DISSERTATION

NOVEL MODULATORS OF BLOOD PRESSURE WITH AGE:

A PHYSIOLOGICAL AND BIOINFORMATICS-BASED APPROACH

Submitted by

Nate P. Bachman

Department of Health and Exercise Science

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Doctoral Committee:

Advisor: Barry Braun
Co-advisor: Thomas J. LaRocca

Adam J. Chicco
Christopher L. Gentile

ABSTRACT

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Systolic blood pressure (SBP) increases with age and is a significant risk factor cardio- and cerebrovascular diseases. While the causes of high blood pressure (hypertension) have been extensively studied, the causes of the age-related rise in blood pressure independent of chronic disease remain unclear. Thus, the identification of novel mechanisms underlying age-related high blood pressure may lead to new strategies to reduce chronic disease risk in older adults. Therefore, the goal of this dissertation was to use both physiological and bioinformatics-based approaches to better elucidate contributors to elevated blood pressure in healthy older adults.

The main findings are that 1) inhibition of Rho-kinase (an enzyme that participates in numerous cellular/regulatory pathways) lowers systemic blood pressure in healthy older adults concomitant with reduced vascular resistance but not improved endothelial function, 2) genes expression patterns in peripheral white blood cells differ in healthy older adults with elevated SBP compared to those with normal SBP and transcriptomic (RNA) changes relate to vascular and immune function, and 3) circulating chemokines and whole blood immune-related transcripts track with elevated SBP in healthy older adults. Taken together, this work shows that Rho-kinase, circulating RNA transcripts, and circulating chemokines may be novel therapeutic targets and/or biomarkers of elevated blood pressure in healthy older adults with untreated hypertension.

ACKNOWLEDGEMENTS

I would like to thank the wonderful volunteers who participated in these studies. Our community members are the ones who make human-based research possible (and fun). This research was supported by my advisors, Drs. Barry Braun and Thomas LaRocca, and resources from Dr. Frank Dinenno. I appreciate Dr. Braun's collaboration and insight on serval different projects over the years. Dr. LaRocca has been fantastic in guiding my analytical thinking, writing, and overall professional development. Drs. Janée Terwoord, Matt Racine, and Nathan Ketelhut were vital to conducting study one and helping me grow as a scientist. Dr. Terwoord played a substantial role in both the technical and intellectual aspects of my scientific training. Meghan Smith provided guidance and expertise with studies two and three. I would also like to thank Dr. Nathan Deming, Jacob Anna, and Brianna Dupre for their assistance with other projects during my time at Colorado State University. Finally, I would like to express my gratitude to my committee members, Drs. Christopher Gentile and Adam Chicco. Throughout my graduate education, Dr. Chicco has been a guiding force by holding stimulating conversations about science (and mountains) driven by curiosity and excitement. Thank you for providing me with this opportunity.

DEDICATION

This dissertation is dedicated to everyone who could see qualities in me that I could not see in myself. Drs. Gerald Smith and Gig Leadbetter, and Brent Alumbaugh helped guide me towards graduate school and, ultimately, to my current career. I am eternally grateful to my peers in graduate school who made work enjoyable each day. Lastly, I dedicate this to my family, who have encouraged me to reach this goal for more than a decade.

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INTRODUCTION

Precise regulation of blood pressure is critical to cardiovascular homeostasis. Indeed, sufficient pressure is needed to maintain adequate perfusion of the brain and other vital organs. However, dysregulation of many processes linked with aging and disease can lead to high blood pressure (arterial hypertension). High blood pressure is a significant risk factor for numerous chronic diseases, including heart failure, stroke, and Alzheimer's Disease (Benjamin et al., 2018).

Blood pressure and chronic disease risk

Blood pressure is an independent predictor of cardiovascular disease (Whelton et al., 2018). In fact, an increase in systolic blood pressure (SBP) by 20 mmHg or diastolic blood pressure (DBP) by 10 mmHg doubles the risk of death from coronary artery disease and stroke (Prospective Studies Collaboration, 2002). In addition, chronically elevated SBP causes increased afterload on the heart, leading to heart failure and end-organ damage in organs such as the brain and kidney (Cohuet & Struijker-Boudier, 2006). As a result, blood pressure is also a strong predictor of dementias (including Alzheimer's disease) and chronic kidney disease. Almost one-half of adults in the United States have high blood pressure based on recent guidelines (Whelton et al., 2018), which translates to a high cost for medical services and a substantial decrease in quality of life for individuals living with hypertension.

Blood pressure definitions

An increase in SBP above 130 mmHg or DBP greater than 80 mmHg is defined as arterial hypertension (Whelton et al., 2018). However, many individuals living with early stages of hypertension (stage 1: 130-139 mmHg/80-90 mmHg) have not been diagnosed with

hypertension or treated using pharmacotherapy. Furthermore, blood pressure guidelines prior to 2017 defined hypertension as SBP > 140 mmHg or DBP > 90 mmHg. Thus, some adults living with hypertension may be unaware of their hypertensive status. Furthermore, adults with untreated hypertension represent the natural continuum of the disease without being influenced by pharmacotherapy. With these considerations in mind, the studies described in this dissertation did not recruit individuals with diagnosed/pharmacologically treated hypertension. Therefore, individuals technically categorized as hypertensive will hereafter be referred to as having elevated or high blood pressure (see studies for details).

Causes of elevated blood pressure

Most cases of hypertension (~90%) are defined as essential, meaning that the observed hypertension is not secondary to another disease; thus, the cause is undefined (Oparil, Zaman, & Calhoun, 2003). Nonetheless, much research has investigated the causes of *primary* hypertension. Known contributors to hypertension include altered vascular signaling and endothelial dysfunction, genetic factors, immune system dysregulation, alterations in renal function and sodium handling, and enhanced sympathetic nervous system activity (Harrison, Coffman, & Wilcox, 2021). Despite this knowledge, therapeutic interventions (primarily pharmaceuticals) still fail to adequately control blood pressure (treatment-resistant hypertension) for nearly 30% of individuals diagnosed with hypertension (Calhoun et al., 2008). *Therefore, defining new therapeutic targets and biomarkers of hypertension is critical to establishing more effective treatment options and a better understanding of the causes of hypertension.*

Aging and elevated blood pressure

Aging is a major unmodifiable risk factor for hypertension. SBP rises with age in nearly all developed societies. This increase typically begins around middle age and occurs at a rate of approximately 7 mmHg per decade (Wolf-Maier et al., 2003). Consequently, most older adults (≥65 y) have hypertension (Benjamin et al., 2018). Given the relationship between aging and SBP, hypertension can be considered a gradually progressive disease rather than a condition with rapid onset. Therefore, physiological and biological changes in the cardiovascular system likely begin long *before* the diagnosis of hypertension. Thus, identifying the factors related to elevated blood pressure in aging will help elucidate mechanisms that ultimately lead to hypertension.

Rho-kinase contributes to hypertension

Rho-kinase is a serine/threonine kinase that contributes to vasculature tone. The enzyme is stimulated by G-protein receptor activation of Rho proteins. The binding of vasoconstrictor molecules such as angiotensin II and endothelin 1 to receptors on the vascular smooth muscle leads to Rho kinase signaling. Subsequently, Rho-kinase acts on myosin light chain to cause vascular smooth muscle contraction (vasoconstriction). In healthy humans, Rho-kinase is important for regulating the basal tone of the resistance vasculature (Büssemaker et al., 2007). Conversely, the enzyme is overactive in hypertensive volunteers (Masumoto et al., 2001). Pre-clinical studies have also demonstrated increased Rho-kinase activity in the vascular wall of hypertensive rodents (Uehata et al., 1997). Additionally, inhibition of Rho-kinase signaling promotes a reduction in SBP in experimental hypertension (Mukai et al., 2001). To date, no studies have investigated the relationship between age-related blood pressure elevation and Rho-kinase signaling. However, the existing data suggest that Rho-kinase dysregulation may be a fundamental process contributing to rising blood pressure with age and, ultimately, hypertension.

Transcriptomics

Transcriptomics is an emerging approach for identifying biological pathways that contribute to age-related blood pressure increases (and perhaps dysregulation of related mechanisms like Rho-kinase function). Transcriptomics involves quantifying RNA transcripts expressed in a target tissue at a single point in time (Lowe, Shirley, Bleackley, Dolan, & Shafee, 2017). Various technologies exist to perform this RNA analysis; however, next-generation RNA sequencing allows for the unbiased examination of all RNA transcripts (>26,000 genes), whereas older technologies (e.g., microarrays) are only capable of detecting a few thousand pre-determined genes/transcripts. The use of next-generation RNA sequencing also enables measurements of coding and non-coding transcripts (e.g., microRNAs [miRNAs]), which have emerged as potential novel contributors to hypertension. This approach allows a "snapshot" of local transcriptional control since RNA expression varies among cell and tissue types. It is commonly applied to blood/peripheral blood cells, which are easily accessible and can reflect "systemic" gene/transcript expression. The blood also is a tissue that transverses the cardiovascular system, carrying molecules that bind to and enter the vascular wall, altering vascular signaling and blood pressure (and therefore has biological relevance in the context of hypertension). Transcriptomics has been used to examine circulating white blood cells in older adults. However, only a few studies have examined associations with blood pressure, and it is unclear how changes to the *entire* transcriptome contribute to age-related elevated blood pressure because no studies have used RNA-sequencing to investigate this topic.

Immune function and hypertension

Importantly, transcriptome studies of blood/circulating cells capture RNA primarily from immune cells. Nearly all immune cells contribute to hypertension (Norlander, Madhur, &

Harrison, 2018). Immune system activation promotes immune cell infiltration and accumulation in the vascular wall, heart, kidneys, and brain. As a result, cytokines (released by these cells) cause inflammation, leading to impaired vasodilation and enhanced vasoconstriction. Inflammation in the central nervous system can also stimulate sympathetic nerve firing, also leading to vasoconstriction. Both mechanisms downstream of inflammation can contribute to elevated blood pressure. The causes for immune cell activation and inflammation resulting in hypertension remain unclear. Established contributors to hypertension such as high dietary sodium intake and angiotensin II likely play a role (Guzik et al., 2007). However, treatment strategies already exist to target these widely accepted contributors to hypertension, yet treatment-resistant hypertension persists. Recently, there has been a growing interest in determining how RNA expression, including non-coding miRNAs, promotes hypertension. Non-coding miRNAs serve an essential function as molecular switches to influence gene expression and are central to immune activation (Marques et al., 2011; Nosalski, McGinnigle, Siedlinski, & Guzik, 2017). It is likely that miRNAs and other transcripts influence immune responses that contribute to hypertension pathogenesis. Indeed, a recent investigation examining whole blood miRNA expression demonstrated that miRNA changes that accompany increasing age are associated with hypertension (Huan et al., 2018). Nonetheless, the link between gene expression patterns, immune system activity, and blood pressure elevation remains uncertain.

Summary and study aims

Hypertension is highly prevalent and contributes to cardiovascular, cerebrovascular, and renal morbidity and mortality. SBP increases with age such that age represents an immutable risk factor for hypertension. Numerous contributors to hypertension have been identified, yet successful treatment of hypertension continues to be a challenge. Changes in vascular signaling,

gene expression, and immune system activity contribute to rises in blood pressure; however, it is unclear how these factors change in otherwise healthy middle-aged and older adults. The establishment of novel targets and biomarkers may lead to new treatment strategies and a better understanding of hypertension pathogenesis to help offset the negative health consequences of hypertension. As such, the goal of these dissertation studies was to investigate novel mediators of higher blood pressure in older adults.

SPECIFIC AIMS

Experiment 1: To determine if Rho-kinase inhibition in healthy older adults leads to a reduction in systemic blood pressure

Experiment 2: To identify novel transcriptomic biomarkers for elevated blood pressure from white blood cells from healthy older adults

Experiment 3: To determine how circulating cytokines/chemokines relate to blood pressure and whole blood gene expression in healthy older adults

CHAPTER 2 – MANUSCRIPT 1

Rho-kinase inhibitor fasudil reduces blood pressure without altering endothelial function in healthy older adults

INTRODUCTION

Cardiovascular aging involves a complex combination of factors, including neurohormonal, humoral, and anatomical changes that contribute to alterations in cardiovascular structure and function. Collectively, these processes promote age-related dysfunction in the cardiovascular system. One major consequence of age-related cardiovascular dysfunction is elevated systolic blood pressure (SBP). In industrialized societies, SBP increases ~7 mmHg per decade in adults over age 40 (Wolf-Maier et al., 2003). Over time, this rise in SBP can lead to overt hypertension and elevated cardiovascular disease (CVD) risk (Najjar, Scuteri, & Lakatta, 2005). Indeed, nearly 70% of adults in the United States over the age of 60 have hypertension (Benjamin et al., 2018). Despite this prevalence, treatment-resistant hypertension (uncontrolled hypertension with ≥ 3 medications) may make up almost 30% of hypertension, and contributors to age-related elevations in SBP remain unclear (Calhoun et al., 2008).

One potential novel mechanism of elevated SBP with age is enhanced Rho-kinase activity. Rho-kinase is a serine/threonine kinase downstream of the Rho family of G proteins, and an important regulator of vascular smooth muscle contractility that has been implicated in development of cardiovascular disease (Masumoto et al., 2001; Nohria et al., 2006; Shimokawa, Sunamura, & Satoh, 2016). Rho-kinase modulates vascular tone directly by phosphorylating myosin light chain to induce vascular smooth muscle contraction and also indirectly through inactivation of myosin light chain phosphatase and by modulating myosin calcium sensitivity

(Kimura et al., 1996; Kureishi et al., 1997; Uehata et al., 1997). This influence on vascular control mechanisms can be observed in vivo during Rho kinase inhibition. Indeed, graded doses of intra-arterial Rho-kinase inhibitor fasudil lead to increases in forearm blood flow and abolish the constrictor response to exogenous endothelin-1 (ET-1) (Büssemaker et al., 2007). Given the influence of Rho-kinase on basal vascular tone, dysregulation of the enzyme is a likely contributor to elevated SBP in humans.

