in NADPH oxidase subunits (p67phox, p47phox, p22phox) compared to HUVECs isolated from NHW individuals.¹² HUVECs isolated from NHB individuals have

been shown to exhibit lower SOD1 activity and total SOD activity, higher protein expression for NADPH oxidase subunits (p47phox, gp91phox, and p22phox), and higher levels of circulating proinflammatory cytokines (e.g., IL-6) compared to HUVECs isolated from NHW individuals, all contributing to a heightened oxidative stress.^{8,165}

To date, only one study, to our knowledge, has delved into PBMCs as a potential player in the racial differences in systemic oxidative stress and its potential contribution to the disparities in CVD prevalence.⁴³ PBMCs isolated from healthy NHB men exhibited a greater protein expression for NADPH oxidase subunits (p47phox and gp91phox) and elevated intracellular O_2^- production compared to PBMCs isolated from healthy NHW men that could contribute to the increased systemic oxidative stress seen in this at-risk population. Limited work has been performed to investigate the source(s) and mechanism(s) contributing the racial differences in oxidative stress in PBMCs. At present there is no data looking at the mitochondria as a contributor in the potential racial differences in PBMCs. Given the role of mitochondria as a source of oxidative, it is important to further explore the role of the mitochondria (ETC complexes, antioxidant defense enzymes, and mitochondrial respiration) as a potential player in the racial differences observed in vascular function and the pathophysiology of CVDs.

Chapter 3: Methods

3.1 Participant Recruitment

Peripheral blood mononuclear cells (PBMCs) were isolated as an ancillary subproject from forty-one participants in the "Neighborhood Disadvantage, Sleep, and Vascular Health" (NDSVH) Study (NCT04576338). Research participants in the NDSVH study were recruited from an NIH National Heart, Lung, and Blood Institute (NHLBI) grant (Dr. Thomas Fuller-Rowell; R15HL140504) which consisted of NHB and NHW college students at Auburn University who were, by design, healthy, young adults free from known cardiometabolic diseases (e.g., medicated hypertension, diabetes, poorly controlled hyperlipidemia) or malignancies. Additional exclusion criteria for participants included: 1) reports of sleep disorders, 2) sleep disorder screening measures above threshold values at screening visit, 3) serious or medical conditions that could interfere with sleep or precluded wearing an actigraph. Participants in the NDSVH study were involved in an experimental laboratory visit that included peripheral vascular testing, a blood draw, participation in eight-days of sleep actigraphy (Phillips Actiwatch Spectrum PLUS) and a sleep diary assessment, concurrent physical activity actigraphy (Actigraph GT3X-BT), and ambulatory blood pressure monitoring (Suntech Oscar2 with SphymoCor technology). For additional detail on data collection procedures see NCT04576338. Prior to any research activities, participants provided written informed consent. Participants were compensated \$110 following the study completion. All study procedures complied with ethical guidelines for human subject's research and received approval from an institutional review board (Protocol AU IRB #20-262 MR 2008).

3.2 PBMCs Isolations

Whole blood was obtained by standard venipuncture using six 10 mL BD K₂EDTA vacutainers (VWR Cat No. BD-366643; Avantor, Radnor PA). Briefly, Human PBMCs were

separated by density-gradient centrifugation using lymphocyte separation media-1077 (LSM; Cat. No. C-44010; PromoCell; Heidelberg, Germany). EDTA anti-coagulated blood was diluted 1:1 of Dulbecco's Phosphate-Buffered Saline without calcium and magnesium (DPBS; Cat. No. 21-031-CM; Corning, Corning, New York) treated with 1% penicillin-streptomycin, then ~30ml of the DPBS-diluted blood was carefully layered on top of 12mL LSM and centrifuged at 650 g for 30 mins at 10°C with the slowest acceleration and deceleration settings. The PBMCs layer was carefully transferred into 25mL DBPS and centrifuged at 150 g for 10 mins at 10°C. PBMCs were then washed twice by discarding the supernatant, resuspending the pellet in 1mL DPBS, and an additional 13mL before centrifuging again. After the second wash, the pellet was resuspended in 2ml DPBS to determine cell count and viability using the Cell Countess II Automated Cell Counter (Thermo Fisher; Waltham, MA). Trypan blue (ca No. T10282; Thermo Fisher) was used for viable and non-viable cell identification. Cell viability was calculated using viable cell count/total cell count. PBMCs underwent one final centrifuge at 150 g for 5 mins at 10°C, 1mL of the supernatant was then discarded, and PBMCs were resuspended in 1mL of freezing media (90% Fetal Bovine Serum + 10% DMSO) to be stored in the Mr. Frosty Freezing Container (Thermo Fisher; Waltham, MA) at -80°C for 24 hours before the vial was transferred into the liquid nitrogen dewar until ready for experimental measures.

3.3 PBMCs Experimental Preparation

PBMCs were removed from the liquid nitrogen dewar, placed in a 37°C water bath for two minutes, and mixed with 1 ml of ThermoPEAK™ X-VIVO™-15 Media (Cat. No. BEBP04-744Q; Lonza, Morriston, NJ) in a sterile cell hood. The PBMCs suspension was then transferred into a 50 ml Falcon tube and an additional 10 ml of media was slowly added. PBMCs were centrifuged at 400 g for 15 minutes at 24°C, supernatant was aspirated, and PBMC pellet was resuspended

with 10 ml media before being centrifuged a second time. After the supernatant was aspirated a second time, PBMCs were resuspended in 12 ml media and placed overnight in the 37°C humidity-controlled incubator with 5% CO₂ for ~14-18 hours. The cap of the tube was loosened in order to allow for gas exchange and the tube was placed at an angle of ~5° above horizontal.

After the overnight incubation, PBMCs were centrifuged at 400 g for 15 minutes at 24°C, the supernatant was aspirated, and the PBMC pellet was resuspended in 800 µl of media. To determine cell density, the cell media suspension was diluted 1:6 of Trypan blue and the Cell Countess II Automated Cell Counter was used. Of the resuspended PBMCs, 400 µl was aliquoted for cell respiration readings and the remaining 400 µl for harvesting protein which are described below.

3.4 Cell Respiration

For cell respiration readings an oximeter chamber (Oxytherm, Hanstech) was used. Chambers were first equilibrated with 300 µl of TheraPEAK™ X-VIVO™-15 Media. Then 400 µl of PBMC suspension was added into the chamber for three-minutes of basal respiration. Basal respiration was immediately followed by the addition of 1µM Oligomycin, an ATP synthase inhibitor, for three-minutes to determine leak respiration. Following leak respiration, 1 µM FCCP, a mitochondrial uncoupler, was added for five-minutes to determine maximal respiration. Finally, 0.5µM Rotenone, a Complex I inhibitor, and 0.5µM Antimycin, a Complex III inhibitor, were subsequently added, with respiration recorded for another five to ten-minutes. With each pharmaceutical agent added to the chamber, respiration readings were recorded until a steady state was reached, usually between three to five minutes. Basal and maximal respiration rates were normalized to viable cell density. Below are the formulas used for additional measurements in

ATP demand, coupling efficiency, spare respiratory capacity, and oxygen consumption rate (OCR) metabolic potential.

$$\text{Basal Respiration} = \frac{\text{Basal Respiration (nmol O}_2\text{/mL/min)}}{\text{Viable Cell Density (1 x 10}^6\text{ cells)}}$$

$$\text{Maximal Respiration} = \frac{\text{Maximal Respiration (nmol O}_2\text{/mL/min)}}{\text{Viable Cell Density (1 x 10}^6\text{ cells)}}$$

$$\text{Coupling Efficiency} = \frac{\text{ATP Demand (nmol O}_2\text{/mL/min/1 x 10}^6\text{ cells)}}{\text{Basal Respiration (nmol O}_2\text{/mL/min/1 x 10}^6\text{ cells)}}$$

$$\text{Leak Respiration} = \frac{\text{Respiration with Oligomycin (nmol O}_2\text{/mL/min)}}{\text{Viable Cell Density (1 x 10}^6\text{ cells)}}$$

$$\text{Non-Mitochondrial Respiration} = \frac{\text{Non-mitochondrial respiration (nmol O}_2\text{/mL/min)}}{\text{Viable Cell Density (1 x 10}^6\text{ cells)}}$$

$$\text{ATP Demand} = \text{Basal Respiration} - \text{Leak Respiration}$$

$$\text{Spare Respiratory Capacity} = \text{Maximal Respiration} - \text{Basal Respiration}$$

$$\text{OCR Metabolic Potential} = \frac{[\text{Maximal Respiration} \times 100]}{\text{Basal Respiration}}$$

3.5 PBMCs Protein Isolation

The remaining 400 μ l PBMC suspension was centrifuged at 15,000 g for 7 mins at 4°C. The pelleted PBMCs were washed three times with 400 μ l of ice-cold PBS and resuspended in 400 μ l PBS before being centrifuged a second time. The pelleted cells were then washed three times with 400 μ l PBS and went through a liquid homogenization with an ice-cold RIPA (Cat. No. 89901; Thermo Fisher) lysis buffer containing a protease and phosphatase inhibitor cocktail (Cat. No. 78446; Thermo Fisher). These samples were placed on ice and on a shaker for 30 minutes before they were centrifuged. Supernatant was immediately stored at -80°C for protein analysis.

3.6 Western Blotting

Protein concentrations of cell lysates were determined using the Pierce BCA (bicinchoninic acid) protein assay kit (Cat. No. 23227; Thermo Fisher). Samples containing 10 μ g of protein were mixed with NuPage LDS Sample Buffer 4X (Cat. No. NP0008; Thermo Fisher) and NuPage Sample Reducing Agent 10X (Cat. No. NP0009; Thermo Fisher). Denaturing of samples was accomplished by heating at 70°C for 10 minutes.

Tris-glycine SDS-PAGE was utilized to separate proteins using 15% TGX gels (Cat. No. 5678084; BioRad, Hercules, CA). All samples were run in duplicate gels, with the ladder containing both a pre-stained Novex Sharp (Cat No. LC5800; Life Technologies, Rockville, MD) and horseradish peroxidase (HRP)-linked ladder MagicMark XP (Cat No. LC5602; Life Technologies) for molecular weight visualization.

Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes using a wet transfer process, blocked with 5% non-fat dry milk (NFDM) for 1 hour at room temperature, and incubated overnight with the primary antibodies of interest on a shaker with gentle agitation at 4-8°C. Primary antibodies used were: rabbit monoclonal anti-p47phox (47 kDa; Cat. No. 4301S, Cell signaling technology, Beverly, MA), mouse monoclonal anti- α -Tubulin (52 kDa; Cat. No. 3873S, Cell signaling technology), mouse monoclonal anti-SOD1 (18 kDa; Cat No 4266S, Cell signaling technology), mouse monoclonal anti-gp91-phox (60 kDa; Cat. No. sc-130543, Santa cruz biotechnology, Dallas, TX), rabbit polyclonal anti-SOD2 (26 kDa; Cat. No. GTX116093, GeneTex, Irvine, CA), rabbit monoclonal anti-SIRT3 (43 kDa; Cat. No. ab217319, Abcam Cambridge, United Kingdom), mouse polyclonal Total OXPHOS WB Antibody Cocktail (complex I, 18 kDa; complex II, 30 kDa; complex III, 48 kDa; complex IV, 40 kDa; complex V, 55 kDa; Cat. No. ab110413, Abcam), and rabbit monoclonal anti-SOD2/MnSOD (Acetyl K68; 24

kDa; Cat. No. ab137037, Abcam). All antibodies were prepared in a 1:1000 dilution in 5% Bovine Serum Albumin (BSA; VWR Cat No. 97061-420; Avantor, Radnor PA).

After an overnight incubation, membranes were washed with Tris-Buffered Saline with Tween (TBST) and incubated with the appropriate secondary antibody, Anti-mouse (Cat. No. 7076S, Cell signaling technology) or Anti-rabbit (Cat. No. 7074P2, Cell signaling technology), at a 1:1000 dilution in 5% BSA and conjugated with horseradish peroxidase (HRP). Proteins were visualized by chemiluminescent detection using an HRP illumination substrate Luminata Forte (Cat. No. WBLUF0100; EMD Millipore, Billerica, MA). The UVP ChemiDoc-it2 imaging system was used to image each PVDF membrane and the Vision Woks software was used to quantify protein expression levels by band densitometry analyses. The densities of the selected protein bands were normalized to the internal control protein, α -Tubulin.

3.7 Statistical Analyses

All study variables were checked for normality using the Kolmogorov-Smirnov test and descriptive statistics were also performed. Non-adjusted differences between groups were analyzed with the student's t-test (for normally distributed data) or the Mann-Whitney U test (non-parametric test for non-normally distributed data). All tests were performed using two-tailed tests, and a significance level of $p \leq 0.05$ was adopted throughout. All analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL). Tables with participant characteristics were reported as mean and standard deviation, while figures were reported as mean, standard deviation, and plotted as individual data points.

Chapter 4: Results

4.1 Demographic Characteristics

A total of forty-one participants completed the blood draws for PBMC isolations. Participant characteristics (age, height, weight, resting blood pressure, glucose levels, triglycerides, and total cholesterol) can be found in Table 1. Participant characteristics were separated by race (Table 2), and no racial differences were found for risk factors of CVD (e.g., blood pressure, glucose). When participant characteristics were separated by sex (Table 3), females had significantly lower body mass ($p=0.02$) and glucose levels ($p=0.05$) than their male counterparts.

4.2 Protein Expression of Antioxidant Modulators of Oxidative Stress

No racial differences were found for protein expression levels of the antioxidant modulators of oxidative stress, which included SOD1 (Fig. 6A, $p=0.280$), SOD2 (Fig. 6B, $p=0.817$), AcSOD2 (Fig. 6C, $p=0.237$), and SIRT3 (Fig. 6D, $p=0.237$).

When comparing sex differences for protein expression of oxidative stress modulators, PBMCs isolated from females exhibited a greater expression of SIRT3 compared to PBMCs isolated from males (Fig. 7D, $p=0.038$). However, no sex differences were found in the expression of SOD1 (Fig. 7A, $p=0.138$), SOD2 (Fig. 7B, $p=0.142$), and AcSOD2 (Fig. 7C, $p=0.187$).

4.3 Protein Expression of Superoxide-Generating NADPH Oxidase Subunits

No racial differences were found in protein expression levels of the NADPH Oxidase subunits gp91phox (Fig. 8A, $p=0.862$) or p47phox (Fig. 8B, $p=0.908$).

Also, no sex differences were found in protein expression levels of the NADPH Oxidase subunits gp91phox (Fig. 9A, $p=0.245$) or p47phox (Fig. 9B, $p=0.111$).