Recent studies have demonstrated the role of increased Rho-kinase signaling in hypertension and the pathogenesis of cardiovascular disease. Rho-kinase inhibition decreases SBP in hypertensive rat models, and intra-arterial administration of the Rho-kinase inhibitor fasudil reduces forearm vascular resistance in hypertensive humans (Masumoto et al., 2001; Uehata et al., 1997). Mechanistically, this reduction in vascular resistance may be mediated in part by an improvement in endothelial function. Rho-kinase can influence endothelial function through down-regulation of endothelial nitric oxide synthase (eNOS) gene expression and diminished eNOS activity (Ming et al., 2002). Accordingly, Rho-kinase activity is negatively correlated with peripheral endothelial function (flow-mediated dilation, FMD) (Soga et al., 2011). Furthermore, short-term treatment with oral fasudil improves FMD in coronary artery disease patients (Nohria et al., 2006). Other clinical studies have linked elevated Rho-kinase activity with aortic stiffening middle-aged and older adults, which may be a cause or consequence of elevated SBP (Boutouyrie, Chowienczyk, Humphrey, & Mitchell, 2021; Lakatta & Levy, 2003; Noma et al., 2007).

To date, no studies have investigated the relationship between Rho-kinase activity and age-related increases in SBP. To test the hypothesis that Rho-kinase contributes to age-related elevations in SBP, we performed a double-blind placebo control crossover study using a

systemic infusion of Rho-kinase inhibitor fasudil in healthy young and older adults. Additionally, since previous studies suggest a role for Rho-kinase in endothelial dysfunction and arterial stiffening, we also measured brachial artery FMD and aortic stiffness with and without systemic Rho-kinase inhibition.

METHODS

Participants

Healthy young (6F/6M, 25 ± 1 y) and older adults (7F/6M, 65 ± 1 y) were recruited from the local community. All participants were non-smokers and free of cardiometabolic disease based on a health history questionnaire and blood chemistries. No participant had been diagnosed with or was being treated for hypertension (defined as SBP > 140 mmHg, DBP > 90 mmHg). Older adults underwent ECG at rest and during a treadmill graded exercise test to screen for undiagnosed cardiovascular disease. All exercise test results were reviewed by a supervising physician. The study procedures were approved by the Colorado State University Institutional Review Board (protocol #16-6361H) and conformed with the Declaration of Helsinki.

Fasudil

Fasudil hydrochloride (LC Laboratories, Woborn, MA, USA) was locally compounded into an injectable solution (10 mg fasudil/1ml sodium chloride 0.9%; Pencol Compounding Pharmacy, Denver, CO). The fasudil solution was subsequently screened for purity, sterility, endotoxins, and fungal presence (Analytical Research Laboratories, Oklahoma City, OK, USA) and deemed safe for human use.

Study Design

Participants visited the laboratory on two occasions and were randomized to receive either fasudil or saline (placebo) during their first study visit in a double-blind, crossover design. Treatments were assigned by a laboratory member not involved in the data collection. Fasudil (60 mg of fasudil/100 ml 0.9% sodium chloride) or saline (100 ml 0.9% sodium chloride) was administered intravenously over a 60 min period via a catheter placed in an antecubital vein (Shibuya, Hirai, Seto, Satoh, & Ohtomo, 2005). Pilot testing in our laboratory demonstrated this dose was well tolerated in healthy subjects. Plasma levels peak immediately after the 60 min venous infusion and fall rapidly within ~30 min when using this concentration of fasudil. Importantly, the active metabolite of fasudil, hydroxyfasudil, remains elevated in the plasma (0.12 ± 0.02 - 0.20 ± 0.05 μg/ml) within the established range for Rho-kinase inhibition for ≥ 2.5 h (Shibuya et al., 2005). A washout period of ≥ 5 days was used between study visits. The data presented in this report were collected as part of a larger investigation (ClinicalTrials.gov identifier NCT03404843).

Subject Preparation

Participants arrived at the laboratory following a ≥12h fast. Sleep duration, physical activity, and dietary information from the prior day were recorded during the first study visit. Participants were instructed to replicate behaviors on the day before their second study visit. Pre-menopausal women were studied during the early follicular phase of the menstrual cycle or the placebo phase of oral contraceptives.

Measurements

All measurements were taken in a temperature-controlled room (~20-22°C) while the participant was in the supine position.(Limberg et al., 2020). For Doppler measurements, the participant's arm was abducted to ~90°.

Heart rate

Heart rate was measured throughout the study visit using 3-lead ECG (Datex-Ohmeda Cardiocap/5).

Peripheral and central blood pressure

SBP and DBP were measured over the brachial artery using an automated arm cuff (SphygmoCor Xcel) immediately following the fasudil or saline infusion (Time 0). Mean arterial pressure (MAP) was calculated from the SBP and DBP measures collected using the Xcel system using the following equation: MAP = DBP + (1/3 x [SBP – DBP]). Central (aortic) blood pressures were calculated from brachial pressures using a generalized transfer function built into the commercial software.(McEniery, Cockcroft, Roman, Franklin, & Wilkinson, 2014; Park et al., 2014) Blood pressure (SBP, DBP, MAP) was reassessed 1 and 2 h after the infusion (Datex-Ohmeda Cardiocap/5). Blood pressure values reflect the average of two measurements for each time point. When MAP was >5 mmHg different between measurements, an additional blood pressure was collected and values associated with >5 mmHg difference in MAP were excluded from analysis.

Vascular stiffness and peripheral vascular tone

Aortic stiffness and central augmentation index (SphygmoCor Xcel) were evaluated immediately following the treatment infusion. Aortic stiffness was assessed by carotid-femoral pulse wave velocity (cfPWV) using a tonometer probe and inflatable thigh cuff (Hwang et al.,

2014). The probe position on the carotid artery was marked and the distance from the probe to the suprasternal notch was measured. The femoral artery was palpated immediately distal to the inguinal ligament and the distance from the femoral artery to the thigh cuff was recorded. The linear distance from the suprasternal notch to the thigh cuff was measured with calipers. The probe and thigh cuff positions were duplicated for the return visit to the laboratory. The distances from the probe to the suprasternal notch and femoral artery to thigh cuff were subtracted from the suprasternal notch to the thigh cuff measurement and used to calculate path length (Hwang et al., 2014). cfPWV was automatically calculated as the quotient of the pulse wave distance (path length) divided by time difference between the upstroke of the carotid and femoral pulses. Each measurement was assessed by quality control standards and two to three measures were collected and averaged.

Following the 2 h blood pressure measurement, brachial artery diameter and mean blood velocity (MBV) were measured using Doppler ultrasound (GE Vivid 7) to calculate forearm blood flow in ml/min (FBF = MBV x π [brachial diameter/2]^2 x 60) (Limberg et al., 2020; Terwoord et al., 2020). All Doppler measures were performed using a 12 Mz linear array transducer probe and a minimum insonation angle of <60° was maintained at all times. Forearm vascular resistance (FVR = MAP/FBF) and conductance (FVC = MAP/FBF x 100) were calculated to determine the effects of acute Rho-kinase inhibition on peripheral vascular tone.

Brachial artery endothelial function

Flow-mediated dilation (FMD) of the brachial artery was assessed as a measure of endothelial function. FMD procedures conformed to established guidelines (Thijssen et al., 2011). Brachial artery diameter and MBV were measured using Doppler ultrasound at rest and for 3 min after 5 min of arterial occlusion. An automated cuff (D.E. Hokanson) was pressurized

to 250 mmHg to occlude arterial inflow. Brachial artery diameter was recorded at end diastole for each cardiac cycle (Vascular Imager) during rest and following occlusion and analyzed offline using a semi-automated software program (Brachial Analyzer). FMD was quantified as the change in diameter from rest to peak dilation (Ab. FMD = peak diameter -resting diameter; % FMD = [peak diameter -resting diameter]/resting diameter x 100) following the release of the occlusion cuff. Forearm blood flow was calculated as described above and the maximal hyperemic response during a single cardiac cycle following occlusion was reported as peak blood flow. To quantify the stimulus for FMD, shear rate (SR = MBV/diameter x 8) was determined for each cardiac cycle and the trapezoidal method was used to calculate the change in SR area-under-curve (AUC) from rest to peak diameter (Parker, Trehearn, & Meendering, 2009; Thijssen et al., 2011) FMD was normalized to SR_{AUC} (% FMD/ SR_{AUC}) to evaluate endothelial function relative to the stimulus for dilation.

Statistics

Data are presented as mean ± SE. Subject characteristics were compared using an unpaired *t*-test. Outcome variables were compared using an analysis of variance (ANOVA) with age as a between-subjects factor and drug as a repeated measure factor. Post-hoc analysis was performed using Tukey's test for multiple comparisons. To determine the influence of blood pressure on aortic stiffness values, cfPWV was also assessed with an analysis of covariance (ANCOVA) using central MAP as a covariate. Statistical significance was set at $P < 0.05$.

RESULTS

Participant characteristics are listed in Table 2.1. The older adults had greater percent body fat than young adults. The older adults also tended to have higher total ($P = 0.058$) and LDL ($P = 0.053$) cholesterol compared to young adults.

Table 2.1.
Participant characteristics.Data are mean ± SEM. *P*-values from unpaired *t*-test.

	Young	Older	*P*-value
n	6F / 6M	7F / 6M	
Age, y	25 ± 1	65 ± 1	< 0.001
BMI, kg/m^2	23.1 ± 0.6	25.2 ± 1.0	0.084
Body fat, %	24.5 ± 2.1	33.6 ± 2.4	0.009
Cholesterol, mg/dl	153 ± 9	182 ± 11	0.058
LDL, mg/dl	83 ± 6	106 ± 9	0.053
HDL, mg/dl	53 ± 4	59 ± 4	0.281
Triglycerides, mg/dl	83 ± 8	89 ± 8	0.633

Blood Pressure

Under control conditions, brachial MAP, SBP, and DBP were elevated in older adults compared to younger adults at 0 and 1 h (Fig. 2.1). Immediately following fasudil administration (time 0), MAP and SBP were reduced and DBP tended to be reduced ($P = 0.051$) compared to the saline condition in older adults. In young adults, fasudil lowered MAP and DBP while SBP was unaffected ($P = 0.09$) at time 0. In older adults, MAP and SBP remained lower 1 and 2 h post fasudil infusion compared to the saline condition such that the age-related differences in MAP and SBP were abolished (Figure 2.1). The DBP response to fasudil treatment followed similar trends in older adults (1 h: $P = 0.053$ vs saline; 2 h: $P < 0.05$ vs saline). MAP, SBP, and DBP returned to control levels within 1 h following fasudil treatment in young adults. Heart rate was not affected by fasudil treatment in either group (Table 2.2).

Central MAP, SBP, and DBP were elevated in older compared to young participants (MAP, 94 ± 2 vs 83 ± 2 mmHg; SBP, 124 ± 3 vs 102 ± 2 mmHg; DBP, 80 ± 1 vs 72 ± 2; all $P < 0.05$) after the saline infusion. Fasudil lowered all central blood pressures in older adults (MAP, 90 ± 2; SBP, 117 ± 3; DBP, 77 ± 2 mmHg; all $P < 0.05$ vs saline). In young participants, central MAP and DBP were decreased by fasudil (MAP, 79 ± 2; DBP, 69 ± 2 mmHg; both $P < 0.05$ vs saline) whereas central SBP was not different (98 ± 2 mmHg, $P = 0.11$ vs saline).

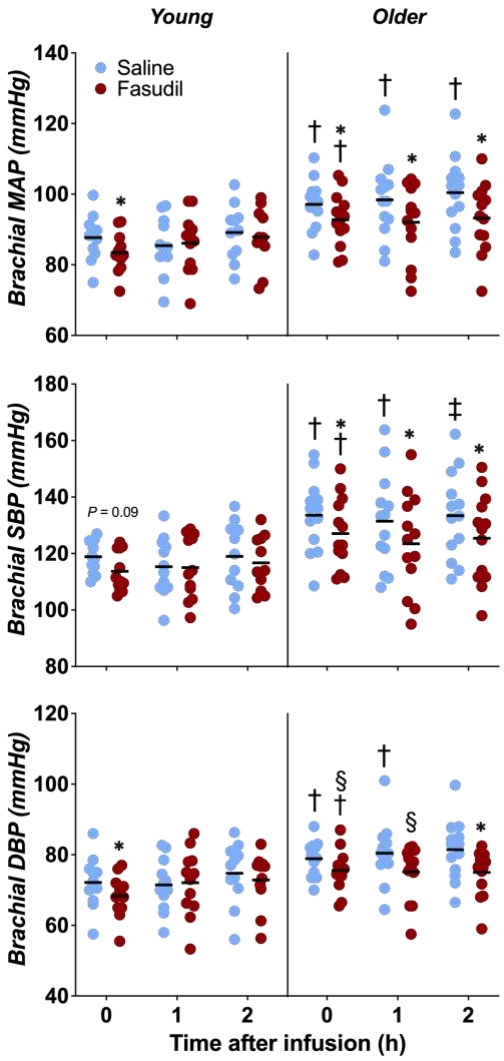


Figure 2.1.
Brachial MAP and SBP were lower immediately after fasudil infusion. In older participants, blood pressure generally remained lower at 1 and 2 h after infusion. In the young group, blood pressure normalized over time and there was no difference between the fasudil and saline conditions. SBP, systolic blood pressure; DBP, diastolic blood pressure. Data are mean ± SE. *$P < 0.05$ vs Saline; § $P = 0.051 – 0.053$ vs Saline; † $P < 0.05$ vs Young; ‡ $P = 0.07$ vs Young.

Table 2.2.
Heart rate, bts/min. Data are mean ± SEM.

Time post-infusion	0h	1h	2h
Young			
Saline	51 ± 3	58 ± 2	56 ± 3
Fasudil	54 ± 2	58 ± 2	57 ± 3
Older			
Saline	51 ± 2	59 ± 2	57 ± 2
Fasudil	55 ± 2	57 ± 2	55 ± 2

Vascular stiffness and peripheral vascular tone

cfPWV was elevated in older compared to young adults under control conditions and the age-related difference persisted after fasudil administration (Figure 2.2). Fasudil reduced cfPWV in older adults but not in young adults ($P = 0.07$). The effect of fasudil on cfPWV was no longer present ($P = 0.89$) in the older adults when central MAP was included as a covariate, indicating that the effect of fasudil to decrease MAP drove the apparent reduction in cfPWV. FVR and FVC were not different between older and young adults under the saline conditions (Figure 2.3). Following the fasudil treatment, FVR was reduced and FVC was increased in the older but not younger adults.

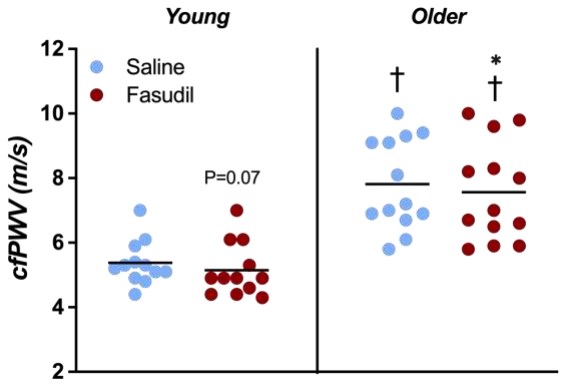


Figure 2.2.
Aortic stiffness assessed by carotid-femoral pulse wave velocity (cfPWV) after systemic saline or fasudil infusion. Data are mean ± SE. * $P < 0.05$ vs Saline; † $P < 0.05$ vs Young.

Brachial artery endothelial function

Fasudil increased brachial diameter at baseline and throughout the FMD trial in the older but not young group (Table 2.3 & Figure 2.4). FMD calculated as an absolute or percent change was not different between groups under saline conditions and remained unchanged by the fasudil treatment. Additional parameters from the FMD trials are provided in Table 2.3 and Figure 2.4.

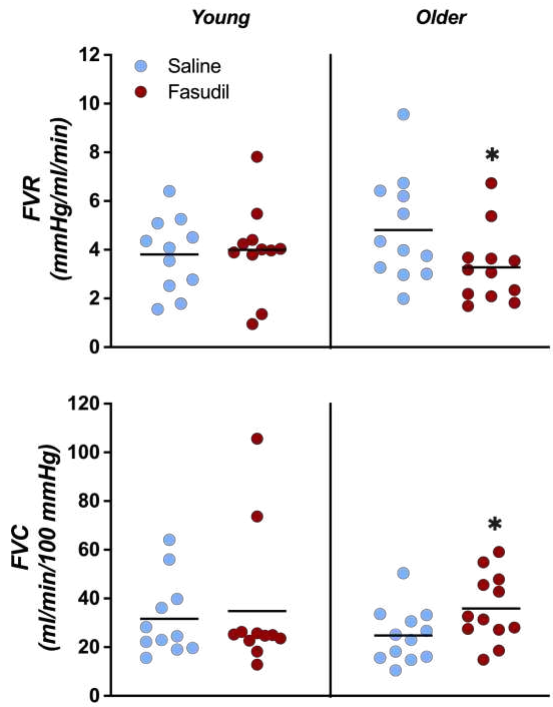


Figure 2.3.
Peripheral vascular tone represented as (A) forearm vascular resistance (FVR) and (B) forearm vascular conductance (FVC). Fasudil altered vascular tone in older but not young adults. Data are mean ± SE. * $P < 0.05$ vs Saline.

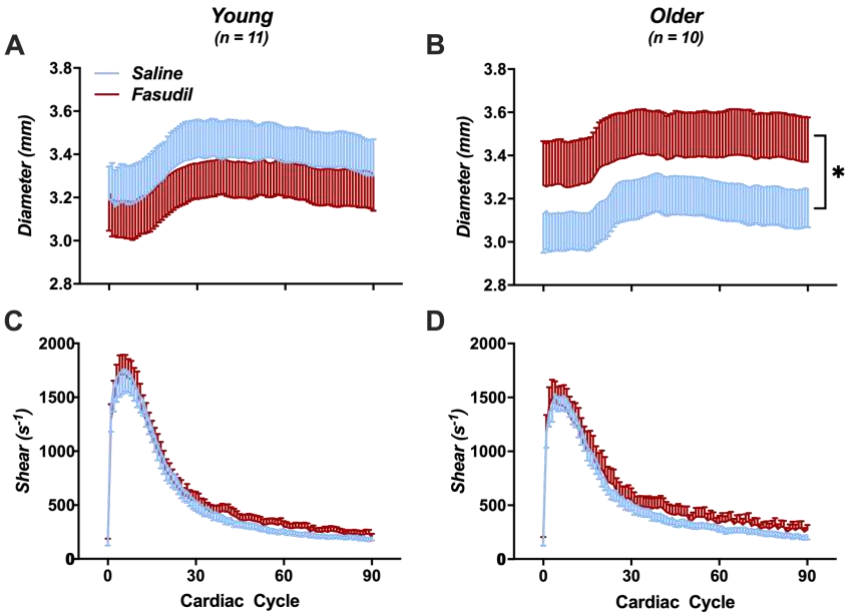


Figure 2.4.
Brachial artery endothelial function determined by flow-mediated dilation (FMD). Brachial artery diameter was increased by fasudil in older (B) but not young adults (A). Shear rate was unaffected by fasudil (C and D). Data are mean ± SE. * $P < 0.05$ vs Saline.

Table 2.3.
Brachial artery endothelial function assessed by flow mediated dilation (FMD). Data are mean ± SEM. * $P < 0.05$ vs saline.

	Young		Older	
	Saline	*Fasudil*	*Saline*	*Fasudil*
Baseline diameter, mm	3.19 ± 0.15	3.22 ± 0.18	3.13 ± 0.18	3.26 ± 0.20*
Peak diameter, mm	3.43 ± 0.17	3.42 ± 0.17	3.34 ± 0.18	3.45 ± 0.21*
Change in diameter, mm	0.25 ± 0.04	0.19 ± 0.02	0.21 ± 0.03	0.19 ± 0.03
Change in diameter, %	7.7 ± 1.2	6.5 ± 1.0	6.8 ± 1.0	5.8 ± 1.0
Resting blood flow, ml/min	27.7 ± 4.7	31.8 ± 8.7	25.6 ± 3.9	32.2 ± 3.7
Peak blood flow, ml/min	336.0 ± 42.2	356.6 ± 58.7	286.6 ± 27.7	286.7 ± 26.5
Normalized FMD, % / s^{-1}	2.4 x 10^{-4} ± 0.4 x 10^{-4}	2.6 x 10^{-4} ± 0.4 x 10^{-4}	2.4 x 10^{-4} ± 0.2 x 10^{-4}	2.4 x 10^{-4} ± 0.3 x 10^{-4}

DISCUSSION

In the present study, we sought to determine if Rho-kinase contributes to elevated blood pressure in older adults using a double-blind placebo control crossover study. Our study is the first to demonstrate an acute reduction in blood pressure in both young and older adults following a systemic infusion of Rho-kinase inhibitor fasudil. Importantly, this reduction in blood pressure (MAP, SBP) persists over time (1 and 2 h after infusion) in older adults, whereas blood pressure normalizes in young adults. We also observed a decrease in aortic stiffness (cfPWV) in the older group, which appears to be mediated by the fasudil-induced reduction in blood pressure. Furthermore, the fasudil treatment influenced peripheral vascular tone by reducing vascular resistance (FVR) but did not alter endothelial function (FMD). Taken together, these data suggest Rho-kinase contributes to age-related blood pressure elevation and may act through enhanced peripheral vasoconstriction.

Preclinical studies have implicated Rho-kinase in the pathogenesis of hypertension. Rho kinase activity is increased in the vasculature of hypertensive rats, and both acute and chronic inhibition of Rho-kinase lowers blood pressure in several models of hypertension (Mukai et al., 2001; Uehata et al., 1997). Interestingly, blood pressure is also reduced in normotensive rats following Rho-kinase inhibition, although this response is attenuated and transient (Uehata et al.,

1997). We observed a similar response in the current study, where blood pressure was lower in young and older adults immediately following the fasudil infusion compared to saline. However, blood pressure returned to values similar to the placebo condition by 1 h post-infusion in the young group while blood pressure remained depressed in the older adults. These results suggest Rho-kinase plays a fundamental role in blood pressure regulation under physiologic conditions but becomes overactive in advancing age.

Central blood pressure refers to the pressure within the aorta, which is a more accurate representation of afterload on the heart compared to brachial blood pressure. There is growing interest in measurement of central blood pressure as it may offer additional information about CVD risk alongside traditional brachial blood pressure. Indeed, a population-based study comparing normotensive and a range of hypertensive volunteers found >70% of participants with high normal blood pressure (SBP = 130 – 139 mmHg, defined as stage I hypertension based on current guidelines) had similar central pressure as hypertensive subjects (McEniery et al., 2008). This demonstrates how elevated central blood pressure in otherwise healthy individuals could represent a "hidden" risk factor, increasing myocardial workload and eventually leading to pathological conditions such as left ventricular hypertrophy. Accordingly, some data suggest that central blood pressure is more strongly related to left ventricle mass and CVD outcomes compared to brachial pressure (Covic, Goldsmith, Panaghiu, Covic, & Sedor, 2000; Roman et al., 2007). Furthermore, central blood pressure may respond more favorably to some hypertensive therapies compared to brachial blood pressure (Williams et al., 2006). These data demonstrate the need for reliable measures to better understand how central pressures are related to CVD risk and affected by therapeutics.

Direct measures of central (aortic) pressure must be obtained invasively using arterial catheters, thus limiting the utility for large-scale trials and clinical applications (McEniery et al., 2014). Non-invasive methods to determine central pressure, like those used in the current study, represent a practical and valid approach for approximating aortic pressures (Park et al., 2014). When examining the effect of fasudil on central pressure, we observed a reduction similar to that of brachial blood pressure in both young and older groups. These data indicate that acute Rho-kinase inhibition does not differentially affect central blood pressure compared to brachial pressure in healthy young and older adults.

Aortic stiffening is another aspect of vascular aging that may be influenced by Rho-kinase signaling. The primary contributors to increased vascular stiffening include reduced elastin and increased collagen deposition in the vascular matrix (Lakatta & Levy, 2003). Rises in arterial pressure may lead to structural damage that promotes elastin fragmentation and collagen production to normalize transmural pressure. Conversely, increased arterial stiffening may limit vascular distension and contribute to elevated blood pressure. It remains unresolved which condition precedes the other; however, hypertension and arterial stiffening often occur simultaneously (Boutouyrie et al., 2021; Lakatta & Levy, 2003). Importantly, arterial stiffness, particularly cfPWV, is predictive of CVD events independent of blood pressure (Sutton-Tyrrell et al., 2005). Thus, cfPWV adds valuable prognostic information about CVD risk in addition to blood pressure. Due to the growing interest in Rho-kinase in cardiovascular pathophysiology and observation that Rho-kinase activity is independently predictive cfPWV, we assessed cfPWV in the presence and absence of fasudil (Noma et al., 2007; Shimokawa et al., 2016). In the present study, cfPWV was lower in older but not young adults following the fasudil treatment compared to the saline condition. However, due to the close relationship between blood pressure

and cfPWV, we wanted to verify that the reduction in cfPWV that we observed could not be attributed to the blood pressure response to fasudil. Indeed, when we accounted for the reduction in central MAP with fasudil, there was no longer a difference in cfPWV between treatments. This relationship between a decline in MAP driving acute changes cfPWV has been demonstrated in pharmacological experiments in humans and supports the findings from the current study that vascular stiffening is a slow process not likely to be influenced by acute Rho-kinase inhibition (Stewart, Millasseau, Kearney, Ritter, & Chowienczyk, 2003). It is important to note that some hypertension therapies can improve vascular stiffening independent of changes in blood pressure, demonstrating the need for further research into the effect of long-term fasudil treatment on cfPWV (Boutouyrie et al., 2021).

Enhanced Rho-kinase activity in the vascular endothelium can lead to diminished NO dilator function and subsequent impaired endothelial function and increased peripheral resistance (Masumoto et al., 2001; Ming et al., 2002; Soga et al., 2011). Aging and cardiovascular diseases are often characterized by reduced endothelial function, which serves as a proxy for NO bioavailability (Celermajer et al., 1994; Green, Dawson, Groenewoud, Jones, & Thijssen, 2014). Furthermore, impaired endothelial function, represented as reduced FMD, precedes the onset of overt cardiovascular disease and predicts CVD risk (Landmesser, Hornig, & Drexler, 2004; Shechter, Shechter, Koren-Morag, Feinberg, & Hiersch, 2014). Therefore, Rho-kinase represents a new therapeutic target for improving FMD. In coronary artery disease patients, four weeks of oral fasudil treatment improved FMD (Nohria et al., 2006). Surprisingly, the acute fasudil treatment in the present study did not improve FMD in the older adults. This discrepancy could be due to a difference in acute versus chronic responses to fasudil treatment. Inhibition of Rho-kinase can lead to increased eNOS mRNA; however, our study visit may have been too

short for these transcripts to increase eNOS protein expression. Alternatively, this lack of effect may be due to similar FMD values between groups at baseline, suggesting no age-related impairment in this cohort of healthy adults. Typically, FMD is lower in older compared to younger adults; however, FMD is also negatively influenced by elevated cardiovascular risk factors such as BMI and blood lipids (Witte et al., 2005). Thus, the health status of our older adult group may have contributed to preserved endothelial function. Future investigations examining the relationship between Rho-kinase activity and older adults may wish to use a priori screening for impaired FMD. This method has been used previously, suggesting diminished FMD is a common but not universal finding in small studies of healthy older adults (Rossman et al., 2018).

Potential Mechanisms and Experimental Considerations

We are somewhat limited in understanding the mechanisms by which systemic fasudil reduces blood pressure in older adults in the current study. However, other investigations using Rho-kinase inhibition provide evidence for speculation. Much of previous work in humans using fasudil has utilized arterial infusions to understand the influence of Rho-kinase on local vascular control rather than systemic hemodynamics. For instance, intra-arterial fasudil promotes forearm blood flow in young adults, indicating a regulatory role of Rho-kinase on vascular tone (Büssemaker et al., 2007). This control mechanism seems to be altered in hypertension, where Rho-kinase activity becomes overactive. Indeed, forearm blood flow is markedly augmented in hypertensive volunteers following the local administration of fasudil (Masumoto et al., 2001). Furthermore, intra-arterial fasudil completely abolishes the vasoconstrictor response to ET-1 in healthy, young adults (Büssemaker et al., 2007). In older adults, protein expression of ET-1 is increased in arterial endothelial cells and the use of an oral ET-1 receptor agonist leads to a

reduction in blood pressure in hypertensive patients (Donato et al., 2009; Krum, Viskoper, Lacourciere, Budde, & Charlon, 1998). Thus, enhanced vasoconstriction and subsequent elevations in blood pressure in older adults may be mediated by Rho-kinase activity downstream of ET-1. Our data demonstrate this link between attenuated peripheral vasoconstriction and blood pressure. Indeed, we observed a reduction in FVR following fasudil treatment concomitant with a reduction in systemic blood pressure. In the current study, we did not utilize ET-1 inhibitors or have any measures of endothelial ET-1 protein expression, limiting our ability to determine potential upstream activation of Rho-kinase. Future studies are needed to determine whether Rho kinase acts as a downstream effector of ET-1 to contribute to age-related increases in blood pressure.