4.4 Protein Expression of Mitochondrial ETC Complexes

When comparing racial differences for protein expression levels of the mitochondrial complexes, PBMCs isolated from NHB individuals exhibited a significantly greater expression for Complex I compared to PBMCs isolated from NHW individuals (Fig. 10A, $p=0.014$). Also, PBMCs isolated from NHB individuals tended to have a lower expression for Complex V compared to PBMCs isolated from NHW individuals, however, it was not significant (Fig. 10E, $p=0.064$). There were no racial differences in the expression of Complex II (Fig. 10B, $p=0.471$), Complex III (Fig. 10C, $p=0.489$), or Complex IV (Fig. 10D, $p=0.198$).

Moreover, no sex differences were found for protein expression levels of the mitochondrial complexes; Complex I (Fig. 11A, $p=0.808$), Complex II (Fig. 11B, $p=0.187$), Complex III (Fig. 11C, $p=0.716$), Complex IV (Fig. 11D, $p=0.435$), or Complex V (Fig. 11E, $p=0.435$).

4.5 PBMC Respiration

When comparing racial differences in cellular respiration, PBMCs isolated from NHB individuals exhibited lower levels of basal respiration, maximal respiration, leak respiration and non-mitochondrial respiration compared to PBMCs isolated from NHW individuals (Fig. 12A $p=0.017$; Fig. 12B, $p=0.057$; Fig. 12D, $p=0.009$; Fig. 12E, $p=0.014$ respectively). Also, PBMCs isolated from NHB individuals tended towards higher levels of OCR Metabolic Potential compared to PBMCs isolated from NHW individuals, nevertheless, it was not significant (Fig. 12H, $p=0.068$). Finally, no racial differences were found in Coupling Efficiency (Fig. 12C, $p=0.129$), Spare Respiratory Capacity (Fig. 12G, $p=0.436$) or ATP Demand (Fig. 12F, $p=0.256$).

Furthermore, no sex differences in cellular respiration were found for Basal Respiration (Fig. 8a, $p=0.989$), Maximal Respiration (Fig. 13B, $p=0.882$), Coupling Efficiency (Fig. 13C, $p=0.173$), Leak Respiration (Fig. 13D, $p=0.616$), Non-Mitochondrial Respiration (Fig. 13E, $p=0.597$), ATP demand (Fig. 13F, $p=0.364$), Spare Respiratory Capacity (Fig. 13G, $p=0.579$), or OCR Metabolic Potential (Fig. 13H, $p=0.680$).

Table 1. Participant Characteristics

	All Participants (N = 41)	
	Mean ± SD	Range
Age (years)	21.4 ± 0.7	20 – 23
Height (cm)	171.6 ± 10.4	155.0 – 191.0
Weight (Kg)	73.7 ± 13.0	52.3 – 104.8
Resting Systolic Blood Pressure (mmHg)	105 ± 8	87 – 119
Resting Diastolic Blood Pressure (mmHg)	63 ± 7	47 – 76
Glucose (mg/dL)	86 ± 9	71 – 109
Triglycerides (mg/dL)	73 ± 25	<45 – 132
Total Cholesterol (mg/dL)	168 ± 30	104 – 243

Values are means ± SD.

Table 2. Participant Characteristics by Race

	NHW (N = 24)		NHB (N = 17)		p Value	Effect Size (Cohen's d)
	Mean ± SD	Range	Mean ± SD	Range		
Age (years)	21.3 ± 0.8	20 – 23	21.6 ± 0.6	21 – 23	0.30	0.36
Height (cm)	172.9 ± 11.0	155.0 – 191.0	169.7 ± 9.6	155.0 – 189.0	0.40	0.31
Weight (Kg)	73.3 ± 12.7	52.3 – 95.7	74.4 ± 13.8	53.3 – 104.8	0.80	.08
Resting Systolic Blood Pressure (mmHg)	104 ± 7	89 – 115	105 ± 9	87 – 119	0.82	.08
Resting Diastolic Blood Pressure (mmHg)	62 ± 7	47 – 74	63 ± 8	51 – 76	0.59	.18
Glucose (mg/dL)	87 ± 9	76 – 107	84 ± 10	71 – 109	0.24	.38
Triglycerides (mg/dL)	74 ± 24	<45 – 122	72 ± 29	<45 – 132	0.65	.06
Total Cholesterol (mg/dL)	170 ± 28	127 – 243	164 ± 34	104 – 219	0.56	.19

Values are means ± SD.

Table 3. Participant Characteristics by Sex

	Males (N = 22)		Females (N = 19)		p Value	Effect Size (Cohen's d)
	Mean ± SD	Range	Mean ± SD	Range		
Age (years)	21.3 ± 0.8	20 – 23	21.3 ± 0.7	20 – 23	0.27	0.32
Height (cm)	173.6 ± 11.6	155.0 – 191.0	169.2 ± 8.6	157.0 – 184.0	0.24	0.42
Weight (Kg)	78.0 ± 13.6	52.3 – 104.8	68.8 ± 10.8	53.3 – 91.9	0.02*	0.70
Resting Systolic Blood Pressure (mmHg)	106 ± 8	89 – 119	103 ± 8	87 – 114	0.31	0.32
Resting Diastolic Blood Pressure (mmHg)	63 ± 8	47 – 76	63 ± 7	51 – 75	1.00	0.002
Glucose (mg/dL)	89 ± 8	75 – 107	83 ± 9	71 – 109	0.05**	0.63
Triglycerides (mg/dL)	74 ± 24	<45 – 122	77 ± 28	<45 – 77	0.51	0.21
Cholesterol (mg/dL)	163 ± 29	104 – 219	173 ± 31	117 – 243	0.29	0.34

Values are means ± SD.

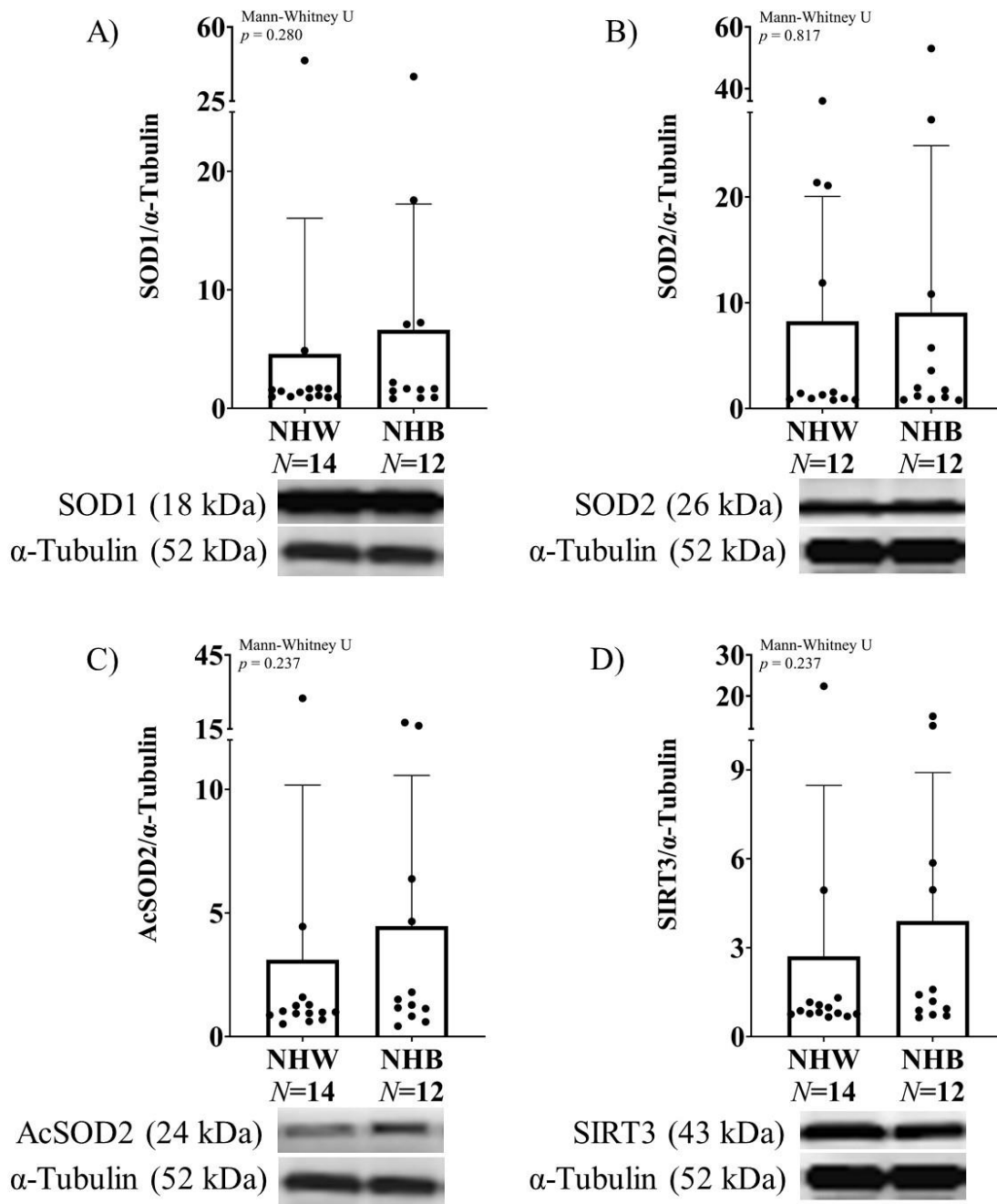


Figure 6. Protein Expression of Antioxidant Modulators of Oxidative Stress in PBMCs isolated from NHW and NHB Individuals.

No racial differences in A) SOD1, B) SOD2, C) AcSOD2, or D) SIRT3 protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment (N = 24 – 26 PBMC protein samples) that were run in duplicate gels.

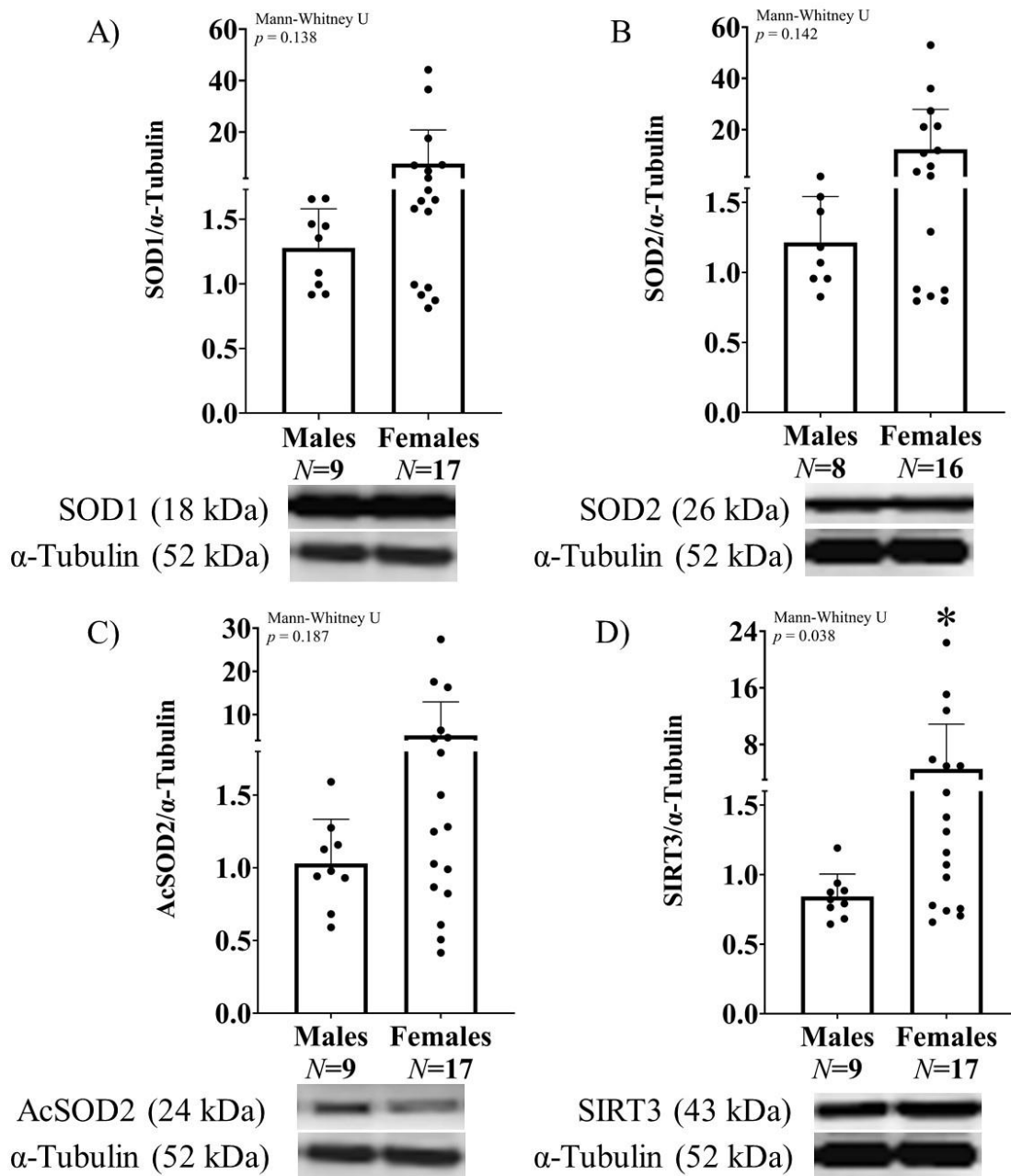


Figure 7. Protein Expression of Antioxidant Modulators of Oxidative Stress in PBMCs isolated from Males and Females.

PBMCs isolated from females exhibited a higher expression in D) SIRT3 compared to PBMCs isolated from males. No sex differences were found for A) SOD1, B) SOD2, or C) AcSOD2 protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment (N = 24-26 PBMC protein samples) that were run in duplicate gels.

* Significantly higher ($p < 0.014$) compared to PBMCs isolated from males.