Another potential explanation of how Rho-kinase inhibition reduces blood pressure is through improved eNOS signaling. Therefore, improved NO bioavailability (and endothelial function) may have contributed to the reduction in FVR we observed following fasudil treatment. Contrary to this line of thinking, we found no difference in brachial artery FMD between the fasudil and placebo conditions in either young or older group. In support of these findings, combined intra-arterial infusion of fasudil and NOS inhibitor NG-monomethyl- L-arginine leads to a diminished but not abolished forearm blood flow response (Noma et al., 2007). Furthermore, a study examining the use of combined fasudil and nitroglycerin (NO donor) treatment on coronary artery dilation found an additive effect of the two drugs compared to two doses of nitroglycerin (Otsuka et al., 2008). Taken together, these data suggest that the observed vasodilation to Rho-kinase inhibition in humans is only due in part to increased NO bioavailability and may not have contributed to the blood pressure reducing effect of fasudil in the current study.

It is important to acknowledge that we did not collect measures of cardiac output in the current study, so we cannot be certain that changes in central hemodynamics were unaffected by Rho-kinase inhibition. Nonetheless, we did not observe any differences in heart rate during the fasudil treatment. Additionally, data in rodents show Rho-kinase activity can impair diastolic function and interfere with cardiomyocyte tension development, indicating that Rho-kinase inhibition may improve indices of cardiac function (Fukui et al., 2008; Vahebi, Kobayashi, Warren, De Tombe, & Solaro, 2005). If this response had occurred with Rho-kinase inhibition in the current study, we would expect that stroke volume and cardiac output would have been augmented with fasudil. Thus, fasudil would have potentially increased blood pressure rather than lowered it. Another factor that may have influenced our results is a reduction in sympathetic outflow. Indeed, Rho-kinase signaling in the brain has been shown to stimulate sympathetically-mediated increases in blood pressure (Ito et al., 2003). Considering the ability of fasudil to cross the blood-brain barrier, we cannot rule out that the reduction in blood pressure with fasudil was not the result of diminished sympathetic nerve firing (Koch et al., 2018). However, the lack of change in heart rate suggests there was no dramatic difference in sympathetic activity between the fasudil and saline conditions.

Finally, we also note recent data that challenge the accuracy of the central pressure measurement technique used in the current study (Schultz et al., 2020). Shultz et al. (2020) found that the brachial cuff-based approach for estimation of central pressures was inaccurate when using the Xcel device compared to invasive readings. While we acknowledge this could create challenges with interpreting central pressure data, it is important to point out that the standard approach to measure brachial blood pressure also differs from invasive recordings (Picone et al., 2017). Importantly, other devices that apply a generalized transfer function to

arterial waveforms to peripheral wave forms (radial applanation tonometry) to derive central pressures have been used to validate the cuff-based device we used in the current study (Park et al., 2014). These non-invasive devices have been successful in predicting CVD risk using central blood pressure estimates (Roman et al., 2007). Collectively, these data support the notion that the potential "inaccuracy" of calculated central pressures from peripheral waveforms may not limit their research and clinical applicability.

Perspectives

The known contributors to age-related increases in blood pressure and hypertension are complex and varied. Here, we show that Rho-kinase contributes to elevated blood pressure in a group of healthy older adults not diagnosed with hypertension. Acute Rho-kinase inhibition with fasudil produced a ~6-7 and ~3-6 mmHg reduction in systolic and diastolic brachial blood pressure, respectively. By comparison, available hypertensive treatments including angiotensin converting enzyme inhibitors and calcium channel blockers may reduce blood pressure by twice as much as our results (J. Wu et al., 2005). The difference in effectiveness could be due to the population (normotensive vs hypertensive) or drug dosage. In support of this idea, intra-arterial fasudil lessens FVR to a greater extent in hypertensive than control participants and this response is dose-dependent (Masumoto et al., 2001). Systemic blood pressure responses to fasudil treatment in rodent models mirror this pattern. A high but not low dose of fasudil reduces systolic blood pressure in hypertensive rats by ~30 mmHg (Mukai et al., 2001). As such, large-scale studies are needed to determine the relative contribution of Rho-kinase to age-related rises in blood pressure and overt hypertension, and the efficacy and ideal dosing of chronic Rho-kinase inhibition to treat these conditions.

CHAPTER 3 – MANUSCRIPT 2

RNA-sequencing identifies a unique transcriptomic signature in peripheral blood cells of older adults with untreated high blood pressure

INRODUCTION

Hypertension affects more than one third of US adults and leads to adverse cardiovascular outcomes including stroke, heart failure, and heart disease (Benjamin et al., 2018). One major contributor to hypertension is aging itself. Blood pressure increases with age (Wolf-Maier et al., 2003), and 25% of older adults with systolic blood pressure (SBP) >120 mmHg (prehypertension or elevated blood pressure) develop overt hypertension within four years of baseline measures (Benjamin et al., 2018). Targeting age-related elevations in blood pressure is an effective strategy for reducing cardiovascular disease risk (Julius et al., 2006); however, the exact biological causes of age-related hypertension are multifactorial and incompletely understood.

Transcriptomics may be a promising approach to identify novel biomarkers and therapeutic targets for treating blood pressure increases with age. Indeed, Huan et al. (2015) found genes previously implicated in blood pressure regulation and many novel genes to be differentially expressed in participants high compared to low blood pressure. In a different study, specific transcriptome signatures were associated with blood pressure changes over time (Zeller et al., 2017). These studies provide evidence that a more detailed description of blood pressure-associated changes in the transcriptome may contribute to our understanding of hypertension pathophysiology. However, the work of Huan et al. (2015) and others (Korkor et al., 2011; Marques et al., 2011; Zeller et al., 2017) utilized microarray analysis techniques, which

include only a limited, pre-selected set of genes (not the entire transcriptome). A more comprehensive method to interrogate the transcriptome is next-generation RNA sequencing (RNA-seq). This approach can, in theory, examine the entire transcriptome (>26,000 genes), allowing for the discovery of unknown contributors to disease. Moreover, although many RNA-seq studies focus on protein-coding transcripts, current methods for "total" transcriptome library preparation have made it possible to include non-coding transcripts in RNA-seq analyses. Importantly, non-coding microRNAs (miRNAs) have been shown to be differentially expressed in hypertensive participants in microarray studies (Marques et al., 2011), and inhibition of specific miRNAs can reduce blood pressure in experimental hypertension (Friese et al., 2013), suggesting other unidentified non-coding RNAs may be dysregulated in states of elevated blood pressure. To date, no studies have been performed using total RNA-seq (which detects both coding and non-coding RNAs) to examine the complete transcriptomic differences between older adults with higher and lower blood pressure.

To address this gap in knowledge, we assessed whole-transcriptome differences using total RNA-seq in peripheral blood mononuclear cells (PBMCs) from healthy middle-aged and older adults with a wide range of SBP. We chose to use PBMCs as they are a common biological sample collected for transcriptomic analyses, and they reflect signaling that influences systemic processes. Importantly, our participants had not been previously diagnosed or treated for hypertension (allowing us to capture in vivo gene expression patterns unaltered by comorbidities or pharmacotherapy). We then validated our findings in a second data set. Using the common upregulated genes in both data sets in participants with higher blood pressure, we were able to explore the biological processes associated with differentially expressed genes in these participants.

METHODS

Participant Characteristics and Stratification

Ten healthy middle-aged and older adults (62-76 y, Table 3.1) who were not previously diagnosed or treated for hypertension were separated into two groups based on SBP ("High" >130 mmHg; "Low" <130 mmHg). All participants were free of cardiometabolic, renal, and other chronic diseases. Participants were matched for age, sex, ethnicity, race (all non-Hispanic white), and aerobic fitness (VO_2 max collected during a graded treadmill test). All study protocols received local institutional review board approval (University of Colorado Boulder) and were conducted in accordance with the Declaration of Helsinki. Each participant completed an informed consent form prior to study participation.

Blood Collection

Whole blood samples were collected from participants for PBMC isolation (described below) following an overnight fast.

Blood Pressure Measurement

Participants visited the laboratory in the morning hours following an overnight fast. SBP and diastolic blood pressure (DBP) were measured at the brachial artery after a period of quiet rest using an automated device (Dinamap XL). Three measurements within 5 mmHg of each other were averaged.

Bioinformatics Analysis

Standard laboratory and analytical techniques previously described (LaRocca, Mariani, Watkins, & Link, 2019; Wahl, Cavalier, Smith, Seals, & LarRocca, 2021) were used for evaluating transcriptomic differences between the High and Low SBP groups. Briefly, PBMCs were isolated from whole blood samples using Ficoll and density gradient centrifugation. PBMCs were lysed with Trizol reagent and RNA was then extracted using a spin column kit

(Direct-Zol, Zymo Research). The recovered RNA was used to prepare total, ribo-depleted sequencing libraries using Takara Pico kits, and sequenced for all transcripts at a core facility (University of Colorado Anschutz Medical Campus Genomics Shared Resource Facility) on an Illumina NovaSeq 6000 sequencer to generate >40M paired end reads per sample. Raw FASTQ files were quality filtered with the fastp program and aligned to the hg38 genome using the STAR aligner to generate gene expression counts.

Differential gene expression analysis was performed using the DESeq2 program to compare transcript differences between the High and Low SBP groups. Over- and under-expressed genes were identified and a log2 fold change of gene expression >1 was used as a cutoff for further group comparisons. All genes that met the cutoff criteria were analyzed for gene ontology (GO) using the GOrilla program (Eden, Navon, Steinfeld, Lipson, & Yakhini, 2009), a single-ranked approach and a false discovery rate (FDR) of <0.1 to determine the biological processes related to differential gene expression. Among significant GO terms, the most specific biological processes were identified as terminal GO nodes in a directed acyclic graph. A weighted gene correlation network analysis (WGCNA) was also performed to identify gene clusters/modules that were most associated with SBP in the entire cohort as previously described (Wahl et al., 2021). For WGCNA, a minimum module size of 50 was used (to capture discrete gene clusters related to blood pressure), and GO analyses of genes in the WGCNA modules were performed using the GOrilla algorithm as described above.

Validation Analysis and Data Set Comparisons

To verify that differential gene expression patterns were not specific to our initial sample, we performed a validation analysis on 20 middle-aged and older adults (60-73 y, Table 4) using the same approach as described above. Additionally, we examined the correlation between the

log2 fold changes of all RNA transcripts between the two data sets, and upregulated biological processes related to increased RNA transcripts were compared. Genes from the common upregulated processes in both data sets were analyzed as potential SBP/DBP predictors using a penalized general regression model.

Statistics

Group characteristic data were compared using unpaired *t*-tests. Differential gene expression was conducted using DESeq2 software with a significance threshold of $P < 0.05$ and FDR <0.1, and significant WGCNA modules were identified as those with $P < 0.05$. Penalized general regression models utilized LASSO shrinkage and were generated using JMP software.

RESULTS

Data Set 1

To evaluate transcriptomic differences between healthy, middle-age and older adults with High and Low SBP, we performed differential gene expression analysis. An abundance of genes were over- (n=2619, FDR<0.1) and under-expressed (n=3289, FDR<0.1) in the High vs. Low group (Figure 3.1). We then selected transcripts with a log2 fold change >1 for GO enrichment analysis, which yielded 63 upregulated and 94 downregulated biological processes associated with higher SBP. Not surprisingly, biological processes related to vascular function ("regulation of vascular associated smooth muscle apoptotic process," "regulation of smooth muscle proliferation") (Brown et al., 2018) and ion channel function ("negative regulation of voltage-gated potassium channel activity") (Nieves-Cintrón, Syed, Nystoriak, & Navedo, 2018) were upregulated. However, the top 20 increased GO terms based on *P*-values consisted of broader biological processes including gene silencing by miRNA and posttranscriptional regulation of

gene expression (Table 3.2). Some general gene control processes were also downregulated; however, numerous immune responses were also decreased (Table 3.3).

Table 3.1.
Participant Characteristics for Data Set 1. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BMI, body mass index; VO_2, oxygen consumption. Data are Avg ± SEM.

	Data Set 1		
	High	Low	*P*-value
n (sex)	5 (3F/2M)	5 (3F/2M)	-
Age, y	69 ± 2	72 ± 2	0.231
SBP (range), mmHg	141 ± 4 (133 – 155)	115 ± 5 (98 – 127)	**0.004**
DBP (range), mmHg	78 ± 3 (71 – 85)	64 ± 5 (53 – 79)	**0.036**
HR, bts/min	73 ± 6	65 ± 4	0.293
BMI, kg/m^2	27.8 ± 1.5	24.7 ± 1.1	0.129
VO_2 Peak, ml/kg/min	24.0 ± 3.5	28.1 ± 2.3	0.355

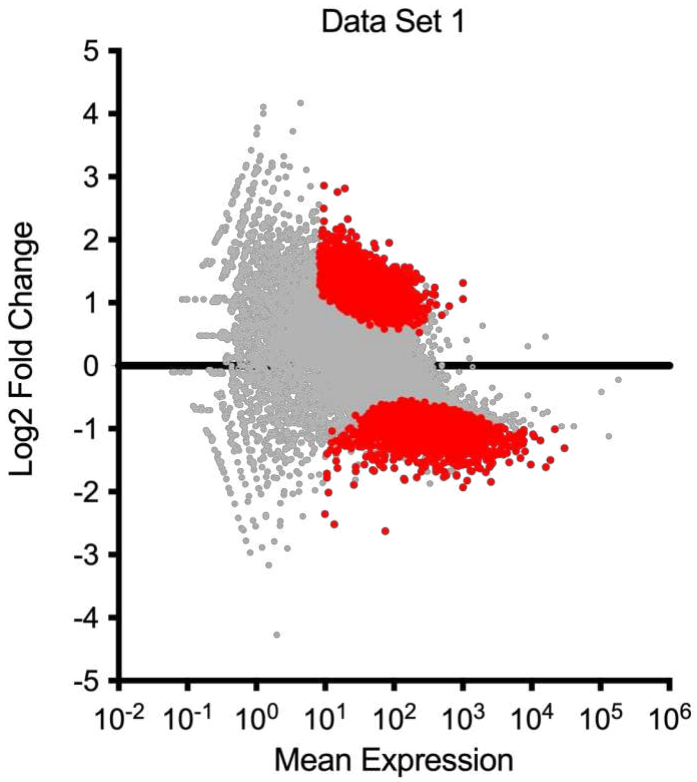


Figure 3.1.
Gene expression profile of High vs Low SBP groups. Relative gene expression in Data Set 1 shown using MA plots (M, log ratio; mean average). Each data point represents a single gene. "Mean Expression" indicates the average transcript abundance across both groups, and log2 fold change indicates relative abundance in the High vs Low group. Statistically significantly over- or under-represented genes are shown in red (FDR<0.1).