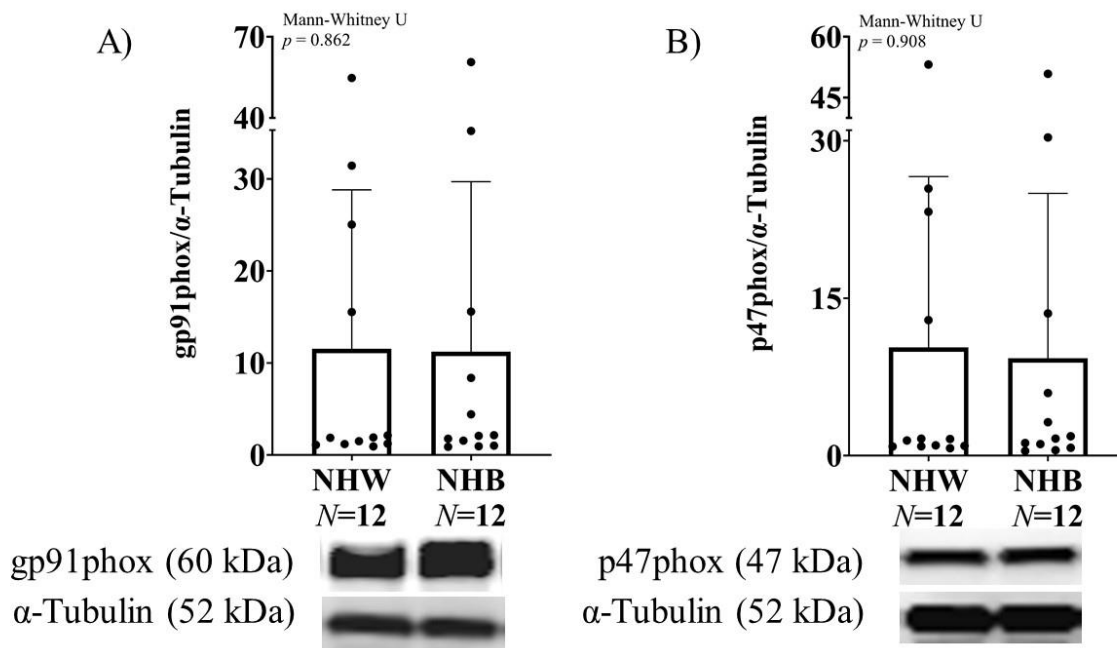


Figure 8. Protein Expression of Superoxide-generating NADPH Oxidase Subunits in PBMCs isolated from NHW and NHB Individuals.

No racial differences for A) gp91phox or B) p47phox protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment (N = 24 PBMC protein samples) that were run in duplicate gels.

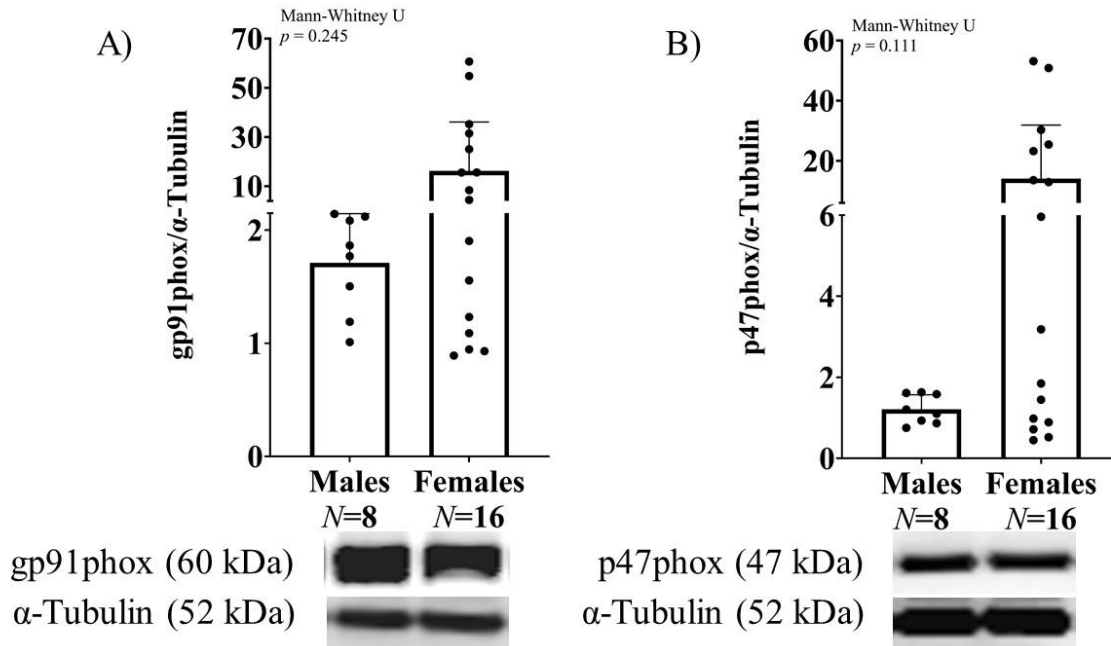
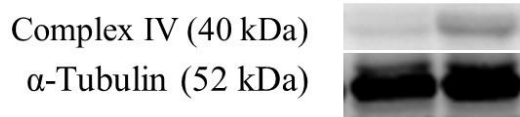
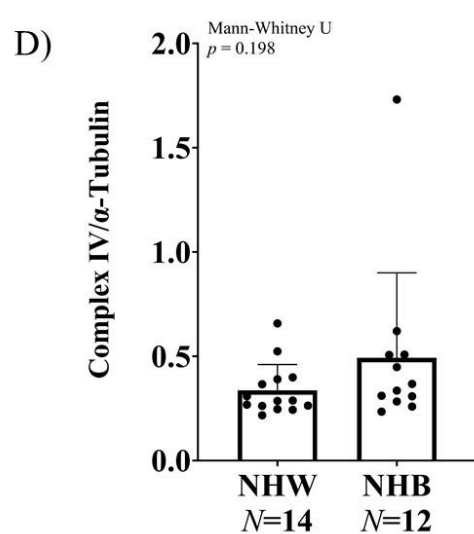
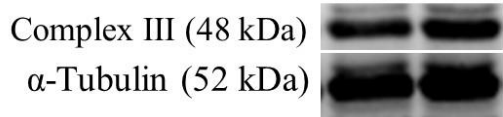
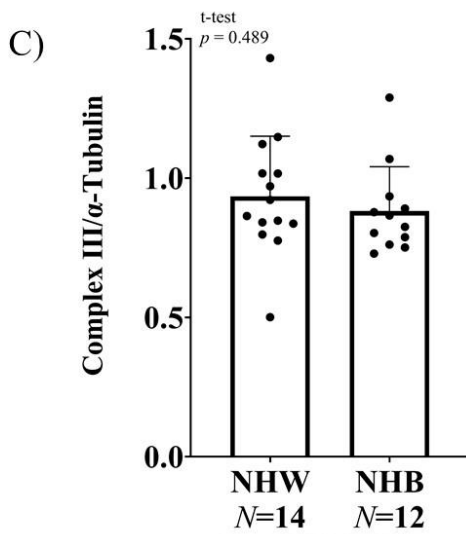
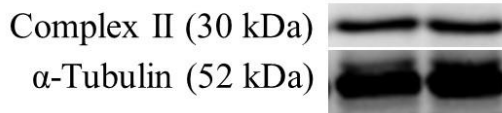
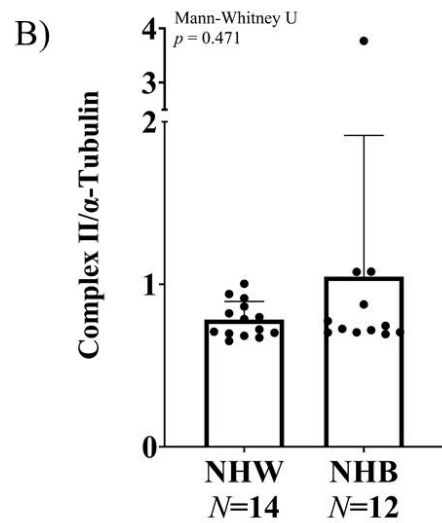
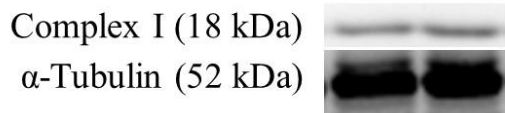
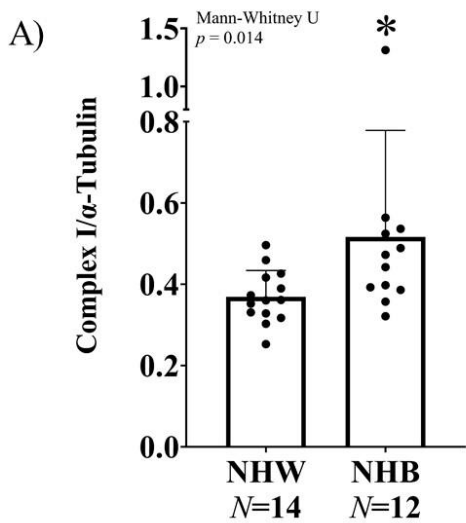


Figure 9. Protein Expression of Superoxide-generating NADPH Oxidase Subunits in PBMCs isolated from Males and Females.

No sex differences were found for A) gp91phox or B) p47phox protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment (N = 24 PBMC protein samples) that were run in duplicate gels.



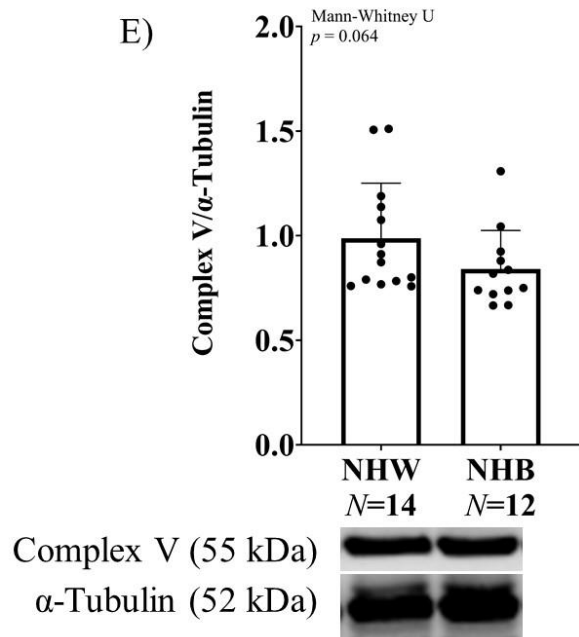
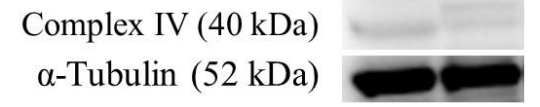
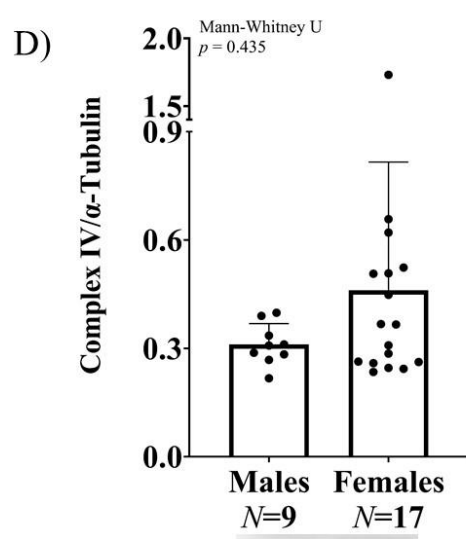
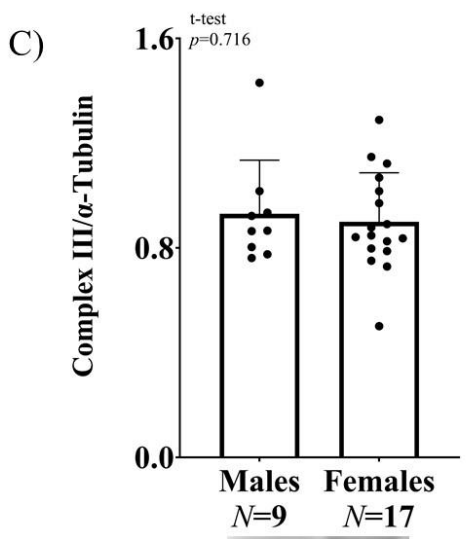
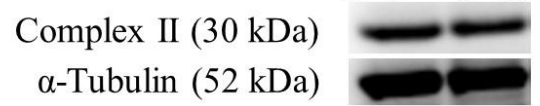
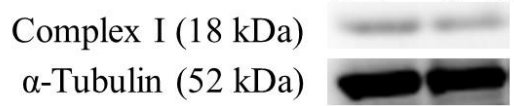
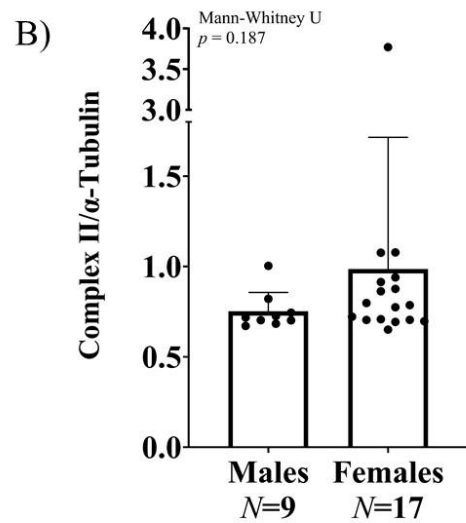
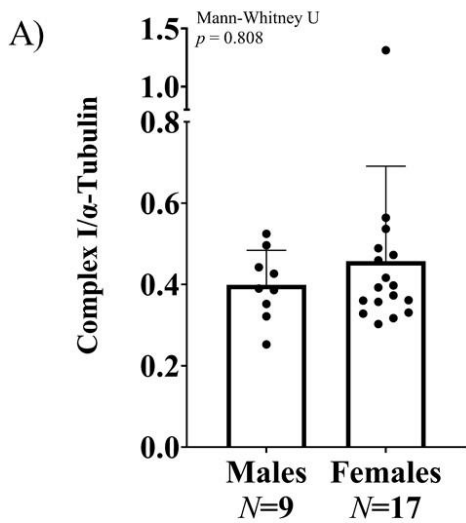


Figure 10. Protein Expression of Mitochondrial Complexes in PBMCs isolated from NHW and NHB Individuals.

PBMCs isolated from NHB individuals demonstrated a higher expression of A) Complex I compared to PBMCs isolated from NHW individuals. No racial differences were exhibited for B) Complex II, C) Complex III, D) Complex IV, or E) Complex V protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment ($N = 26$ PBMC protein samples) that were run in duplicate gels.

* Significantly higher ($p = 0.014$) compared to PBMCs isolated from NHW individuals.