Table 3.2.
Top 20 Upregulated GO Enrichment Terms in High SBP group

GO Term	Description	P-value	FDR	Enrichment
GO:0035195	gene silencing by miRNA	5.20E-36	4.39E-32	4.41
GO:0016441	posttranscriptional gene silencing	9.17E-36	3.87E-32	4.39
GO:0035194	posttranscriptional gene silencing by RNA	9.17E-36	2.58E-32	4.39
GO:0031047	gene silencing by RNA	8.46E-35	1.78E-31	4.29
GO:0040029	regulation of gene expression, epigenetic	1.22E-33	2.05E-30	4.17
GO:0016458	gene silencing	1.22E-33	1.71E-30	4.17
GO:0010608	posttranscriptional regulation of gene expression	4.12E-33	4.96E-30	4.03
GO:0010629	negative regulation of gene expression	2.05E-20	2.17E-17	4.44
GO:0010605	negative regulation of macromolecule metabolic process	2.30E-17	2.15E-14	3.62
GO:0009892	negative regulation of metabolic process	3.44E-16	2.90E-13	3.42
GO:0010468	regulation of gene expression	4.73E-11	3.63E-08	2.45
GO:0048519	negative regulation of biological process	3.95E-10	2.78E-07	2.19
GO:0006396	RNA processing	3.21E-08	2.09E-05	5.23
GO:0060255	regulation of macromolecule metabolic process	2.01E-07	1.21E-04	2.01
GO:0019222	regulation of metabolic process	1.55E-06	8.71E-04	1.9
GO:1904046	negative regulation of vascular endothelial growth factor production	5.28E-06	2.79E-03	9.36
GO:0001818	negative regulation of cytokine production	5.72E-06	2.84E-03	3.63
GO:0001817	regulation of cytokine production	1.08E-05	5.04E-03	5.23
GO:0050728	negative regulation of inflammatory response	1.97E-05	8.77E-03	15.04
GO:0042984	regulation of amyloid precursor protein biosynthetic process	2.30E-05	9.72E-03	9.86

Table 3.3.
Top 20 Downregulated GO Enrichment Terms in Low SBP group

GO Term	Description	P-value	FDR	Enrichment
GO:0035195	gene silencing by miRNA	4.79E-10	3.92E-06	3.49
GO:0016441	posttranscriptional gene silencing	5.50E-10	2.25E-06	3.18
GO:0035194	posttranscriptional gene silencing by RNA	5.50E-10	1.50E-06	3.18
GO:0002682	regulation of immune system process	8.63E-10	1.76E-06	1.33
GO:0002263	cell activation involved in immune response	9.70E-10	1.59E-06	1.38
GO:0002376	immune system process	1.14E-09	1.55E-06	1.24
GO:0002366	leukocyte activation involved in immune response	1.35E-09	1.58E-06	1.37
GO:0002274	myeloid leukocyte activation	1.96E-09	2.00E-06	1.39
GO:0031047	gene silencing by RNA	3.63E-09	3.30E-06	3.03
GO:0045321	leukocyte activation	9.22E-09	7.54E-06	1.32
GO:0002252	immune effector process	1.29E-08	9.57E-06	1.32
GO:0042119	neutrophil activation	1.45E-08	9.91E-06	1.39
GO:0002283	neutrophil activation involved in immune response	1.56E-08	9.83E-06	1.4
GO:0001775	cell activation	1.78E-08	1.04E-05	1.32
GO:0007166	cell surface receptor signaling pathway	2.11E-08	1.15E-05	1.27
GO:0036230	granulocyte activation	2.61E-08	1.33E-05	1.39
GO:0045055	regulated exocytosis	2.91E-08	1.40E-05	1.34
GO:0007165	signal transduction	3.06E-08	1.39E-05	1.19
GO:0043312	neutrophil degranulation	3.25E-08	1.40E-05	1.39
GO:0002275	myeloid cell activation involved in immune response	3.38E-08	1.38E-05	1.38

Validation Analysis: Data Set 2

To confirm our findings, we repeated our analysis in a second data set (Table 3.4). Again, we observed a distinct gene expression profile very similar to our findings in Data Set 1 (Figure 3.2), although few genes reached statistical significance. Despite this difference in significance compared to Data Set 1, we found a remarkably strong correlation (r=0.79, $P<0.0001$; Figure 3.3) between the gene expression profiles of the two data sets, suggesting that a common PBMC gene expression signature is associated with elevated SBP. To investigate the biological processes that might underlie this observation, we repeated the GO enrichment analysis in Data Set 2 and found that 60 biological processes were increased and none decreased (likely due to the less pronounced gene expression differences in this data set). When the two groups were compared, there were 34 common biological processes increased in both data sets (Figure 3.4). Among these, the most specific biological processes (terminal GO nodes) included "negative regulation of voltage-gate potassium activity," "miRNA mediated inhibition of translation, gene silencing by miRNA," and "RNA processing."

Table 3.4.
Participant Characteristics for Data Set 2. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BMI, body mass index; VO_2, oxygen consumption. Data are Avg ± SEM.

	Data Set 2		
	High	Low	*P*-value
n (sex)	10 (5F/5M)	10 (5F/5M)	-
Age, y	66 ± 1	65 ± 1	0.427
SBP (range), mmHg	140 ± 2 (130 – 150)	108 ± 2 (98 – 115)	**< 0.001**
DBP (range), mmHg	77 ± 2 (66 – 88)	69 ± 2 (60 – 79)	**0.014**
HR, bts/min	58 ± 3	57 ± 3	0.685
BMI, kg/m^2	24.7 ± 1.0	22.6 ± 1.0	0.156
VO_2, ml/kg/min	27.2 ± 3.2	32.5 ± 2.3	0.198

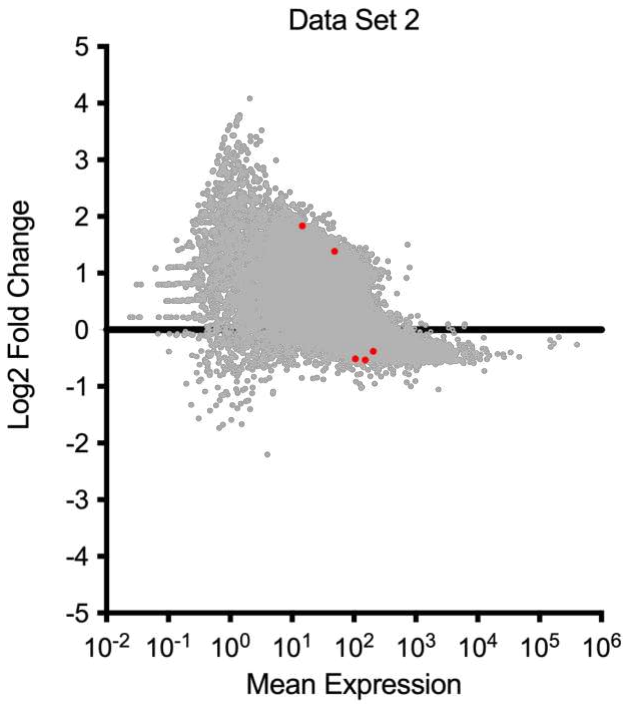


Figure 3.2.
Gene expression profile of High vs Low SBP groups. Relative gene expression in Data Set 2 shown using MA plots (M, log ratio; mean average). Each data point represents a single gene. "Mean Expression" indicates the average transcript abundance across both groups, and log2 fold change indicates relative abundance in the High vs Low group. Statistically significantly over- or under-represented genes are shown in red (FDR<0.1).

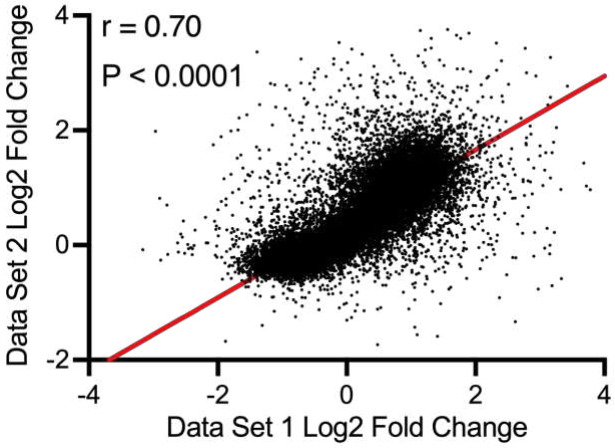


Figure 3.3.
Relationship between the gene expression profiles of High vs Low participants each data set. The gene expression (log2 fold expression changes in High vs Low SBP groups) of Data Set 1 and 2 were strongly correlated.

Figure 3.4.
Common increased biological processes were observed in both data sets. Overexpressed genes contributed to 63 and 60 enriched GO terms in Data Set 1 and 2, respectively. Thirty-four of these increased processes were present in both data sets.

To determine if common overexpressed genes in both data sets were predictive of blood pressure, we extracted the genes from the common GO terms. Interestingly, 171 of the 205 genes examined were miRNAs. However, a penalized regression analysis of these genes did not yield any significant predictors of SBP. We also examined the top 1000 significant genes from Data Set 1 and, again, found no significant regression-based associations with SBP.

Multiple Regression and Network Analysis

Because we did not find any direct/individual correlates of blood pressure in our above analyses, we looked for associations/correlations among gene expression and blood pressure more broadly. First, we conducted a penalized regression analysis including *all genes/transcripts* and blood pressure. This did not yield any significant predictor equations for SBP. Therefore, we next conducted an unbiased network analysis to identify clusters (modules) of genes related to SBP/DBP. Using the WGCNA program and normalized gene counts, as well as all available clinical data (SBP, DBP, age, BMI, VO_2max, etc.), we were able to identify several gene modules that were significantly correlated with both SBP and DBP (Figure 3.5). A GO/KEGG analysis (more specific to signaling pathways vs. generic processes) of these modules showed

that pathways positively associated with both SBP and DBP (those in the red module) included primarily “mineral absorption” reflected by genes from the metallothionein (MTH) and solute carrier (SLC) families. In addition, DBP was positively associated with pathways like “steroid hormone biosynthesis” and “ascorbate metabolism”, both of which were represented by numerous genes from the UDP Glucuronosyltransferase family (enzymes critical to kidney function). DBP was also negatively related (brown module/gene cluster) to pathways like “T cell differentiation” and “B cell signaling”, suggesting a possible role for immune function.

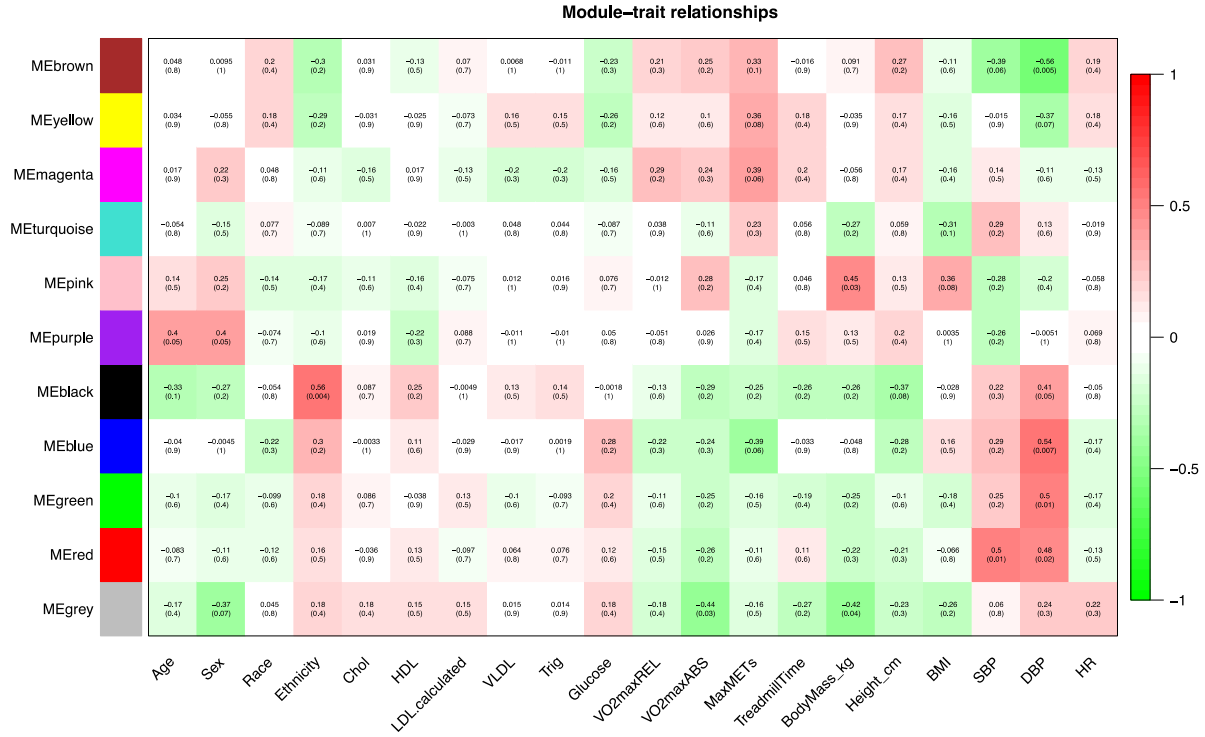


Figure 3.5.
Gene clusters associated with blood pressure and other clinical variables. Heatmap showing results of a Weighted Gene Correlation Network Analysis to identify clusters of genes (eigen-modules, ME) related to clinical variables, including blood pressure, in all subjects in Data Set 1. Gene modules are indicated by arbitrary color names. Pearson’s r-values are listed in each heatmap block with *P*-values in parentheses below.

DISCUSSION

Our study is the first to investigate transcriptome differences in circulating white blood cells using RNA-seq in healthy middle-aged and older adults with High and Low SBP. Here, we show that high SBP in older adults is associated with a unique differential gene expression

pattern. Importantly, participants were carefully matched for age, sex, resting heart rate, BMI, and aerobic fitness to avoid potential confounding effects on SBP and gene expression. Interestingly, a highly similar pattern of gene expression was also present in a second group of High vs Low SBP middle-aged and older adults, and the differentially expressed genes were strongly correlated between the two data sets (suggesting that the patterns we observed are biologically meaningful). We also observed 34 biological processes that were increased in both data sets, which included 171 upregulated miRNAs out of the 205 overexpressed genes contributing to those processes. Nonetheless, these genes did not correlate with SBP nor did the top 1000 overexpressed genes from Data Set 1.

The lack of significance when assessing the contribution of one thousand genes to blood pressure is likely due to the highly conservative method of regression analysis (penalized regression). Indeed, high levels of multicollinearity among candidate predictor genes creates challenges when using a penalized regression that includes genes that interact with each other or influence common biological processes. However, we did find numerous moderate correlations when we examined associations between individual genes from the group of significant differentially expressed genes and SBP. For instance, genes that positively correlated with SBP included C1QTNF4 (r=0.79, $P<0.01$) and C10orf99 (r=0.63, $P<0.05$). Both genes are linked to inflammatory processes, which are a prominent feature of hypertension pathogenesis (discussed below). Furthermore, C10orf99 has been shown to be predictive of the autoimmune condition psoriasis, another condition characterized by inflammation (Guo et al., 2014). Upregulation of C10or99 may increase transcription factor NF- κB activity, a known contributor to hypertension and vascular dysfunction (C. Chen et al., 2018; Pierce, Lesniewski, Lawson, Beske, & Seals, 2009; K. I. S. Wu & Schmid-Schönbein, 2011).

Importantly, the transcripts analyzed in the current study were isolated from PBMCs, which consist of several white blood cell types including lymphocytes and monocytes. This approach provides some advantages. First, collection of whole blood is common in many clinical studies. Compared to transcriptomic investigations that use invasive tissues biopsies (Basu et al., 2017; Marques et al., 2011), our approach to collecting samples for transcriptomic analysis provides an accessible option for future studies. Additionally, immune function is well-established as a causal factor in hypertension (Norlander et al., 2018). Indeed, nearly all immune cells, including those that make up PBMCs, contribute to hypertension. For instance, mice lacking B and T cells have an attenuation in blood pressure elevation in an experimental model of hypertension (Guzik et al., 2007). In humans, T cells obtained from hypertensive volunteers have a pro-inflammatory phenotype (Youn et al., 2013), a salient feature of hypertension pathogenesis. Interestingly, we observed transcriptomic evidence that numerous aspects of immune function were decreased (downregulated GO terms) in the High group in Data Set 1. These data suggest that increased SBP is associated with compromised rather than overactive immune responses. Indeed, it has been observed that activation of myeloid-derived suppressor cells occurs in experimental hypertension to restrict inflammation and immune activity (Shah et al., 2015). Thus, it is likely that some immune processes are increased while others are simultaneously decreased in hypertension, which is consistent with our observations on GO terms in Data Set 1. Further studies are needed to determine the precise functional changes that follow immune transcriptomic patterns accompanying elevated SBP and hypertension in humans.

A unique insight we gained from examining the terminal GO nodes (the most specific biological processes identified in our analyses) was the modulation of gene silencing by miRNA.

We were able to detect this due to our use of total RNA-seq, which includes the assessment of a large set of non-coding transcripts. Among individual genes contributing to gene silencing in Data Set 1, numerous miRNAs (miR665, miR134, miR1-1, miR224, miR661, miR21, and others) that were present have pro or anti-oncogenic properties (OncomiR Cancer Database) (Sarver, Sarver, Yuan, & Subramanian, 2018). This observation could be a result of the fact that miRNAs can have multiple targets, many of which have yet to be characterized, but previous work does indicate a potential link between blood pressure and future cancer risk (Seretis et al., 2019). However, this connection is still uncertain, and it is unclear if the expression of miRNAs that influence oncogenic processes are caused by or the consequence of high blood pressure. Other terminal GO nodes modulated by gene expression patterns in Data Set 1 also included established vascular processes/changes that are characteristic of hypertension including negative regulation of voltage-gated potassium channels and regulation of vascular associated smooth muscle cell apoptotic processes. Voltage-gated potassium channels are essential in controlling vascular smooth muscle cell tone through hyperpolarization, and they become impaired in hypertension, leading to enhanced vasoconstriction (Nieves-Cintrón et al., 2018). Vascular smooth muscle cell apoptosis is likely the result of vascular remodeling that accompanies hypertension (Brown et al., 2018). Collectively, these data support previous findings while also providing a new appreciation of novel transcripts associated modulated biological processes in middle-aged and older humans with elevated SBP.

The use of network analyses is another way to evaluate how gene expression relates to biological processes that influence blood pressure. This approach avoids some of the pitfalls of regression analysis described above by assessing correlations between individual genes to identify patterns/groups of genes that are over- or under-expressed together. Thus, the goal is to

determine gene groups that relate to each other and blood pressure. These groups of genes are then organized in "modules", which are analyzed for GO enrichment to interpret biological relevance. In our analysis, we found several gene modules related blood pressure, and genes in the modules reflected a positive relationship between ascorbate metabolism and DBP. Ascorbate (vitamin C) is an anti-oxidant molecule that has been inversely correlated with SBP and DBP (Ran et al., 2020). Oxidative stress is an established contributor to hypertension and is known to increase with age (Taddei et al., 2001). Thus, high levels of circulating antioxidants, including ascorbate, may have a protective role against hypertension. In the current study, it remains unclear if the network analysis results indicate transcriptional dysregulation reflective of increased ascorbate degradation that promotes a rise in blood pressure in older adults.

Our network analysis also identified an association between modules related to adaptive immune responses, including T cell differentiation and B cell signaling, and blood pressure. As previously mentioned, studies of immune dysregulation in experimental models of hypertension have established a casual-relationship between T and B cell activity and increasing blood pressure (X. H. Chen et al., 2018; Guzik et al., 2007; Norlander et al., 2018). Other pioneering research in this area also demonstrated that thymus transplantation from a normotensive to hypertensive rat reduced SBP in the recipient, indicating the importance of T cell involvement in maintenance of hypertension (Ba, Takeichi, Kodama, & Kobayashi, 1982). Interestingly, we found that T cell differentiation was negatively associated with DBP. This finding may be due to the opposing influence that different subtypes of T cells have on blood pressure. For instance, Chen and colleagues (2018) demonstrated that removal of regulatory T cells from the blood, spleen, and kidney from angiotensin II-infused rodents lead to increased blood pressure, supporting a known protective role of regulatory T cells against hypertension. Further research

measuring both gene expression and circulating chemokines (critical inducers of T cell differentiation) may provide more insights into upstream signals that promote T cell activity that may protect against or promote hypertension (Hughes & Nibbs, 2018).

Experimental Considerations and conclusion

We chose to group our participants into "High" (>130 mmHg) and "Low" (<130 mmHg) SBP groups to determine patterns of gene expression in participants who would generally be classified as hypertensive and normotensive. It is important to note that current hypertension guidelines would categorize some of the Low participants in Data Set 1 (n=2) as having elevated SBP (>120/<130 mmHg) (Whelton et al., 2018). Thus, some of these individuals did not truly have low blood pressure. Nonetheless, no participant had a clinical hypertension diagnosis, and high blood pressure is often not treated until SBP reaches or exceeds 130 mmHg (Whelton et al., 2018). Overall, our approach allowed us to assess differentially expressed genes in individuals who may have increased cardiovascular risk (High group) despite being otherwise healthy. Using this High and Low approach, we were able to identify clear gene expression patterns that persisted across data sets. These data reveal that transcriptomic dysregulation may be a key feature of hypertension pathogenesis.

CHAPTER 4 – MANUSCRIPT 3

Circulating chemokine and RNA measures of immune function correspond with but do not predict high blood pressure in older adults

INTRODUCTION

Hypertension is a multifactorial disease. Contributors include enhanced sympathetic nervous system activity, impaired sodium handling, and elevated levels of circulating angiotensin II (Harrison et al., 2021). Collectively, these factors along with numerous others including genetic changes give rise to essential hypertension. The relative contribution of each individual factor to increasing blood pressure remains unclear, and the contributions likely vary among individuals. Importantly, many hypertensive stimuli, including sympathetic nerve activity, high sodium levels, and elevated angiotensin II levels, are known to promote inflammatory processes implicated in the development of hypertension (Norlander et al., 2018).

Numerous inflammatory mediators are also known to promote hypertension directly. One well-established marker of inflammation is c-reactive protein (CRP), which has been shown to be elevated in essential hypertension as well as cardiovascular disease (Bautista, Vera, Arenas, & Gamarra, 2005; Bisoendial, Boekholdt, Vergeer, Stroes, & Kastelein, 2010; Rabkin, Langer, Ur, Calciu, & Leiter, 2013). Other inflammatory cytokines that are associated with elevated blood pressure include interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). For instance, a study of middle-aged hypertensive adults demonstrated that participants with hypertension had ~100% greater circulating IL-6 compared to healthy controls (Bautista et al., 2005). A current theory that integrates these observations with other known mechanisms of hypertension suggests that hypertensive stimuli such as sympathetic nerve firing trigger both

adaptive and innate immune responses that promote immune cell infiltration to various target tissues such as the vasculature and kidney, which ultimately leads to a rise in blood pressure (Norlander et al., 2018). However, much of this work has been performed in experimental models of hypertension, and it remains unclear what other biological signals may accompany increased immune activity in hypertensive humans.

As previous work has linked gene expression patterns with inflammation and hypertension, analyses of the transcriptome may hold promise for identifying immune/hypertension mediators (Basu et al., 2017; Lin et al., 2017). The transcriptome refers to all RNA transcripts (>26,000) present in a tissue sample at a given time (Lowe et al., 2017). Importantly, slight changes in gene expression patterns can have a sizable influence on protein expression and cellular function, so global, "whole-transcriptome" analyses are particularly important. Some studies that have examined transcriptome differences in the context of hypertension have assessed RNA expression from peripheral blood mononuclear cells (PBMCs) (Marques, Booth, & Charchar, 2015). PBMCs represent an easily accessible and relevant tissue sample since PBMCs consist of numerous immune cells implicated in hypertension pathogenesis (Norlander et al., 2018). However, whole blood contains other cell types and immune-related RNA transcripts that freely circulate and are not captured by PBMC analysis (Van Der Sijde et al., 2020). Therefore, transcriptomic analyses specific to PBMCs may fail to detect key molecular signals that contribute to hypertension. Conversely, whole blood transcriptomics provides a means of measuring all circulating RNA. While this technique has been utilized before, some studies that have used "transcriptome-wide" analyses are limited to assessing the expression of a few thousand genes, potentially missing novel modulators of blood pressure (Marques et al., 2011; Zeller et al., 2017). Conversely, next-generation RNA sequencing (RNA-

seq) is an analytic method that allows for determination of all RNA transcripts (i.e., whole transcriptome) (Lowe et al., 2017).

To date, no studies using RNA-seq on whole blood have been performed in hypertensive older adults free of comorbidities or pharmacological treatments for high blood pressure. However, systemic inflammation and blood pressure both increase with age, suggesting that this could be a promising area for research on hypertension (Assar, Angulo, & Rodríguez-Mañas, 2016; Wolf-Maier et al., 2003). Indeed, systolic blood pressure (SBP) rises with age beginning in midlife. First line treatments for early stages of hypertension (Stage I: SBP 130-139 mmHg) include lifestyle modification like engaging in regular physical activity and reducing sodium consumption (Whelton et al., 2018). Thus, it is common for individuals with stage I hypertension to refrain from taking blood pressure reducing medications. Furthermore, some individuals are unaware of their hypertension status, potentially leading to more advanced hypertension prior to receiving pharmacological treatment. These individuals represent the natural continuum of the disease and provide an opportunity to study mechanisms of age-related elevation in blood pressure without confounding effects of pharmacotherapy. Therefore, studying older individuals with and without elevated blood pressure (i.e., untreated/diagnosed hypertension) may be a good strategy for identifying alterations in blood pressure control that correspond to inflammatory and transcriptomic changes.

Several investigations have examined the role of cytokine signaling (e.g. TNF-a, IL-6) in experimental hypertension (Norlander et al., 2018; Nosalski et al., 2017). However, less focus has been placed on circulating chemokines, which are important mediators of immune cell infiltration to vascular, renal, and brain tissues (Hughes & Nibbs, 2018). Furthermore, prior investigations into the role of chemokines in hypertension include animal models of hypertension

or patient populations with comorbidities (e.g., metabolic disease) (Antonelli et al., 2012; Mikolajczyk et al., 2021). Therefore, it is yet to be established whether chemokine activity is increased in healthy older adults with high blood pressure. It also remains unclear whether dysregulated transcriptional control coincides with enhanced systemic inflammation in older adults with elevated blood pressure. Thus, the purpose of this study was to examine circulating markers of inflammation and whole blood RNA expression using RNA-seq in heathy older adults with elevated (untreated hypertension) and normal blood pressure. We hypothesized that circulating chemokines would be higher in older adults with elevated blood pressure, and that this would coincide with over- and under-expression of genes associated with immune functions.

METHODS

Participants

The current study utilized a subset of older adults (>65 y) recruited for a larger study examining factors related aging and brain health. All participants completed an informed consent form approved by the institution review board (University of Colorado Anschutz School of Medicine). The study procedures described below were in compliance with the Declaration of Helsinki. Data included for analysis included healthy older adults without a previous diagnosis of hypertension or treatment with anti-hypertensive medication. Participants were divided into "High" (SBP $\geq$ 130 mmHg) and "Low" (SBP $\leq$ 120 mmHg) blood pressure groups for analysis. Additional participant characteristic data are listed in Table 1.

Blood Pressure Measurement

Blood pressure was measured in the morning hours after an overnight fast. Systolic and diastolic brachial pressures were collected using an automated machine (Dinamap XL). Three measurements with 5 mmHg of each other were averaged.