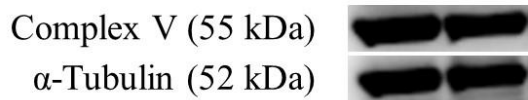
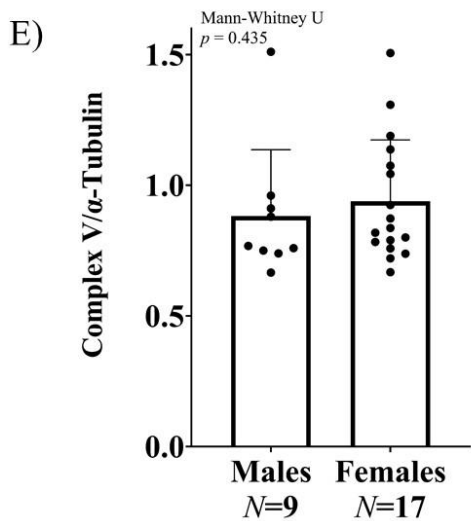
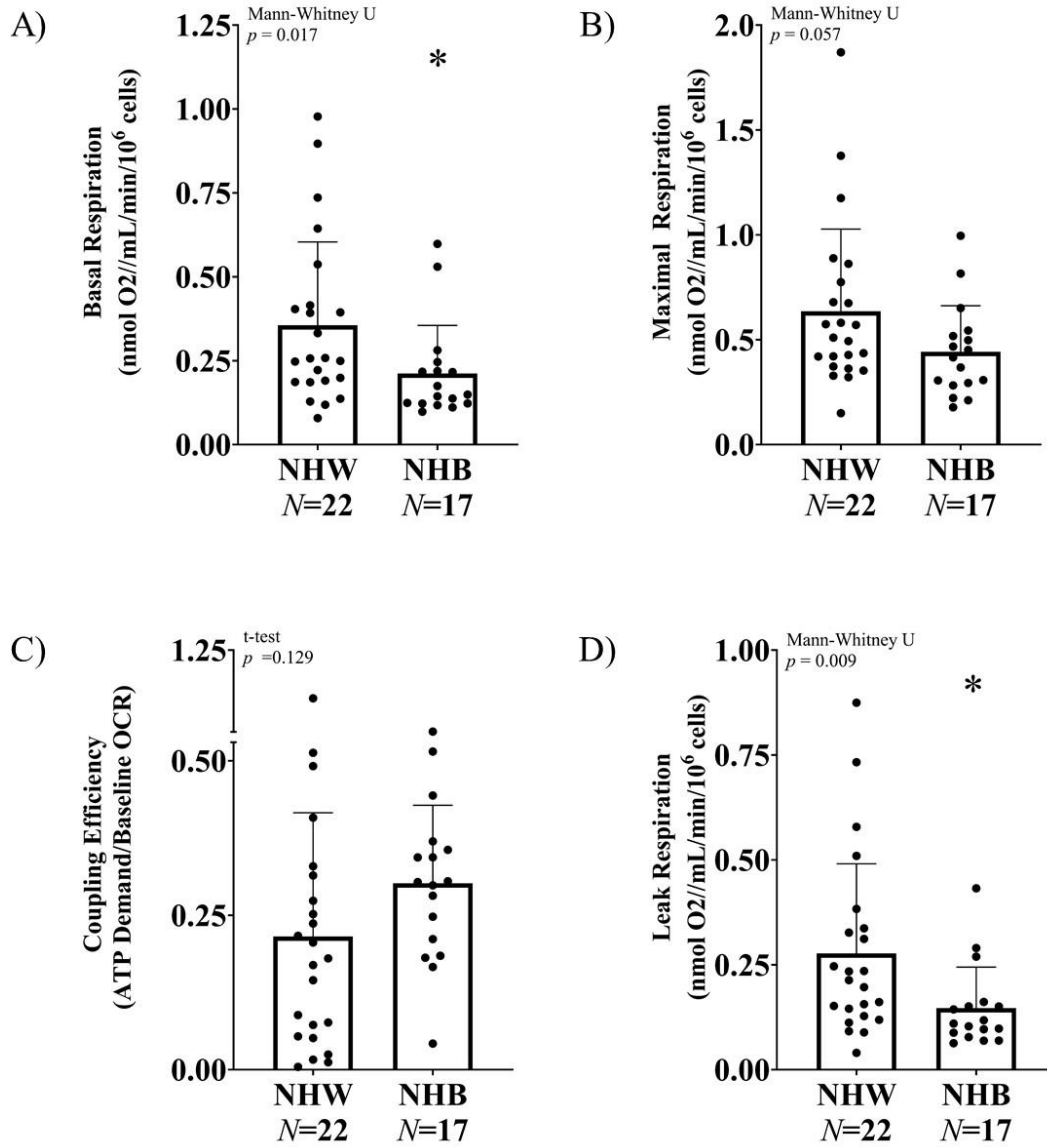


Figure 11. Protein Expression of Mitochondrial Complexes in PBMCs isolated from Males and Females.

No sex differences were found for A) Complex I, B) Complex II, C) Complex III, D) Complex IV, or E) Complex V protein expression in PBMCs. Densitometric quantifications were normalized to housekeeping protein (α -Tubulin). Data are represented as mean \pm SD from one independent experiment (N = 26 PBMC protein samples) that were run in duplicate gels.



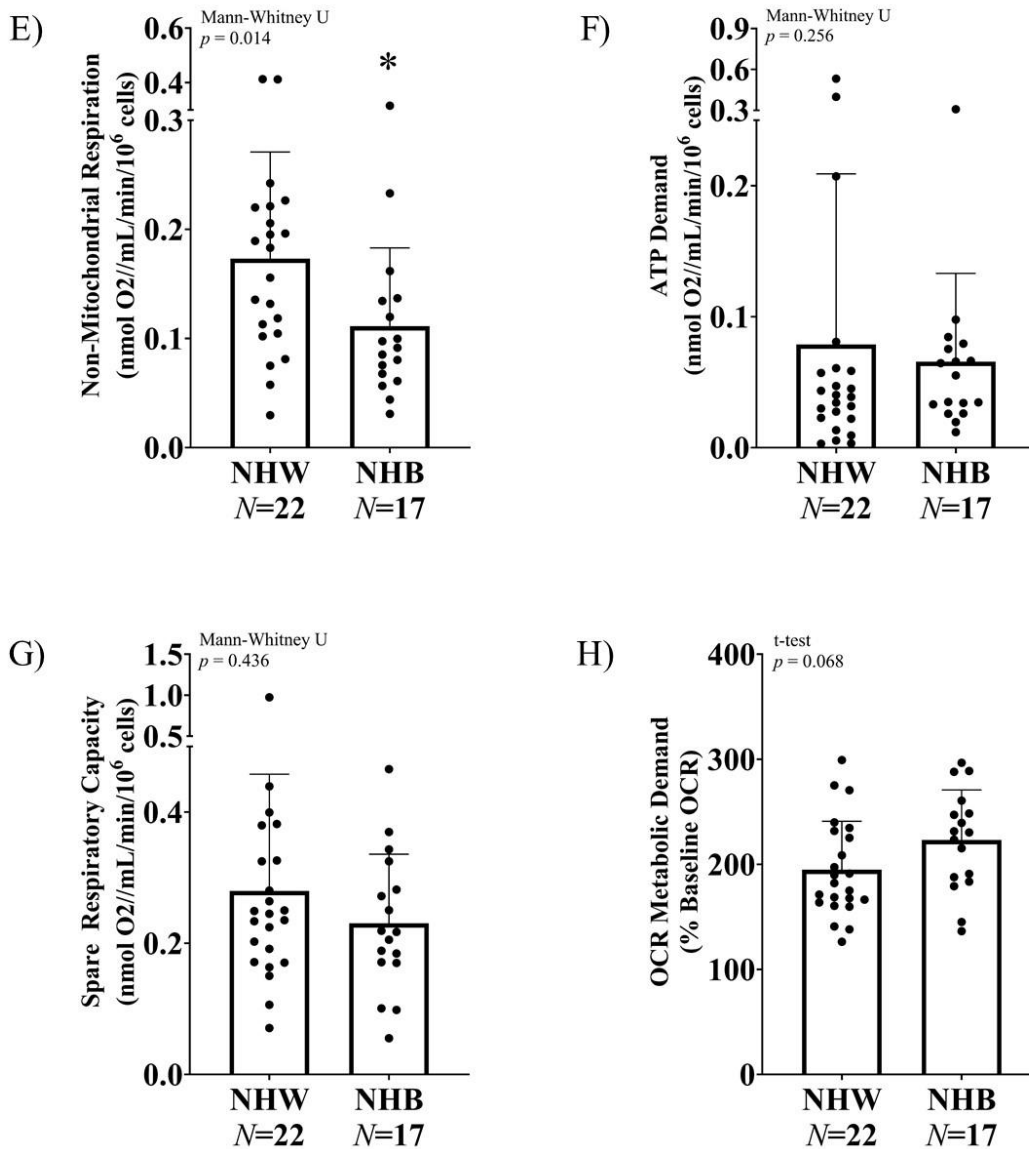
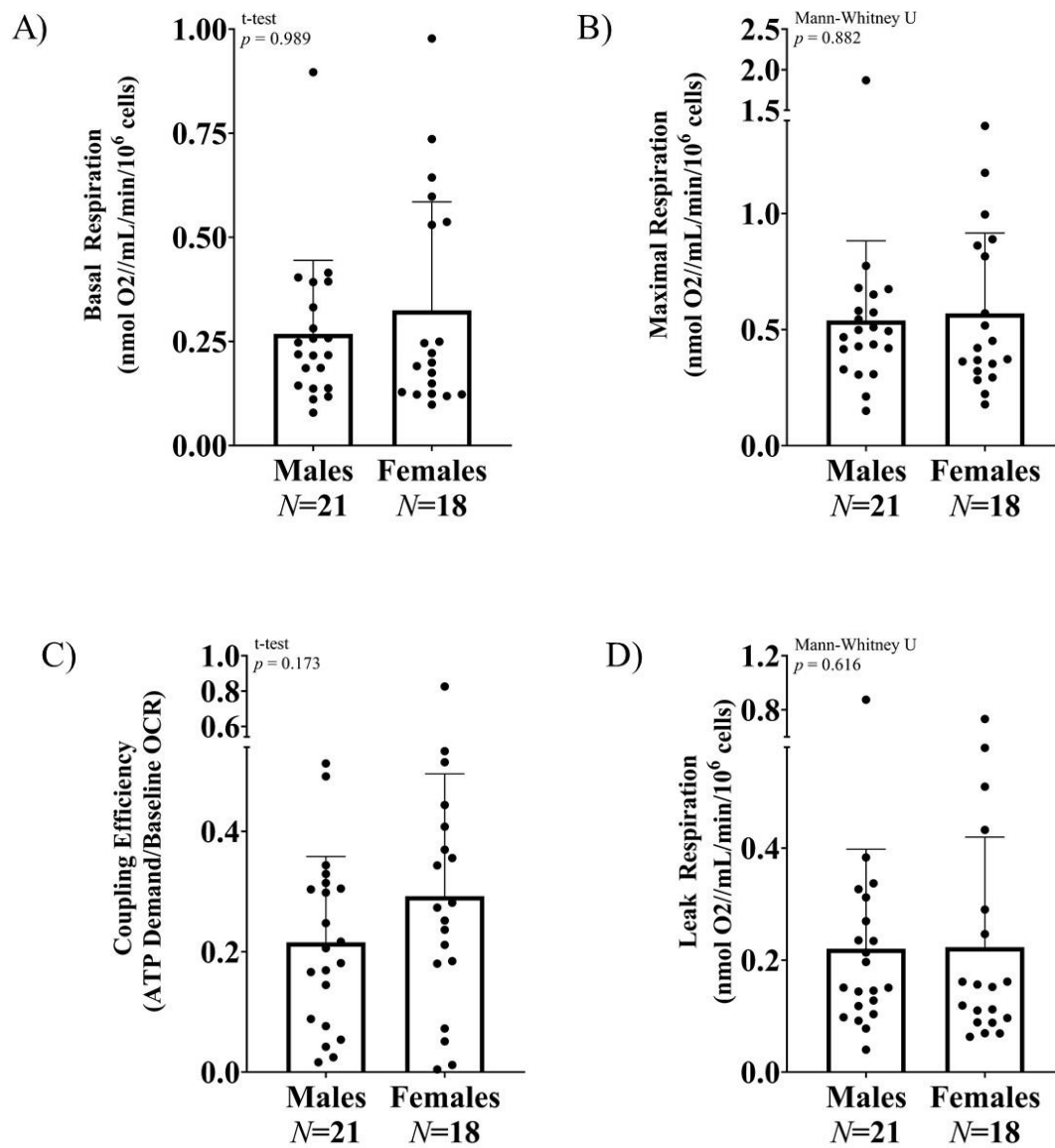


Figure 12. Respiration measures in PBMCs isolated from NHW and NHB Individuals.

PBMCs isolated from NHB individuals demonstrated a significantly lower A) Basal Respiration, D) Leak Respiration, and E) Non-Mitochondrial Respiration compared to PBMCs isolated from NHW individuals. No racial differences in B) Maximal Respiration, C) Coupling Efficiency, F) ATP Demand, G) Spare Respiratory Capacity, and H) OCR Metabolic Potential in PBMCs. Respiration measures were normalized to viable cells. Data are represented as mean \pm SD from one independent experiment (N = 39 PBMC samples).

* Significantly lower ($p < 0.05$) compared to PBMCs isolated from NHW individuals.