Blood Collection

Venous blood samples were collected from participants for whole blood RNA isolation in the morning after an overnight fast.

Chemokine and Inflammatory Protein Analysis

Data from 32 participants (15 Low/17 High) was used for group comparisons of circulating chemokines and inflammatory protein analysis. Plasma samples were analyzed for chemokines and inflammatory proteins using high-sensitivity digital enzyme-linked immunoassays (Simoa platform ELISAs) at the Human Immune Monitoring Share Resource Core Facility at the University of Colorado School of Medicine. Chemokines selected for analysis included eotaxin, eotaixn-3, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), monocyte chemoattractant protein 4 (MCP-4), macrophage-derived chemokine (MDC), macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein 1 beta (MIP-1β), and thymus and activation-regulated chemokine (TARC). C-reactive protein (CRP) and serum amyloid alpha (SAA) were also measured.

Bioinformatics Analysis

Whole venous blood was stored in blood tubes containing RNA-stabilizing reagent (PAXgene tubes, BD Biosciences) and kept at room temperature (~20°C) for 2 h. Blood samples were then stored at -80°C until removal for analysis. RNA was isolated using PAXgene-specific RNA spin column isolation kits (Qiagen) according to manufacturer's instructions and quality control tested on TapeStation bioanalyzer (Agilent). All samples had RNA integrity scores of 8-10. RNA recovered from whole blood includes RNA from red blood cells, peripheral blood mononuclear cells, other immune cells, and plasma. This RNA was then globin-depleted and used to generate ribo-depleted sequencing libraries using Tecan total RNA kits. Sequencing was

performed at a core facility (University of Colorado Anschutz Medical Campus Genomics Shared Resource Facility) on an Illumina NovaSeq 6000 sequencer to yield >40M paired end reads per sample. Raw FASTQ files were quality filtered with the fastp program and aligned to the hg38 genome using the STAR aligner to generate gene expression counts.

Differential gene expression was assessed using DESeq2 to compare the High and Low groups. All over- and under-expressed genes were assessed for gene ontology (GO) enrichment using the GOrilla web-based program (Eden et al., 2009). Single-ranked lists were entered into the program and a false-discovery rate (FDR) <0.1 was used to select significant terminal biological processes (nodes) associated with over- and under-expressed genes. Finally, a weighted gene correlation network analysis (WGCNA) was also performed to identify gene clusters/modules that were most associated with blood pressure and inflammatory markers as previously described (Wahl et al., 2021). For WGCNA, a minimum module size of 50 was used (to capture discrete gene clusters related to blood pressure), and GO analyses of genes in the WGCNA modules were performed using the GOrilla algorithm as described above.

Statistics

Participant characteristics, circulating chemokines and inflammatory protein values were compared with unpaired *t*-tests. Pearson correlations (GraphPad Prism) were generated to determine the association between circulating chemokines and SBP. Differential gene expression was analyzed using the DESeq2 package from Bioconductor in R-studio. A generalized regression model was run using JMP software to determine whether circulating genes were predictive of SBP. LASSO shrinkage was used for the regression.

RESULTS

Both SBP and diastolic blood pressure (DBP) were greater in the High compared to Low group. There were no other differences between groups. Participant characteristics are listed in Table 4.1.

Table 4.1.
Participant Characteristics. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BMI, body mass index. Data are Avg ± SEM.

	Low	High	*P*-value
n (sex)	15 (12F/3M)	17 (11F/6M)	-
Age, y	67 ± 2	70 ± 1	0.113
SBP (range), mmHg	111 ± 2 (95 – 118)	137 ± 1 (130 – 150)	**<0.001**
DBP (range), mmHg	66 ± 2 (53 – 75)	79 ± 2 (64 – 90)	**<0.001**
HR, bts/min	67 ± 3	63 ± 2	0.257
Height, cm	166.0 ± 2.6	165.6 ± 2.3	0.911
Mass, kg	73 ± 4	74 ± 3	0.811
BMI, kg/m^2	26.5 ± 1.2	26.9 ± 1.0	0.787

Chemokine and Inflammatory Protein Analysis

Overall, no difference was observed in any chemokine between High and Low participants. However, a trend (*P*=0.061) was observed for greater levels of circulating eotaxin in the High group. Group comparisons of chemokine concentrations are shown in Figure 4.1. Inflammatory proteins CRP and SAA were also not different between the High and Low groups (CRP: 1.66 ± 0.35 vs 2.04 ± 0.53 mg/l, *P*=0.54; SAA: 3.87 ± 0.46 vs 5.14 ± 2.70 mg/l, *P*=0.62). Despite the lack of marked group differences in chemokines, we observed a positive association between eotaxin and SBP (r=0.38, *P*=0.033), MDC and SBP (r=0.40, *P*=0.023), and MIP-1α and SBP (r=0.48, *P*=0.021). Additional correlations are shown in Figure 4.2.

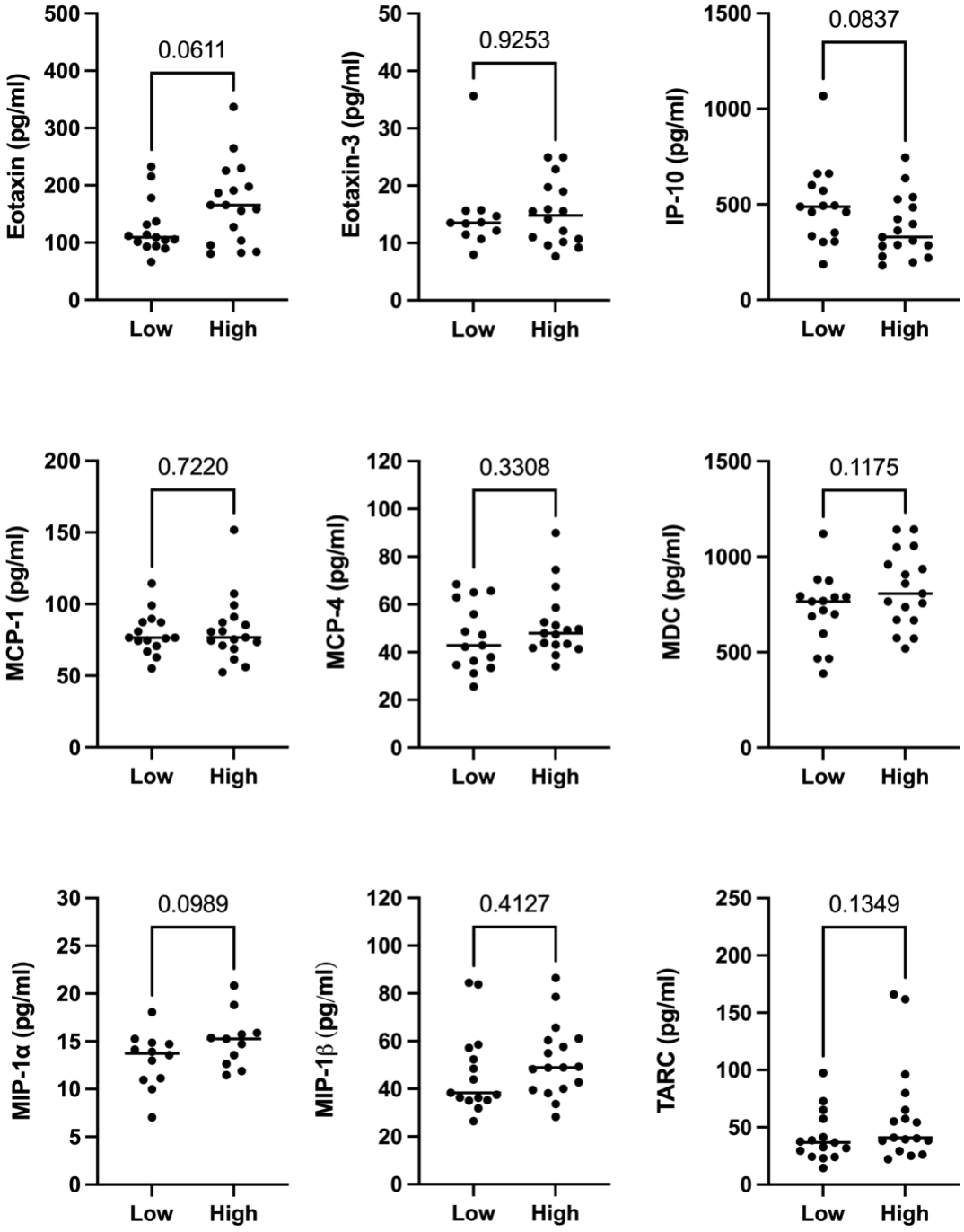


Figure 4.1.
Circulating chemokines in older adults with low (SBP <120 mmHg) and high blood pressure (SBP >130 mmHg). SBP, systolic blood pressure, IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MCP-4, monocyte chemoattractant protein 4; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1 alpha; MIP-1β, macrophage inflammatory protein 1 beta; TARC, thymus and activation-regulated chemokine.

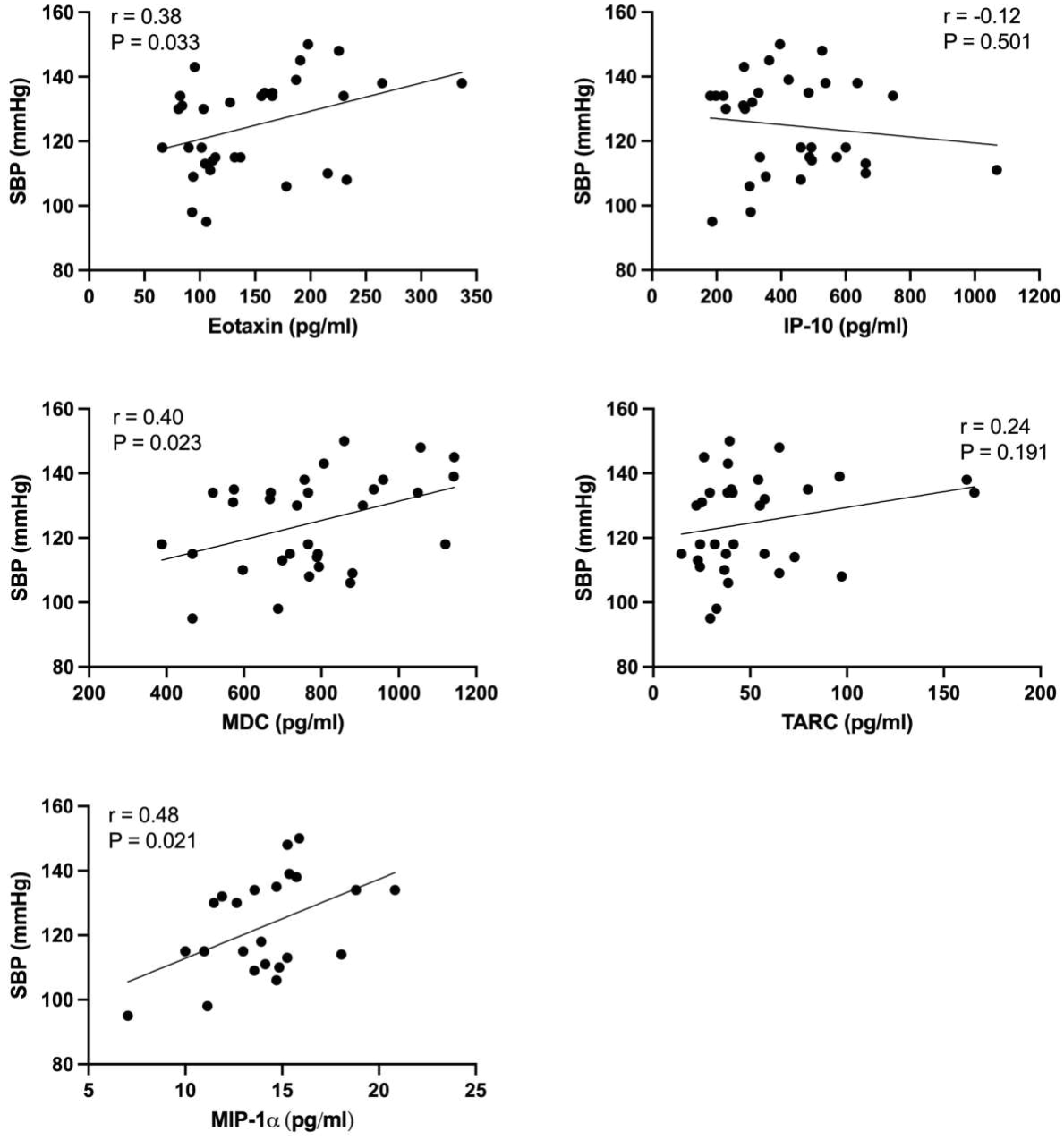


Figure 4.2.
Correlations between select chemokines and SBP in Low and High participants. SBP, systolic blood pressure, IP-10, interferon gamma-induced protein 10; MDC, macrophage-derived chemokine; MIP-1α, macrophage inflammatory protein 1 alpha; TARC, thymus and activation-regulated chemokine.

Bioinformatics: Differential Gene Expression Analyses

A subset of participants from each group were chosen for differential gene expression analysis. For this analysis, five participants from each group were carefully matched for clinical traits (Table 4.2) to reduce the influence of group differences on gene expression. However, no genes were significantly over- or under-expressed in the High vs Low group (Fig. 4.3). Collectively, neither the non-significant over- nor under-expressed genes were predictive of blood pressure.

Table 4.2.
Participant Characteristics for the bioinformatics analysis. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BMI, body mass index. Data are Avg ± SEM.