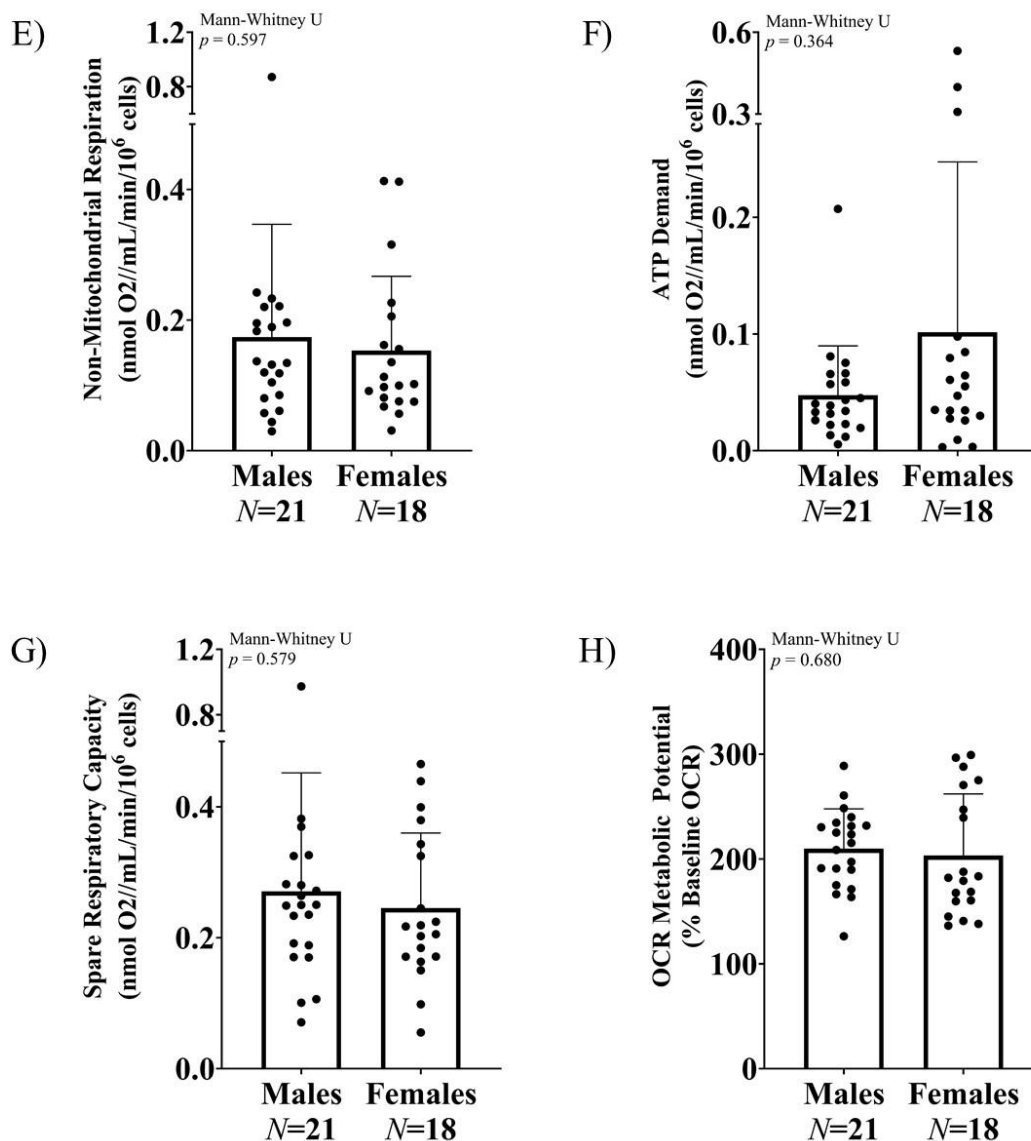


Figure 13. Respiration measures in PBMCs isolated from Males and Females.

No sex differences were found for A) Basal Respiration, B) Maximal Respiration, C) Coupling Efficiency, D) Leak Respiration, E) Non-Mitochondrial Respiration, F) ATP Demand, G) Spare Respiratory Capacity, or H) OCR Metabolic Potential. Respiration measures were normalized to viable cells. Data are represented as mean \pm SD from one independent experiment (N = 39 PBMC samples).

Chapter 5: Discussion

The main findings of this study are the following: 1) There were no racial differences in the antioxidant modulators of oxidative stress or superoxide-generating NADPH oxidase subunits, 2) PBMCs isolated from NHB individuals exhibited a significantly higher expression of Complex I and 3) PBCMs isolated from NHB individuals had lower respiration (basal, leak, and non-mitochondrial) rates compared to PBMCs isolated from NHW individuals. These results did not support our hypothesis of a lower expression of the antioxidant modulators of oxidative stress or a higher expression of superoxide-generating NADPH oxidase subunits in PBMCs isolated from NHB individuals. However, our findings do not preclude mitochondrial function from being a potential contributor to the racial differences observed in systemic oxidative stress, as we did not measure mitochondrial-derived ROS production, mitochondrial damage, or mitochondrial polarity.

Immune cells have gained attention for their role in CVD, specifically as primary contributors to systemic oxidative stress.²¹ One mediator to the PBMC-derived contribution to systemic ROS is NADPH oxidase. In response to norepinephrine (NE) treatment, PBMCs isolated from healthy young men exhibit an increase in monocyte adhesion to ECs, intracellular O_2^- production, and NADPH oxidase subunit (gp91phox, p22phox, p67phox) gene expression in a time-dependent manner.¹⁷⁰ This increase in O_2^- production was mediated by α -adrenergic receptors and was attenuated with NADPH oxidase inhibitors. Along with NE, increases in NADPH oxidase activation are also mediated by Ang II and ET-1.^{84,171} In NT and HTN NHB Individual, circulating NE and ET-1 levels are higher than NHW individuals.^{167,168,170} In addition, the racial differences in PBMC-derived O_2^- levels, differences in NADPH oxidase subunits (gp91phox and p47phox) can potentially upregulate NADPH oxidase activity and contribute to the systemic oxidative

stress.⁴³ Therefore, we hypothesized racial differences in the protein expression of this superoxide-generating enzyme; however, our results failed to support this hypothesis (Fig.8A and 8B). While no racial differences were found in the protein expression of NADPH oxidase subunits, our experiments lacked a measure of NADPH oxidase activity. Thus, measuring NADPH oxidase activity could have provided further insight by measuring activity as opposed to protein expression. For example, if we were to find that PBMCs from NHB adults exhibited greater NADPH oxidase activity these findings would align with the previous studies looking at the NADPH oxidase pathway and PBMC-derived superoxide production.⁴³ It is important to also point out this enzyme remains inactive within a cell until it is exposed to microorganisms, inflammatory mediators, or specific agonists that initiate the translocation and assembly of the cytosolic subunits to create an active enzyme.²⁰ For example, we may have uncovered a potential racial difference if we activated our PBMC samples *in vitro* (e.g., LPS). Our participant cohort consisted of young healthy college age adults that did not exhibit any racial differences in the risk factors of CVD (Table 2; e.g., BP, lipids, or glucose levels) and that may have important implications in our findings. It is possible that the PBMCs isolated from our participants were largely in a naïve state, without an agonist or inflammatory mediator to activate NADPH oxidase and alter protein expression. If our cohort did exhibit racial disparities in CVD risk factors, as seen in many studies, or if we had exposed our cohort to an acute challenge (e.g., a high fat meal or second-hand smoke) then we may have uncovered potential disparities at the PBMC level.^{1,3,4,172,173}

Another avenue in which oxidative stress can rein its deleterious effects in PBMCs and on vascular function is to inhibit the antioxidant enzyme protein expression or activity. PBMCs isolated from older patients (55-80 years) with metabolic syndrome (MetS) and without MetS, found patients with MetS to exhibit lower SOD activity in the plasma and a higher expression of

circulating leukocytes, neutrophils, lymphocytes, and monocytes.¹⁷⁴ Further, HUVECs isolated from NHB individuals have also shown lower SOD1 activity and total SOD activity that contributes to the heightened oxidative stress compared to HUVECs isolated from NHW individuals.^{8,165} SIRT3 is an important regulator SOD2 function/activity, and the depletion of SIRT3 in mice resulted in hyperacetylation of SOD2 (AcSOD2), increased infiltration of T cells and macrophages, increased vascular permeability, and vascular inflammation.⁹² Diminished SIRT3 expression and hyperacetylation of SOD2 was found in arterioles isolated from patients with essential HTN compared to arterioles isolated from NT subjects. SIRT3 expression can be hindered by activation of the renin-angiotensin aldosterone system, high AngII exposure, and inflammation.^{89,175} However, we found no racial differences in SIRT3 (Fig. 6D) or AcSOD2 (Fig. 6C) protein expression in PBMCs isolated from young adults. There were also no apparent racial differences in SOD 1 (Fig. 6A) or SOD2 (Fig. 6B) protein expression. AngII-induced HT is associated with the S-glutathionylation of SIRT3 which inhibits its deacetylation activity, increases acetylation of SOD2, and reduces SOD2 activity.³²⁻³⁶ While no racial differences were found on total protein expression for SIRT3, further research on S-glutathionylation of SIRT3 and SOD activity could provide further insight on the SOD pathway and potential racial differences in combatting oxidative stress.¹⁷⁶

The mitochondria are also implicated as another major generator of ROS, mainly as a result of the premature leakage of electrons at Complex I and III.²³⁻²⁸ To recap, Complex I-derived ROS is exclusively released into the mitochondria, while Complex III-derived ROS is also released into the intermembrane space and transverse into the cytosol via voltage-dependent anion channels.^{19,27,31,85} With oxidative stress, the mitochondria also become a target for ROS which can result in oxidative damage to the ETC complexes.^{48,49} In the present study, we found PBMCs

isolated from NHB individuals to exhibit a greater protein expression for Complex I compared to PBMCs isolated from NHW individuals (Fig. 10A). The higher expression of Complex I in PBMCs isolated from NHB individuals could increase the proton flux across the inner membrane and/or increase ROS production within the mitochondrial matrix. This in turn could influence SOD2 activity, initiate oxidative damage to the ETC complexes, respiration rates, and result in mitochondrial dysfunction. However, our experiments lacked markers of mitochondrial damage (e.g., 4HNE, Oxyblot) and O_2^- production to clarify the physiological significance of increased Complex I that we uncovered in PBMCs from young NHB adults and dampens our ability to delineate potential racial differences in the isolated PBMCs. Nonetheless, our concurrent findings of both increased Complex I expression and reduced cellular respiration in PBMCs from young NHB adults suggests that the increased Complex I expression did not contribute to augmented cellular respiration, and if anything may have been a compensatory response¹⁷⁷

Oxidative stress can also play an important role in derangements of mitochondrial function with decreased mitochondrial respiration, transmembrane potential, and overproduction of ROS in PBMCs isolated from patients with early-stage CHF.⁴⁴⁻⁴⁶ Leak respiration represents the proton leak across the mitochondrial inner membrane^{98,110,111} The lower leak respiration (Fig. 12D) in PBMCs isolated from NHB individuals could complement the higher Complex I protein expression, in which O_2^- produced is being retained in the mitochondrial matrix and potentially contributing to the racial differences in basal respiration (Fig. 12A) as well. The addition of the pharmacological agents to derive non-mitochondrial respiration abolishes all mitochondrial-associated respiration, to determine non-mitochondrial O_2 -consuming processes (e.g., cyclooxygenases, lipoxygenases, NADPH oxidases, endoplasmic reticulum, xanthine oxidase). Importantly, greater non-mitochondrial O_2 -consumption is usually associated with inflammation

and is a negative indicator of bioenergetic health.^{98,110,111,114,115} PBMCs isolated from NHB individuals exhibited a lower non-mitochondrial respiration rate, which could represent a lower activity of non-mitochondrial O₂-consuming enzymes (e.g., NADPH oxidase).

As part of our sub-study on potential sex differences, there were no sex differences in the expression of NADPH oxidase subunits, mitochondrial complexes, or PBMC respiration. However, PBMCs isolated from females exhibited a greater expression of the mitochondrial deacetylase (SIRT3) compared to PBMCs isolated from males. PBMCs isolated from females exhibited greater SIRT3 (Fig. 7D) protein expression with no sex differences in the cascading protein expression of antioxidants AcSOD2 (Fig 7C), SOD2 (Fig. 7B), or SOD1 (Fig. 7A). Premenopausal women are somewhat protected against the development of CVDs compared to men, with CVDs presenting 10 years later in women compared to men.¹⁷⁶ It is of interest to examine S-glutathionylation of SIRT3 and SOD activity between sexes, in PBMCs isolated from premenopausal women and its role in CVD protection.

There are limitations to the current study. While PBMCs have been a widely used model for assessing immune regulation, our *in vitro* experiments lacked any perturbations that would activate the PBMCs. Moreover, our *in vitro* experiments lacked the environmental stimuli that could be presented in an *in vivo* condition and can play a role in how these immune cells respond to different stimuli.⁴⁰ Factors that can influence PBMCs reactivity include nutritional status, hormone levels, and inflammation.⁴⁰ It is still not known how PBMCs respond to physiological stimuli (e.g., stressful situations, acute sickness, food consumption) that may be present during blood draws and can further contribute to inter-experimental variations.⁴⁰ As stated earlier in the discussion, our participant population for PBMC isolations were from young healthy college-aged students and this could have masked potential racial differences in the community, this includes

facing wealth inequities and social disparities which are also known players in CV health.^{63–66} By expanding the sample population to middle-aged and older adults, we would have likely unveiled racial disparities in BP and CVD risk factors missing in our cohort of young college students and provide further insight on the role of racial disparities in mitochondrial function in PBMCs. Also, within PBMCs subsets, differences in the protein expression of ETC complexes may impact the regulation and function of mitochondrial metabolism. For example, monocytes exhibit a greater expression of Complex IV (subunit I) compared to lymphocytes.¹⁷⁷ This further supports the need to further isolate PBMCs into subsets (e.g., lymphocytes only, monocytes only) to determine potential racial differences in these ETC complex expression and cell respiration.⁴⁰ PBMCs primarily rely on oxidative phosphorylation to meet their metabolic needs in their inactive state but appear to shift their metabolic phenotype towards glycolysis when activated by inhibiting oxidative phosphorylation.^{142–147} These metabolic shifts in PBMCs could further aggravate mitochondrial function and increase mitochondrial-derived ROS. Future research should determine if activated PBMCs' and their metabolic phenotype contribute to racial differences in oxidative stress. Another important note for respiration readings in intact cells is that rates can be standardized to cell number or protein concentration, instead of mitochondrial concentrations for isolated mitochondria.⁹⁸ As a result, potential changes in mitochondrial density within intact cells could profoundly affect cellular respiration. We acknowledge the drawbacks to this model; hence our results should be interpreted with caution.

In conclusion, mitochondrial function in PBMCs could be a potential contributor to higher systemic oxidative stress in NHB individuals. While no racial differences were found in the redox regulating proteins, we propose other aspects of PBMC-derived ROS such as antioxidant activity

(e.g., SOD1, total SOD), stimulated NADPH oxidase and O_2^- production, or changes in metabolic phenotype in activated PBMCs as targets for future research.

References

1. Mensah, G. A., Mokdad, A. H., Ford, E. S., Greenlund, K. J. & Croft, J. B. State of Disparities in Cardiovascular Health in the United States. *Circulation* 111, 1233–1241 (2005).
2. Virani, S. S., Alonso, A., Aparicio, H. J., Benjamin, E. J., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Cheng, S., Delling, F. N., Elkind, M. S. V., Evenson, K. R., Ferguson, J. F., Gupta, D. K., Khan, S. S., Kissela, B. M., Knutson, K. L., Lee, C. D., Lewis, T. T., Liu, J., Loop, M. S., Lutsey, P. L., Ma, J., Mackey, J., Martin, S. S., Matchar, D. B., Mussolino, M. E., Navaneethan, S. D., Perak, A. M., Roth, G. A., Samad, Z., Satou, G. M., Schroeder, E. B., Shah, S. H., Shay, C. M., Stokes, A., VanWagner, L. B., Wang, N.-Y., Tsao, C. W. & Subcommittee, O. behalf of the A. H. A. C. on E. and P. S. C. and S. S. Heart Disease and Stroke Statistics—2021 Update: A Report From the American Heart Association. *Circulation* 143, e254–e743 (2021).
3. Fryar, C. D., Chen, T.-C. & Li, X. Prevalence of uncontrolled risk factors for cardiovascular disease: United States, 1999-2010. *Nchs Data Brief* 1–8 (2012).
4. Benjamin, E. J., Muntner, P., Alonso, A., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Chang, A. R., Cheng, S., Das, S. R., Delling, F. N., Djousse, L., Elkind, M. S. V., Ferguson, J. F., Fornage, M., Jordan, L. C., Khan, S. S., Kissela, B. M., Knutson, K. L., Kwan, T. W., Lackland, D. T., Lewis, T. T., Lichtman, J. H., Longenecker, C. T., Loop, M. S., Lutsey, P. L., Martin, S. S., Matsushita, K., Moran, A. E., Mussolino, M. E., O’Flaherty, M., Pandey, A., Perak, A. M., Rosamond, W. D., Roth, G. A., Sampson, U. K. A., Satou, G. M., Schroeder, E. B., Shah, S. H., Spartano, N. L., Stokes, A., Tirschwell, D. L., Tsao, C. W., Turakhia, M. P., VanWagner, L. B., Wilkins, J. T., Wong, S. S. & Virani, S. S. Heart Disease and Stroke Statistics—2019 Update: A Report From the American Heart Association. *Circulation* 139, e56–e528 (2019).
5. Verma, S. & Anderson, T. J. Fundamentals of Endothelial Function for the Clinical Cardiologist. *Circulation* 105, 546–549 (2002).
6. Morimoto, Y., Conroy, S. M., Ollberding, N. J., Kim, Y., Lim, U., Cooney, R. V., Franke, A. A., Wilkens, L. R., Hernandez, B. Y., Goodman, M. T., Henderson, B. E., Kolonel, L. N., Marchand, L. L. & Maskarinec, G. Ethnic differences in serum adipokine and C-reactive protein levels: the multiethnic cohort. *Int J Obesity* 38, 1416 (2014).
7. Khera, A., McGuire, D. K., Murphy, S. A., Stanek, H. G., Das, S. R., Vongpatanasin, W., Wians, F. H., Grundy, S. M. & Lemos, J. A. de. Race and Gender Differences in C-Reactive Protein Levels. *J Am Coll Cardiol* 46, 464–469 (2005).
8. Fearheller, D. L., Park, J., Sturgeon, K. M., Williamson, S. T., Diaz, K. M., Veerabhadrapa, P. & Brown, M. D. Racial Differences in Oxidative Stress and Inflammation: In Vitro and In Vivo. *Clin Transl Sci* 4, 32–37 (2011).

9. Cook, M. D., Heffernan, K. S., Ranadive, S., Woods, J. A. & Fernhall, B. Effect of Resistance Training on Biomarkers of Vascular Function and Oxidative Stress in Young African American and Caucasian men. *J Hum Hypertens* 27, 388–392 (2013).
10. Rodriguez-Perez, A. I., Borrajo, A., Rodriguez-Pallares, J., Guerra, M. J. & Labandeira-Garcia, J. L. Interaction between NADPH-oxidase and Rho-kinase in angiotensin II-induced microglial activation. *Glia* 63, 466–482 (2015).
11. Brown, M. D. & Fearheller, D. L. Are There Race-Dependent Endothelial Cell Responses to Exercise? *Exercise Sport Sci R* 41, 44–54 (2013).
12. Kalinowski, L., Dobrucki, I. T. & Malinski, T. Race-Specific Differences in Endothelial Function. *Circulation* 109, 2511–2517 (2004).
13. Heffernan, K. S., Jae, S. Y., Wilund, K. R., Woods, J. A. & Fernhall, B. Racial differences in central blood pressure and vascular function in young men. *Am J Physiol-heart C* 295, H2380–H2387 (2008).
14. Brown, M. D., Fearheller, D. L., Thakkar, S., Veerabhadrapa, P. & Park, J.-Y. Racial differences in tumor necrosis factor- α -induced endothelial microparticles and interleukin-6 production. *Vasc Heal Risk Management* 7, 541–550 (2011).
15. Cai, H. & Harrison, D. G. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circ Res* 87, 840–844 (2000).
16. Brocq, M. L., Leslie, S. J., Milliken, P. & Megson, I. L. Endothelial Dysfunction From Molecular Mechanisms to Measurement, Clinical Implications, and Therapeutic Opportunities. *Antioxid Redox Sign* 10, 1631–1674 (2008).
17. Lin, K.-T., Xue, J.-Y., Sun, F. F. & Wong, P. Y.-K. Reactive Oxygen Species Participate in Peroxynitrite-Induced Apoptosis in HL-60 Cells. *Biochem Bioph Res Co* 230, 115–119 (1997).
18. Beckman, J. S. & Koppenol, W. H. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol-cell Ph* 271, C1424–C1437 (1996).
19. Krumova, K. & Cosa, G. Singlet Oxygen: Applications in Biosciences and Nanosciences, Volume 1. *Compr Ser Photochem Photobiological Sci* 1, 1–21 (2016).
20. Lambeth, J. D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4, 181–189 (2004).
21. Wenzel, P., Knorr, M., Kossmann, S., Stratmann, J., Hausding, M., Schuhmacher, S., Karbach, S. H., Schwenk, M., Yogev, N., Schulz, E., Oelze, M., Grabbe, S., Jonuleit, H., Becker, C., Daiber, A., Waisman, A. & Münzel, T. Lysozyme M-Positive Monocytes Mediate Angiotensin II-Induced Arterial Hypertension and Vascular Dysfunction. *Circulation* 124, 1370–1381 (2011).

22. Bedard, K. & Krause, K.-H. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol Rev* 87, 245–313 (2007).
23. Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J Physiology* 552, 335–344 (2003).
24. Finkel, T. Signal transduction by reactive oxygen species. *J Cell Biology* 194, 7–15 (2011).
25. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem J* 417, 1–13 (2009).
26. O'Malley, Y., Fink, B. D., Ross, N. C., Prisinzano, T. E. & Sivitz, W. I. Reactive Oxygen and Targeted Antioxidant Administration in Endothelial Cell Mitochondria*. *J Biol Chem* 281, 39766–39775 (2006).
27. Muller, F. L., Liu, Y. & Remmen, H. V. Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane*. *J Biol Chem* 279, 49064–49073 (2004).
28. Osellame, L. D., Blacker, T. S. & Duchon, M. R. Cellular and molecular mechanisms of mitochondrial function. *Best Pract Res Clin Endocrinol Metabolism* 26, 711–723 (2012).
29. Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L. & Brand, M. D. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates☆. *Redox Biol* 1, 304–312 (2013).
30. Shahidi, F. & Zhong, Y. Novel antioxidants in food quality preservation and health promotion. *Eur J Lipid Sci Tech* 112, 930–940 (2010).
31. Kirkman, D. L., Robinson, A. T., Rossman, M. J., Seals, D. R. & Edwards, D. G. Mitochondrial contributions to vascular endothelial dysfunction, arterial stiffness, and cardiovascular diseases. *Am J Physiol-heart C* 320, H2080–H2100 (2021).
32. Prasad, N., Ramteke, P., Dholia, N. & Yadav, U. C. S. Immunity and Inflammation in Health and Disease. *Sect Vi New Perspectives Futur Dir* 341–362 (2018). doi:10.1016/b978-0-12-805417-8.00027-5
33. Faraci, F. M. & Didion, S. P. Vascular Protection. *Arteriosclerosis Thrombosis Vasc Biology* 24, 1367–1373 (2004).
34. Ohashi, M., Runge, M. S., Faraci, F. M. & Heistad, D. D. MnSOD Deficiency Increases Endothelial Dysfunction in ApoE-Deficient Mice. *Arteriosclerosis Thrombosis Vasc Biology* 26, 2331–2336 (2006).
35. Qiu, X., Brown, K., Hirschey, M. D., Verdin, E. & Chen, D. Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. *Cell Metab* 12, 662–667 (2010).

36. Dikalova, A. E., Itani, H. A., Nazarewicz, R. R., McMaster, W. G., Flynn, C. R., Uzhachenko, R., Fessel, J. P., Gamboa, J. L., Harrison, D. G. & Dikalov, S. I. Sirt3 Impairment and SOD2 Hyperacetylation in Vascular Oxidative Stress and Hypertension. *Circ Res* 121, 564–574 (2017).
37. Kluge, M. A., Fetterman, J. L. & Vita, J. A. Mitochondria and Endothelial Function. *Circ Res* 112, 1171–1188 (2013).
38. Quintero, M., Colombo, S. L., Godfrey, A. & Moncada, S. Mitochondria as signaling organelles in the vascular endothelium. *Proc National Acad Sci* 103, 5379–5384 (2006).
39. Pourahmad, J. & Salimi, A. Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics. *Iranian J Pharm Res Ijpr* 14, 979 (2015).
40. Kleiveland, C. R. *The Impact of Food Bioactives on Health in vitro and ex vivo models*. (Cham (CH): Springer, 2015). doi:10.1007/978-3-319-16104-4_15
41. Dean, L. *Blood Groups and Red Cell Antigens*. (National Center for Biotechnology Information (US), 2005). at <<https://www.ncbi.nlm.nih.gov/books/NBK2263/>>
42. Hirahara, K., Poholek, A., Vahedi, G., Laurence, A., Kanno, Y., Milner, J. D. & O’Shea, J. J. Mechanisms underlying helper T-cell plasticity: Implications for immune-mediated disease. *J Allergy Clin Immun* 131, 1276–1287 (2013).
43. Deo, S. H., Holwerda, S. W., Keller, D. M. & Fadel, P. J. Elevated peripheral blood mononuclear cell-derived superoxide production in healthy young black men. *Am J Physiol-heart C* 308, H548–H552 (2015).
44. Coluccia, R., Raffa, S., Ranieri, D., Micaloni, A., Valente, S., Salerno, G., Scrofani, C., Testa, M., Gallo, G., Pagannone, E., Torrisi, M. R., Volpe, M. & Rubattu, S. Chronic heart failure is characterized by altered mitochondrial function and structure in circulating leucocytes. *Oncotarget* 9, 35028–35040 (2018).
45. Shirakawa, R., Yokota, T., Nakajima, T., Takada, S., Yamane, M., Furihata, T., Maekawa, S., Nambu, H., Katayama, T., Fukushima, A., Saito, A., Ishimori, N., Dela, F., Kinugawa, S. & Anzai, T. Mitochondrial reactive oxygen species generation in blood cells is associated with disease severity and exercise intolerance in heart failure patients. *Sci Rep-uk* 9, 14709 (2019).
46. Kong, C.-W., Hsu, T.-G., Lu, F.-J., Chan, W.-L. & Tsai, K. Leukocyte mitochondria depolarization and apoptosis in advanced heart failure: clinical correlations and effect of therapy. *J Am Coll Cardiol* 38, 1693–1700 (2001).
47. Li, P., Wang, B., Sun, F., Li, Y., Li, Q., Lang, H., Zhao, Z., Gao, P., Zhao, Y., Shang, Q., Liu, D. & Zhu, Z. Mitochondrial respiratory dysfunctions of blood mononuclear cells link with cardiac disturbance in patients with early-stage heart failure. *Sci Rep-uk* 5, 10229 (2015).

48. Daiber, A. Redox signaling (cross-talk) from and to mitochondria involves mitochondrial pores and reactive oxygen species. *Biochimica Et Biophysica Acta Bba - Bioenergetics* 1797, 897–906 (2010).
49. Doughan, A. K., Harrison, D. G. & Dikalov, S. I. Molecular Mechanisms of Angiotensin II–Mediated Mitochondrial Dysfunction. *Circ Res* 102, 488–496 (2008).
50. Loo, B. van der, Labugger, R., Skepper, J. N., Bachschmid, M., Kilo, J., Powell, J. M., Palacios-Callender, M., Erusalimsky, J. D., Quaschnig, T., Malinski, T., Gygi, D., Ullrich, V. & Lüscher, T. F. Enhanced Peroxynitrite Formation Is Associated with Vascular Aging. *J Exp Medicine* 192, 1731–1744 (2000).
51. Kokoszka, J. E., Coskun, P., Esposito, L. A. & Wallace, D. C. Increased mitochondrial oxidative stress in the Sod2 (+/–) mouse results in the age-related decline of mitochondrial function culminating in increased apoptosis. *Proc National Acad Sci* 98, 2278–2283 (2001).
52. Li, Y., Huang, T.-T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., Chan, P. H., Wallace, D. C. & Epstein, C. J. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11, 376–381 (1995).
53. Asimakis, G. K., Lick, S. & Patterson, C. Postischemic Recovery of Contractile Function is Impaired in SOD2^{+/-} but Not SOD1^{+/-} Mouse Hearts. *Circulation* 105, 981–986 (2002).
54. Fearheller, D., Park, Rizzo, Kim & Brown, M. Racial differences in the responses to shear stress in human umbilical vein endothelial cells. *Vasc Heal Risk Management* Volume 7, 425–431 (2011).
55. Alfatni, A., Riou, M., Charles, A.-L., Meyer, A., Barnig, C., Andres, E., Lejay, A., Talha, S. & Geny, B. Peripheral Blood Mononuclear Cells and Platelets Mitochondrial Dysfunction, Oxidative Stress, and Circulating mtDNA in Cardiovascular Diseases. *J Clin Medicine* 9, 311 (2020).
56. Williams, H., Mack, C. D., Li, S. C. H., Fletcher, J. P. & Medbury, H. J. Nature versus Number: Monocytes in Cardiovascular Disease. *Int J Mol Sci* 22, 9119 (2021).
57. Miotto, P. M., McGlory, C., Holloway, T. M., Phillips, S. M. & Holloway, G. P. Sex differences in mitochondrial respiratory function in human skeletal muscle. *Am J Physiology-regulatory Integr Comp Physiology* 314, R909–R915 (2018).
58. Silaidos, C., Pilatus, U., Grewal, R., Matura, S., Lienerth, B., Pantel, J. & Eckert, G. P. Sex-associated differences in mitochondrial function in human peripheral blood mononuclear cells (PBMCs) and brain. *Biol Sex Differ* 9, 34 (2018).
59. Junker, A., Wang, J., Gouspillou, G., Ehinger, J. K., Elmér, E., Sjövall, F., Fisher-Wellman, K. H., Neuffer, P. D., Molina, A. J. A., Ferrucci, L. & Picard, M. Human studies of mitochondrial

biology demonstrate an overall lack of binary sex differences: A multivariate meta-analysis. *Faseb J* 36, e22146 (2022).

60. Ventura-Clapier, R., Moulin, M., Piquereau, J., Lemaire, C., Mericskay, M., Veksler, V. & Garnier, A. Mitochondria: a central target for sex differences in pathologies. *Clin Sci* 131, 803–822 (2017).

61. Tsao, C. W., Aday, A. W., Almarzooq, Z. I., Alonso, A., Beaton, A. Z., Bittencourt, M. S., Boehme, A. K., Buxton, A. E., Carson, A. P., Commodore-Mensah, Y., Elkind, M. S. V., Evenson, K. R., Eze-Nliam, C., Ferguson, J. F., Generoso, G., Ho, J. E., Kalani, R., Khan, S. S., Kissela, B. M., Knutson, K. L., Levine, D. A., Lewis, T. T., Liu, J., Loop, M. S., Ma, J., Mussolino, M. E., Navaneethan, S. D., Perak, A. M., Poudel, R., Rezk-Hanna, M., Roth, G. A., Schroeder, E. B., Shah, S. H., Thacker, E. L., VanWagner, L. B., Virani, S. S., Voecks, J. H., Wang, N.-Y., Yaffe, K., Martin, S. S. & Subcommittee, on behalf of the A. H. A. C. on E. and P. S. C. and S. S. Heart Disease and Stroke Statistics—2022 Update: A Report From the American Heart Association. *Circulation* CIR0000000000001052 (2022). doi:10.1161/cir.0000000000001052

62. Fuller-Rowell, T. E., Curtis, D. S., El-Sheikh, M., Chae, D. H., Boylan, J. M. & Ryff, C. D. Racial disparities in sleep: the role of neighborhood disadvantage. *Sleep Med* 27, 1–8 (2016).

63. Gaglioti, A. H., Xu, J., Rollins, L., Baltrus, P., O’Connell, L. K., Cooper, D. L., Hopkins, J., Botchwey, N. D. & Akintobi, T. H. Neighborhood Environmental Health and Premature Death From Cardiovascular Disease. *Prev Chronic Dis* 15, E17 (2018).

64. Bhatnagar, A. Environmental Determinants of Cardiovascular Disease. *Circ Res* 121, 162–180 (2017).

65. Martínez-García, M., Salinas-Ortega, M., Estrada-Arriaga, I., Hernández-Lemus, E., García-Herrera, R. & Vallejo, M. A systematic approach to analyze the social determinants of cardiovascular disease. *Plos One* 13, e0190960 (2018).

66. Insull, W. The Pathology of Atherosclerosis: Plaque Development and Plaque Responses to Medical Treatment. *Am J Medicine* 122, S3–S14 (2009).

67. Badimon, L., Peña, E., Arderiu, G., Padró, T., Slevin, M., Vilahur, G. & Chiva-Blanch, G. C-Reactive Protein in Atherothrombosis and Angiogenesis. *Front Immunol* 9, 430 (2018).

68. Libby, P., Ridker, P. M. & Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature* 473, 317–325 (2011).

69. Flack, J. M., Ferdinand, K. C. & Nasser, S. A. Epidemiology of Hypertension and Cardiovascular Disease in African Americans. *J Clin Hypertens* 5, 5–11 (2003).

70. Traub, O. & Berk, B. C. Laminar Shear Stress. *Arteriosclerosis Thrombosis Vasc Biology* 18, 677–685 (1998).

71. GRIMM, H., KRETZSCHMAR, J., COOK, M. D. & BROWN, M. D. The Effects of Exercise, Aspirin, and Celecoxib in an Atherogenic Environment. *Medicine Sci Sports Exerc* 50, 2033–2039 (2018).
72. Green, D. J., Maiorana, A., O’Driscoll, G. & Taylor, R. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiology* 561, 1–25 (2004).
73. Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D. A., Sim, A. T. & Kemp, B. E. Coordinated Control of Endothelial Nitric-oxide Synthase Phosphorylation by Protein Kinase C and the cAMP-dependent Protein Kinase. *J Biol Chem* 276, 17625–17628 (2001).
74. Verma, S., Devaraj, S. & Jialal, I. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes Atherothrombosis. *Circulation* 113, 2128–2151 (2006).
75. Davies, P. F. Flow-Mediated Endothelial Mechanotransduction. *Physiological Reviews* 75, 519–560 (1995).
76. Pacher, P., Beckman, J. S. & Liaudet, L. Nitric Oxide and Peroxynitrite in Health and Disease. *Physiol Rev* 87, 315–424 (2007).
77. Zou, M.-H., Shi, C. & Cohen, R. A. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 109, 817–826 (2002).
78. Laursen, J. B., Somers, M., Kurz, S., McCann, L., Warnholtz, A., Freeman, B. A., Tarpey, M., Fukai, T. & Harrison, D. G. Endothelial Regulation of Vasomotion in ApoE-Deficient Mice. *Circulation* 103, 1282–1288 (2001).
79. Crabtree, M. J. & Channon, K. M. Synthesis and recycling of tetrahydrobiopterin in endothelial function and vascular disease. *Nato Sci S A Lif Sci* 25, 81–88 (2011).
80. Cooke, M. S., Evans, M. D., Dizdaroglu, M. & Lunec, J. Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J* 17, 1195–1214 (2003).
81. Yakes, F. M. & Van Houten, B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc National Acad Sci* 94, 514–519 (1997).
82. Cho, K.-J., Seo, J.-M. & Kim, J.-H. Bioactive lipoxygenase metabolites stimulation of NADPH oxidases and reactive oxygen species. *Mol Cells* 32, 1–5 (2011).
83. Veith, A. & Moorthy, B. Role of cytochrome P450s In the generation and metabolism of reactive oxygen species. *Curr Opin Toxicol* 7, 44–51 (2018).
84. Cave, A. C., Brewer, A. C., Narayanapanicker, A., Ray, R., Grieve, D. J., Walker, S. & Shah, A. M. NADPH Oxidases in Cardiovascular Health and Disease. *Antioxid Redox Sign* 8, 691–728 (2006).

85. Brand, M. D. The sites and topology of mitochondrial superoxide production. *Exp Gerontol* 45, 466–472 (2010).
86. Zhou, R.-H., Vendrov, A. E., Tchivilev, I., Niu, X.-L., Molnar, K. C., Rojas, M., Carter, J. D., Tong, H., Stouffer, G. A., Madamanchi, N. R. & Runge, M. S. Mitochondrial Oxidative Stress in Aortic Stiffening With Age. *Arteriosclerosis Thrombosis Vasc Biology* 32, 745–755 (2011).
87. Tao, R., Vassilopoulos, A., Parisiadou, L., Yan, Y. & Gius, D. Regulation of MnSOD Enzymatic Activity by Sirt3 Connects the Mitochondrial Acetylome Signaling Networks to Aging and Carcinogenesis. *Antioxid Redox Sign* 20, 1646–1654 (2014).
88. Hirschev, M. D., Shimazu, T., Huang, J. & Verdin, E. Chapter 8 Acetylation of Mitochondrial Proteins. *Methods Enzymol* 457, 137–147 (2009).
89. Capettini, L. S. A., Montecucco, F., Mach, F., Stergiopoulos, N., Santos, R. A. S. & Silva, R. F. da. Role of Renin-Angiotensin System in Inflammation, Immunity and Aging. *Curr Pharm Design* 18, 963–970 (2012).
90. Chaudhry, K. N., Chavez, P., Gasowski, J., Grodzicki, T. & Messerli, F. H. Hypertension in the elderly: Some practical considerations. *Clev Clin J Med* 79, 694–704 (2012).
91. Lanza, I. R., Short, D. K., Short, K. R., Raghavakaimal, S., Basu, R., Joyner, M. J., McConnell, J. P. & Nair, K. S. Endurance Exercise as a Countermeasure for Aging. *Diabetes* 57, 2933–2942 (2008).
92. Dikalova, A. E., Pandey, A., Xiao, L., Arslanbaeva, L., Sidorova, T., Lopez, M. G., IV, F. T. B., Verdin, E., Auwerx, J., Harrison, D. G. & Dikalov, S. I. Mitochondrial Deacetylase Sirt3 Reduces Vascular Dysfunction and Hypertension While Sirt3 Depletion in Essential Hypertension Is Linked to Vascular Inflammation and Oxidative Stress. *Circ Res* 126, 439–452 (2020).
93. Friedman, J. R. & Nunnari, J. Mitochondrial form and function. *Nature* 505, 335–343 (2014).
94. Kühlbrandt, W. Structure and function of mitochondrial membrane protein complexes. *Bmc Biol* 13, 89 (2015).
95. Gabaldón, T. & Huynen, M. A. Shaping the mitochondrial proteome. *Biochimica Et Biophysica Acta Bba - Bioenergetics* 1659, 212–220 (2004).
96. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat Rev Mol Cell Bio* 11, 655–667 (2010).
97. Rossman, M. J., Gioscia-Ryan, R. A., Clayton, Z. S., Murphy, M. P. & Seals, D. R. Targeting mitochondrial fitness as a strategy for healthy vascular aging. *Clin Sci* 134, 1491–1519 (2020).
98. Brand, M. D. & Nicholls, D. G. Assessing mitochondrial dysfunction in cells. *Biochem J* 435, 297–312 (2011).

99. Bayrhuber, M., Meins, T., Habeck, M., Becker, S., Giller, K., Villinger, S., Vonnrhein, C., Griesinger, C., Zweckstetter, M. & Zeth, K. Structure of the human voltage-dependent anion channel. *Proc National Acad Sci* 105, 15370–15375 (2008).
100. Ryan, M. T. & Hoogenraad, N. J. Mitochondrial-Nuclear Communications. *Annu Rev Biochem* 76, 701–722 (2007).
101. Zhou, Q., Zhai, Y., Lou, J., Liu, M., Pang, X. & Sun, F. Thiabendazole inhibits ubiquinone reduction activity of mitochondrial respiratory complex II via a water molecule mediated binding feature. *Protein Cell* 2, 531–542 (2011).
102. Crofts, A. R. The Cytochrome bc₁ Complex: Function in the Context of Structure. *Annu Rev Physiol* 66, 689–733 (2004).
103. Liu, J., Qin, L. & Ferguson-Miller, S. Crystallographic and online spectral evidence for role of conformational change and conserved water in cytochrome oxidase proton pump. *Proc National Acad Sci* 108, 1284–1289 (2011).
104. Rich, P. Chemiosmotic coupling: The cost of living. *Nature* 421, 583–583 (2003).
105. Porter, R. K. & Brand, M. D. Mitochondrial proton conductance and H⁺/O ratio are independent of electron transport rate in isolated hepatocytes. *Biochem J* 310, 379–382 (1995).
106. Brand, M. D., Chien, L. F. & Diolez, P. Experimental discrimination between proton leak and redox slip during mitochondrial electron transport. *Biochem J* 297, 27–29 (1994).
107. Hafner, R. P. & Brand, M. D. Effect of protonmotive force on the relative proton stoichiometries of the mitochondrial proton pumps. *Biochem J* 275, 75–80 (1991).
108. Chance, B. & Williams, G. R. RESPIRATORY ENZYMES IN OXIDATIVE PHOSPHORYLATION I. KINETICS OF OXYGEN UTILIZATION. *J Biol Chem* 217, 383–393 (1955).
109. Rose, S., Frye, R. E., Slattery, J., Wynne, R., Tippett, M., Pavliv, O., Melnyk, S. & James, S. J. Oxidative Stress Induces Mitochondrial Dysfunction in a Subset of Autism Lymphoblastoid Cell Lines in a Well-Matched Case Control Cohort. *Plos One* 9, e85436 (2014).
110. Chacko, B. K., Kramer, P. A., Ravi, S., Benavides, G. A., Mitchell, T., Dranka, B. P., Ferrick, D., Singal, A. K., Ballinger, S. W., Bailey, S. M., Hardy, R. W., Zhang, J., Zhi, D. & Darley-Usmar, V. M. The Bioenergetic Health Index: a new concept in mitochondrial translational research. *Clin Sci Lond Engl* 1979 127, 367–373 (2014).
111. Hill, B. G., Benavides, G. A., Lancaster, J. R., Ballinger, S., Dell’Italia, L., Zhang, J. & Darley-Usmar, V. M. Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biol Chem* 393, 1485–1512 (2012).

112. Cheng, J., Nanayakkara, G., Shao, Y., Cueto, R., Wang, L., Yang, W. Y., Tian, Y., Wang, H. & Yang, X. Mitochondrial Dynamics in Cardiovascular Medicine. *Adv Exp Med Biol* 982, 359–370 (2017).
113. Brookes, P. S., Land, J. M., Clark, J. B. & Heales, S. J. R. Peroxynitrite and Brain Mitochondria: Evidence for Increased Proton Leak. *J Neurochem* 70, 2195–2202 (1998).
114. Underwood, E., Redell, J. B., Zhao, J., Moore, A. N. & Dash, P. K. A method for assessing tissue respiration in anatomically defined brain regions. *Sci Rep-uk* 10, 13179 (2020).
115. Rose, S., Carvalho, E., Diaz, E. C., Cotter, M., Bennuri, S. C., Azhar, G., Frye, R. E., Adams, S. H. & Børshiem, E. A comparative study of mitochondrial respiration in circulating blood cells and skeletal muscle fibers in women. *Am J Physiol-endoc M* 317, E503–E512 (2019).
116. Dranka, B. P., Hill, B. G. & Darley-Usmar, V. M. Mitochondrial reserve capacity in endothelial cells: The impact of nitric oxide and reactive oxygen species. *Free Radical Bio Med* 48, 905–914 (2010).
117. Yadava, N. & Nicholls, D. G. Spare Respiratory Capacity Rather Than Oxidative Stress Regulates Glutamate Excitotoxicity after Partial Respiratory Inhibition of Mitochondrial Complex I with Rotenone. *J Neurosci* 27, 7310–7317 (2007).
118. Choi, S. W., Gerencser, A. A. & Nicholls, D. G. Bioenergetic analysis of isolated cerebrocortical nerve terminals on a microgram scale: spare respiratory capacity and stochastic mitochondrial failure. *J Neurochem* 109, 1179–1191 (2009).
119. Dranka, B. P., Benavides, G. A., Diers, A. R., Giordano, S., Zelickson, B. R., Reily, C., Zou, L., Chatham, J. C., Hill, B. G., Zhang, J., Landar, A. & Darley-Usmar, V. M. Assessing bioenergetic function in response to oxidative stress by metabolic profiling. *Free Radical Bio Med* 51, 1621–1635 (2011).
120. Giordano, S., Lee, J., Darley-Usmar, V. M. & Zhang, J. Distinct Effects of Rotenone, 1-methyl-4-phenylpyridinium and 6-hydroxydopamine on Cellular Bioenergetics and Cell Death. *Plos One* 7, e44610 (2012).
121. Schneider, L., Giordano, S., Zelickson, B. R., Johnson, M. S., Benavides, G. A., Ouyang, X., Fineberg, N., Darley-Usmar, V. M. & Zhang, J. Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress. *Free Radical Bio Med* 51, 2007–2017 (2011).
122. Zelickson, B. R., Benavides, G. A., Johnson, M. S., Chacko, B. K., Venkatraman, A., Landar, A., Betancourt, A. M., Bailey, S. M. & Darley-Usmar, V. M. Nitric oxide and hypoxia exacerbate alcohol-induced mitochondrial dysfunction in hepatocytes. *Biochimica Et Biophysica Acta Bba - Bioenergetics* 1807, 1573–1582 (2011).

123. Sansbury, B. E., Jones, S. P., Riggs, D. W., Darley-Usmar, V. M. & Hill, B. G. Bioenergetic function in cardiovascular cells: The importance of the reserve capacity and its biological regulation. *Chem-biol Interact* 191, 288–295 (2011).
124. Hill, B. G., Dranka, B. P., Zou, L., Chatham, J. C. & Darley-Usmar, V. M. Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal. *Biochem J* 424, 99–107 (2009).
125. Patel, V. K., Williams, H., Li, S. C. H., Fletcher, J. P. & Medbury, H. J. Monocyte inflammatory profile is specific for individuals and associated with altered blood lipid levels. *Atherosclerosis* 263, 15–23 (2017).
126. Tarique, A. A., Logan, J., Thomas, E., Holt, P. G., Sly, P. D. & Fantino, E. Phenotypic, Functional, and Plasticity Features of Classical and Alternatively Activated Human Macrophages. *Am J Resp Cell Mol* 53, 676–688 (2015).
127. Chon, H., Gaillard, C. A. J. M., Meijden, B. B. van der, Dijkstra, H. M., Kraaijenhagen, R. J., Leenen, D. van, Holstege, F. C. P., Joles, J. A., Bluysen, H. A. R., Koomans, H. A. & Braam, B. Broadly Altered Gene Expression in Blood Leukocytes in Essential Hypertension Is Absent During Treatment. *Hypertension* 43, 947–951 (2004).
128. Chon, H., Verhaar, M. C., Koomans, H. A., Joles, J. A. & Braam, B. Role of Circulating Karyocytes in the Initiation and Progression of Atherosclerosis. *Hypertension* 47, 803–810 (2006).
129. Dai, D.-F., Rabinovitch, P. S. & Ungvari, Z. Mitochondria and Cardiovascular Aging. *Circ Res* 110, 1109–1124 (2012).
130. Eirin, A., Lerman, A. & Lerman, L. O. Enhancing Mitochondrial Health to Treat Hypertension. *Curr Hypertens Rep* 20, 89 (2018).
131. Szewczyk, A., Jarmuszkiewicz, W., Koziel, A., Sobieraj, I., Nobik, W., Lukasiak, A., Skup, A., Bednarczyk, P., Drabarek, B., Dymkowska, D., Wrzosek, A. & Zablocki, K. Mitochondrial mechanisms of endothelial dysfunction. *Pharmacol Rep* 67, 704–710 (2015).
132. Widlansky, M. E., Wang, J., Shenouda, S. M., Hagen, T. M., Smith, A. R., Kizhakekuttu, T. J., Kluge, M. A., Weihrauch, D., Gutterman, D. D. & Vita, J. A. Altered mitochondrial membrane potential, mass, and morphology in the mononuclear cells of humans with type 2 diabetes. *Transl Res* 156, 15–25 (2010).
133. Shenouda, S. M., Widlansky, M. E., Chen, K., Xu, G., Holbrook, M., Tabit, C. E., Hamburg, N. M., Frame, A. A., Caiano, T. L., Kluge, M. A., Duess, M.-A., Levit, A., Kim, B., Hartman, M.-L., Joseph, L., Shirihai, O. S. & Vita, J. A. Altered Mitochondrial Dynamics Contributes to Endothelial Dysfunction in Diabetes Mellitus. *Circulation* 124, 444–453 (2011).
134. Kizhakekuttu, T. J., Wang, J., Dharmashankar, K., Ying, R., Gutterman, D. D., Vita, J. A. & Widlansky, M. E. Adverse Alterations in Mitochondrial Function Contribute to Type 2 Diabetes

Mellitus-Related Endothelial Dysfunction in Humans. *Arteriosclerosis Thrombosis Vasc Biology* 32, 2531–2539 (2012).

135. Park, S.-Y., Gifford, J. R., Andtbacka, R. H. I., Trinity, J. D., Hyngstrom, J. R., Garten, R. S., Diakos, N. A., Ives, S. J., Dela, F., Larsen, S., Drakos, S. & Richardson, R. S. Cardiac, skeletal, and smooth muscle mitochondrial respiration: are all mitochondria created equal? *Am J Physiol-heart C* 307, H346–H352 (2014).

136. Blouin, A., Bolender, R. P. & Weibel, E. R. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J Cell Biology* 72, 441–455 (1977).

137. Hom, J. & Sheu, S.-S. Morphological dynamics of mitochondria — A special emphasis on cardiac muscle cells. *J Mol Cell Cardiol* 46, 811–820 (2009).

138. Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, N., Schroder, H. D., Boushel, R., Helge, J. W., Dela, F. & Hey-Mogensen, M. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiology* 590, 3349–3360 (2012).

139. Oldendorf, W. H., Cornford, M. E. & Brown, W. J. The large apparent work capability of the blood-brain barrier: A study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Ann Neurol* 1, 409–417 (1977).

140. Culic, O., Gruwel, M. L. & Schrader, J. Energy turnover of vascular endothelial cells. *Am J Physiol-cell Ph* 273, C205–C213 (1997).

141. Chacko, B. K., Kramer, P. A., Ravi, S., Johnson, M. S., Hardy, R. W., Ballinger, S. W. & Darley-Usmar, V. M. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Laboratory Investigation J Technical Methods Pathology* 93, 690–700 (2013).

142. Macintyre, A. N. & Rathmell, J. C. Activated lymphocytes as a metabolic model for carcinogenesis. *Cancer Metabolism* 1, 5 (2013).

143. Roos, D. & Loos, J. A. Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes II. Relative importance of glycolysis and oxidative phosphorylation on phytohaemagglutinin stimulation. *Exp Cell Res* 77, 127–135 (1973).

144. Rathmell, J. C., Heiden, M. G. V., Harris, M. H., Frauwirth, K. A. & Thompson, C. B. In the Absence of Extrinsic Signals, Nutrient Utilization by Lymphocytes Is Insufficient to Maintain Either Cell Size or Viability. *Mol Cell* 6, 683–692 (2000).

145. Krauss, S., Brand, M. D. & Buttgerit, F. Signaling Takes a Breath – New Quantitative Perspectives on Bioenergetics and Signal Transduction. *Immunity* 15, 497–502 (2001).

146. Rodríguez-Prados, J.-C., Través, P. G., Cuenca, J., Rico, D., Aragonés, J., Martín-Sanz, P., Cascante, M. & Boscá, L. Substrate Fate in Activated Macrophages: A Comparison between Innate, Classic, and Alternative Activation. *J Immunol* 185, 605–614 (2010).
147. O’Neill, L. A. J. & Hardie, D. G. Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* 493, 346–355 (2013).
148. Dikalov, S. I., Nazarewicz, R. R., Bikineyeva, A., Hilenski, L., Lassègue, B., Griendling, K. K., Harrison, D. G. & Dikalova, A. E. Nox2-Induced Production of Mitochondrial Superoxide in Angiotensin II-Mediated Endothelial Oxidative Stress and Hypertension. *Antioxid Redox Sign* 20, 281–294 (2014).
149. Gioscia-Ryan, R. A., LaRocca, T. J., Sindler, A. L., Zigler, M. C., Murphy, M. P. & Seals, D. R. Mitochondria-targeted antioxidant (MitoQ) ameliorates age-related arterial endothelial dysfunction in mice. *J Physiology* 592, 2549–2561 (2014).
150. Cadenas, S. Mitochondrial uncoupling, ROS generation and cardioprotection. *Biochimica Et Biophysica Acta Bba - Bioenergetics* 1859, 940–950 (2018).
151. Chan, S. H. H. & Chan, J. Y. H. Mitochondria and Reactive Oxygen Species Contribute to Neurogenic Hypertension. *Physiology* 32, 308–321 (2017).
152. Corte, V. D., Tuttolomondo, A., Pecoraro, R., Raimondo, D. D., Vassallo, V. & Pinto, A. Inflammation, Endothelial Dysfunction and Arterial Stiffness as Therapeutic Targets in Cardiovascular Medicine. *Curr Pharm Design* 22, 4658–4668 (2016).
153. Mozos, I., Malainer, C., Horbańczuk, J., Gug, C., Stoian, D., Luca, C. T. & Atanasov, A. G. Inflammatory Markers for Arterial Stiffness in Cardiovascular Diseases. *Front Immunol* 8, 1058 (2017).
154. Park, S. & Lakatta, E. G. Role of Inflammation in the Pathogenesis of Arterial Stiffness. *Yonsei Med J* 53, 258–261 (2012).
155. Powell-Wiley, T. M., Baumer, Y., Baah, F. O., Baez, A. S., Farmer, N., Mahlobo, C. T., Pita, M. A., Potharaju, K. A., Tamura, K. & Wallen, G. R. Social Determinants of Cardiovascular Disease. *Circ Res* 130, 782–799 (2022).
156. Phillips, J. E. & Klein, W. M. P. Socioeconomic Status and Coronary Heart Disease Risk: The Role of Social Cognitive Factors. *Soc Personality Psychology Compass* 4, 704–727 (2010).
157. (CDC), C. for D. C. and P. Vital signs: avoidable deaths from heart disease, stroke, and hypertensive disease - United States, 2001-2010. *Mmwr Morbidity Mortal Wkly Rep* 62, 721–7 (2013).
158. Akwo, E. A., Kabagambe, E. K., Jr., F. E. H., Blot, W. J., Bachmann, J. M., Wang, T. J., Gupta, D. K. & Lipworth, L. Neighborhood Deprivation Predicts Heart Failure Risk in a Low-

Income Population of Blacks and Whites in the Southeastern United States. *Circulation Cardiovasc Qual Outcomes* 11, e004052 (2018).

159. Roux, A. V. D., Merkin, S. S., Arnett, D., Chambless, L., Massing, M., Nieto, F. J., Sorlie, P., Szklo, M., Tyroler, H. A. & Watson, R. L. Neighborhood of Residence and Incidence of Coronary Heart Disease. *New Engl J Medicine* 345, 99–106 (2001).

160. Adler, N. E. & Newman, K. Socioeconomic Disparities In Health: Pathways And Policies. *Health Affair* 21, 60–76 (2017).

161. Williams, D. R., Mohammed, S. A., Leavell, J. & Collins, C. Race, socioeconomic status, and health: Complexities, ongoing challenges, and research opportunities. *Ann Ny Acad Sci* 1186, 69–101 (2010).

162. Bell, C. N., Thorpe, R. J., Bowie, J. V. & LaVeist, T. A. Race disparities in cardiovascular disease risk factors within socioeconomic status strata. *Ann Epidemiol* 28, 147–152 (2018).

163. Ski, C. F., King-Shier, K. M. & Thompson, D. R. Gender, socioeconomic and ethnic/racial disparities in cardiovascular disease: A time for change. *Int J Cardiol* 170, 255–257 (2014).

164. RAMSAY, S. E., MORRIS, R. W., WHINCUP, P. H., PAPACOSTA, O., RUMLEY, A., LENNON, L., LOWE, G. & WANNAMETHEE, S. G. Socioeconomic inequalities in coronary heart disease risk in older age: contribution of established and novel coronary risk factors. *J Thromb Haemost* 7, 1779–1786 (2009).

165. Fearheller, D. L., Diaz, K. M., Sturgeon, K. M., Williamson, S. T. & Brown, M. D. Racial Differences in the Time-Course Oxidative Stress Responses to Acute Exercise. *J Exerc Physiology Online* 14, 49–59 (2011).

166. Taherzadeh, Z., Brewster, L. M., Montfrans, G. A. V. & VanBavel, E. Function and Structure of Resistance Vessels in Black and White People. *J Clin Hypertens* 12, 431–438 (2010).

167. Ergul, S., Parish, D. C., Puett, D. & Ergul, A. Racial Differences in Plasma Endothelin-1 Concentrations in Individuals With Essential Hypertension. *Hypertension* 28, 652–655 (1996).

168. Treiber, F. A., Jackson, R. W., Davis, H., Pollock, J. S., Kapuku, G., Mensah, G. A. & Pollock, D. M. Racial Differences in Endothelin-1 at Rest and in Response to Acute Stress in Adolescent Males. *Hypertension* 35, 722–725 (2000).

169. Veerabhadrapa, P., Diaz, K. M., Fearheller, D. L., Sturgeon, K. M., Williamson, S., Crabbe, D. L., Kashem, A., Ahrensfield, D. & Brown, M. D. Enhanced blood pressure variability in a high cardiovascular risk group of African Americans: FIT4Life Study. *J Am Soc Hypertens* 4, 187–195 (2010).

170. Deo, S. H., Jenkins, N. T., Padilla, J., Parrish, A. R. & Fadel, P. J. Norepinephrine increases NADPH oxidase-derived superoxide in human peripheral blood mononuclear cells via α -

adrenergic receptors. *Am J Physiology-regulatory Integr Comp Physiology* 305, R1124–R1132 (2013).

171. Montezano, A. C. & Touyz, R. M. Oxidative stress, Noxs, and hypertension: Experimental evidence and clinical controversies. *Ann Med* 44, S2–S16 (2012).

172. Brothers, R. M., Fadel, P. J. & Keller, D. M. Racial disparities in cardiovascular disease risk: mechanisms of vascular dysfunction. *Am J Physiol-heart C* 317, H777–H789 (2019).

173. Graham, G. Disparities in Cardiovascular Disease Risk in the United States. *Curr Cardiol Rev* 11, 238–245 (2015).

174. Monserrat-Mesquida, M., Quetglas-Llabrés, M., Capó, X., Bouzas, C., Mateos, D., Pons, A., Tur, J. A. & Sureda, A. Metabolic Syndrome Is Associated with Oxidative Stress and Proinflammatory State. *Antioxidants* 9, 236 (2020).

175. Storcka, A., Führlinger, G., Seper, M., Wang, L., Jew, M., Leisser, A. & Wolzt, M. E. coli Endotoxin Modulates the Expression of Sirtuin Proteins in PBMC in Humans. *Mediat Inflamm* 2013, 876943 (2013).

176. Wake, R. & Yoshiyama, M. Gender Differences in Ischemic Heart Disease. *Recent Patents Cardiovasc Drug Discov* 4, 234–240 (2009).

177. Kramer, P. A., Ravi, S., Chacko, B., Johnson, M. S. & Darley-USmar, V. M. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. *Redox Biol* 2, 206–210 (2014).