	Low	High	*P*-value
n (sex)	5 (3F/2M)	5 (3F/2M)	-
Age, y	68 ± 1	68 ± 2	1.000
SBP (range), mmHg	112 ± 2 (106 – 118)	141 ± 3 (131 – 150)	**<0.001**
DBP (range), mmHg	65 ± 4 (53 – 75)	83 ± 6 (64 – 95)	**0.026**
HR, bts/min	64 ± 5	61 ± 4	0.568
Height, cm	171.6 ± 4.5	169.3 ± 3.0	0.688
Mass, kg	84 ± 5	77 ± 7	0.408
BMI, kg/m^2	28.7 ± 1.9	26.6 ± 1.6	0.421

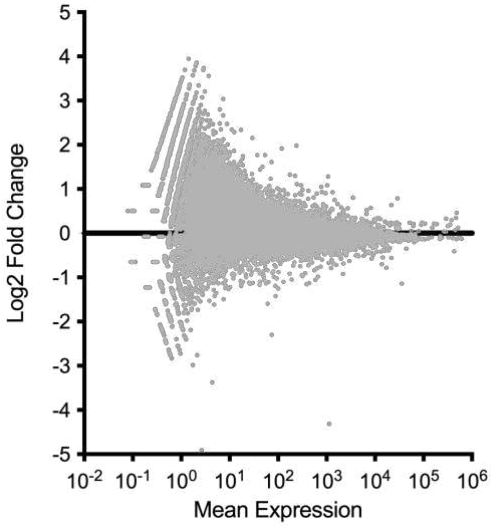


Figure 4.3.
Gene expression profile of High vs Low SBP groups. Relative gene expression shown using MA plots (M, log ratio; mean average). Each data point represents a single gene. "Mean Expression" indicates the average transcript abundance across both groups, and log2 fold change indicates relative abundance in the High vs Low group. Statistically significantly over- or under-represented genes are shown in red (FDR<0.1).

All genes were assessed for GO enrichment, yielding 10 specific biological processes (terminal GO nodes) associated with the over-expressed genes and 41 processes related to the under-expressed genes. Interestingly, the top increased biological processes (Fig. 4.4A) included detection of a chemical stimulus involved in a sensory perception of smell, gene silencing by miRNA, and G protein-coupled signaling pathway. Other increased processes included regulation of vascular smooth muscle cell migration and positive regulation of epinephrine secretion. Top decreased processes (Fig. 4.4B) also included gene silencing by miRNA and detection of chemical stimulus involved in sensory perception of smell, and G protein-coupled signaling pathways. Additional decreased processes included negative regulation of vascular endothelial growth factor production and chemokine-mediated signaling pathways.

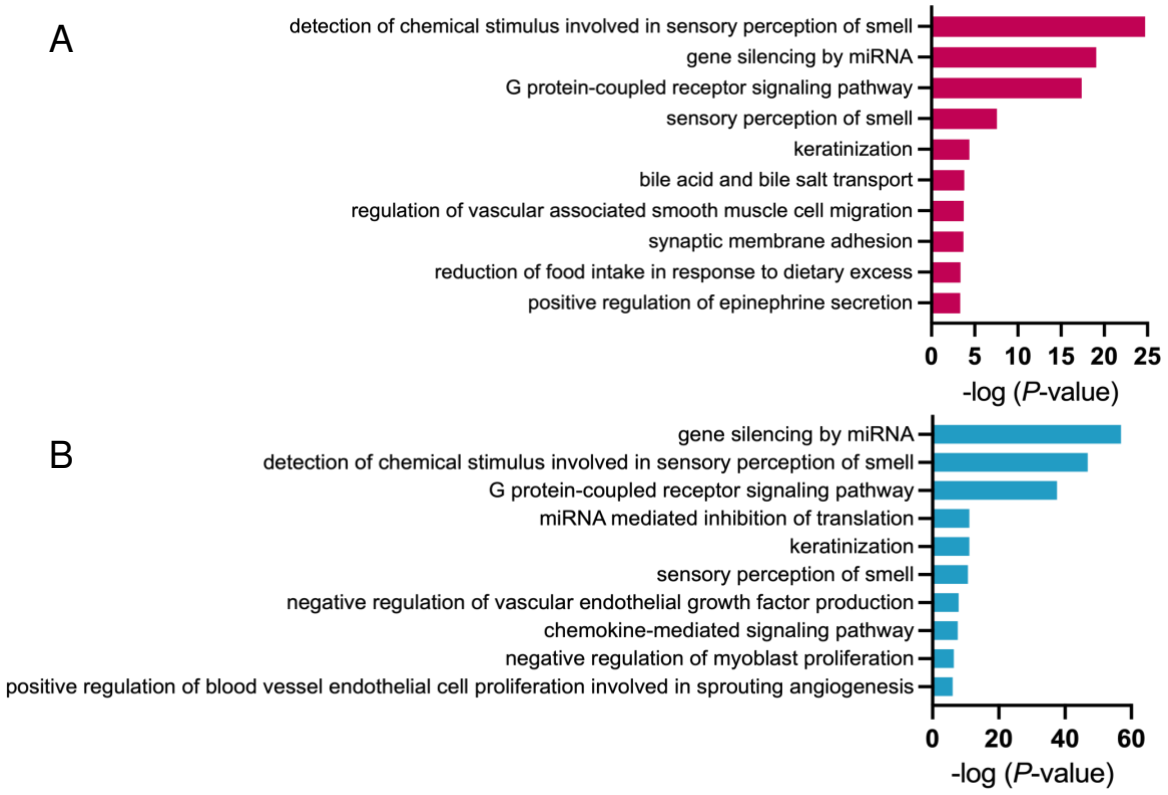
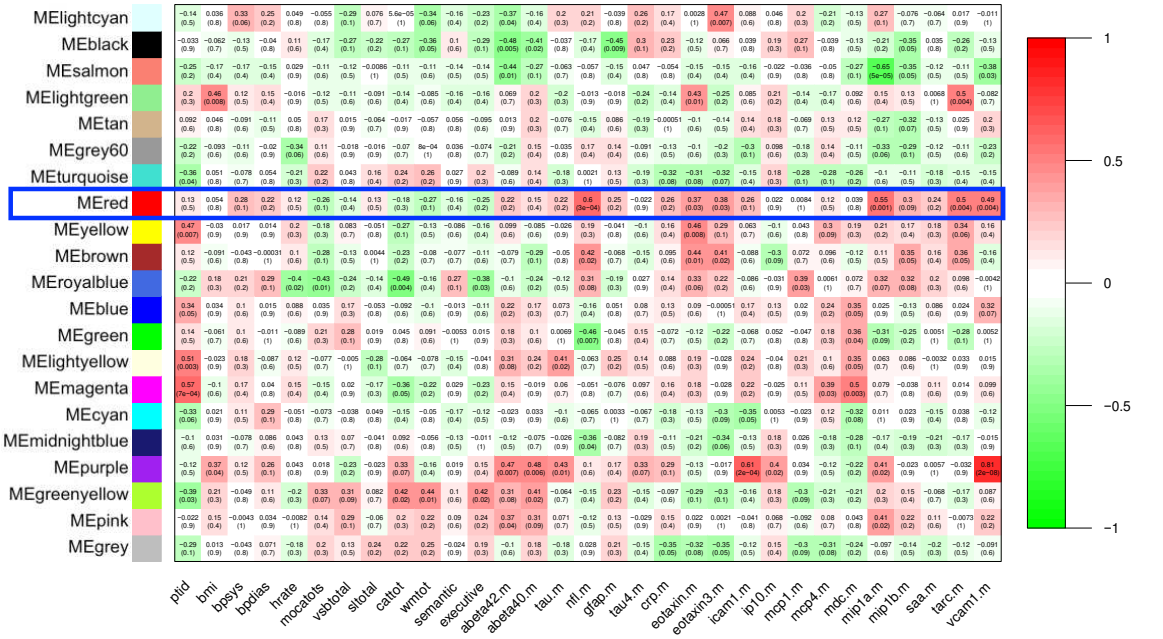


Figure 4.4
Increased (A) and decreased (B) biological processes in High vs. Low SBP subjects determined from gene ontology (GO) enrichment.

Bioinformatics: Network Analysis

To determine if gene expression differences might track with blood pressure (rather than being differentially expressed with High vs. Low SBP) we conducted an unbiased network analysis to identify clusters (modules) of genes related to SBP, DBP, and other clinical traits. Using the WGCNA program and normalized gene counts, as well as all available clinical data and inflammatory markers, we identified several gene modules related to a variety of traits (Fig. 5.5). Although none of these modules were especially related to blood pressure, one (red) was positively related to numerous inflammatory markers, including eotaxins and several cellular adhesion molecules. GO analysis of genes in this module identified multiple biological processes related to inflammation, marked by numerous GO terms including "leukocyte activation", "immune response" and "cytokine production".

Figure 4.5.



Gene clusters associated with blood pressure, clinical traits and inflammatory markers. Heatmap showing results of a Weighted Gene Correlation Network Analysis (WGCNA) to identify clusters of genes (eigen-modules, ME) related to clinical variables (including blood pressure) and ELISA-based markers of inflammation in all subjects. Gene modules are indicated by arbitrary color names. Pearson's r-values are listed in each heatmap block with *P*-values in parentheses below.

DISCUSSION

The purpose of this study was to examine circulating inflammatory markers and gene expression in the context of elevated blood pressure in older adults. To our knowledge, this is the first study to look at both an extensive array of chemokines and whole transcriptome gene expression in healthy older adults with elevated and normal blood pressure. Due to the established role of inflammation in hypertension pathogenesis, we hypothesized that circulating chemokines would be higher in older adults with elevated blood pressure. Contrary to our hypothesis, we found no significant differences in circulating chemokines between the High and Low group when examining nine different chemokines. Despite this lack of differences, we found several positive correlations between SBP and individual chemokines. Additionally, we sought to determine if a concomitant changes in the circulating, whole-blood transcriptome accompanied elevated blood pressure. Although we found no statistically significant over- or under-expressed genes in the subset of samples used for differential gene expression analysis, we did find a cluster of genes related to leukocyte activation and cytokine signaling that tracked with SBP and a number of immune/inflammatory markers. Taken together, these data suggest that wide-spread inflammation does not clearly explain elevated blood pressure in healthy older adults, but rather the contribution of inflammation to elevated blood pressure with aging is more nuanced.

Chemokines are a subtype of cytokines that are vital to promoting chemotaxis. This characteristic makes chemokines key mediators of immune cell infiltration into target tissues. In the context of hypertension, immune cell infiltration has been proposed as a fundamental process that interferes with normal physiological function in tissues such as the brain, kidney, and vasculature with the ultimate consequence of promoting dysfunction that leads to hypertension (Mikolajczyk et al., 2021). For instance, a prolonged angiotensin II infusion leads to increased T

cell accumulation, TNF- α, and chemokine RANTES in the vascular wall in a rodent model of hypertension (Guzik et al., 2007). Thus, hypertensive stimuli can trigger immune responses that increase chemokine signaling that promotes further inflammation and hypertension. Currently, the directional and temporal aspects of these processes are not well understood.

Given this significance of chemokines, we were interested in determining whether these immune signals were present in healthy older adults with elevated blood pressure. Previous work in humans has primarily focused on cytokines and inflammatory proteins like TNF- α, IL-6, and CRP. Furthermore, most of the work examining chemokines has been limited to animal models of hypertension or studies using hypertensive adults with other comorbidities such as diabetes, which is also characterized by chronic systemic inflammation (Antonelli et al., 2012; Guzik et al., 2007; Norlander et al., 2018; Rabkin et al., 2013). In the current study, we were somewhat surprised to find that circulating chemokines were not different between the High and Low groups (although some trends were observed). Nonetheless, we did observe moderate correlations between SBP and eotaxin, MDC, and MIP-1α. Eotaxin stimulates eosinophil trafficking and has long been recognized as a chemokine involved in allergic responses including asthma and allergic rhinitis (Elsner, Escher, & Forssmann, 2004; Matthews et al., 1998). Eotaxin has also been associated with coronary artery disease severity, indicating its potential role in vascular inflammation in humans (Emanuele et al., 2006). Additional studies are needed to elucidate the role of eotaxin in essential hypertension.

Macrophage activity is central to cardiovascular inflammation and dysregulation. In obesity and diabetes, macrophages take on a pro-inflammatory phenotype (M1) that leads to increased cytokine release and conditions such as vascular dysfunction (Hotamisligil, 2017; Taube, Schlich, Sell, Eckardt, & Eckel, 2012). Not surprisingly, we found a positive association

between SBP and MDC and SBP and MIP-1α. Previous work has demonstrated the anti-hypertensive effect of reducing macrophage presence following renal injury in experimental hypertension (Huang et al., 2018). Interestingly, one study in an experimental model of hypertension demonstrated greater M2 macrophage (repair and maintenance state) accumulation in the vascular wall during after 14 days of angiotensin II infusion compared to M1 presence after the first seven days (Moore et al., 2015). Therefore, it remains unclear what specific cellular conditions in vivo promote a restorative vs damaging macrophage response. Considering the temporal aspect of the phenotypic switching in the study mentioned above, macrophage type may vary depending on the duration of hypertensive stimuli. Future studies are needed to determine the interaction between macrophage subtype and macrophage chemokine release in healthy older adults with elevated blood pressure.

We chose to assess circulating levels of CRP and SAA as these are well-established markers of systemic inflammation. Indeed, both molecules are acute-phase proteins that respond to inflammatory stimuli and are markedly increased during on-going infections and in the presence of chronic disease like atherosclerosis (Ansar & Ghosh, 2013; Bisoendial et al., 2010). Previous investigations have demonstrated a positive correlation between CRP and SBP in hypertensive patients and increased levels of CRP and SAA in hypertensive compared to normotensive individuals (Bautista et al., 2005; Rabkin et al., 2013). In the present study, neither protein was elevated in the High compared to the Low group. These data are in support of our chemokine/blood pressure findings, which did not indicate widespread inflammation in the High group. Additionally, other investigations have included hypertensive participants with comorbidities (diabetes, dyslipidemia) and risk factors (smoking) that likely contribute to the enhanced expression of inflammatory proteins (Bautista et al., 2005; Rabkin et al., 2013).

The choice of biological sample for transcriptomic evaluation is vital because genetic profiles vary by tissue type (Basu et al., 2017). In the current study, we chose to use whole blood since it is a tissue that traverses the entire cardiovascular system and perfuses numerous organs such as the vasculature, kidneys, and brain that are integral to hypertension pathogenesis. Often, PBMCs are used for RNA analyses to establish transcriptomic dysregulation in disease states (Marques et al., 2015). This approach is physiologically relevant because various leukocytes such as T and B cells that are included in PBMC analyses have been shown to participate in the development of hypertension (Norlander et al., 2018). However, additional immune cells including neutrophils that are not captured by PBMC isolation have also been associated with hypertension in humans and rodents (Jhuang et al., 2019; Nosalski et al., 2017). Indeed, a recent investigation comparing gene expression in PBMCs and whole blood in participants with asthma found more differentially expressed transcripts in whole blood compared to PBMCs when assessing transcriptomic differences in asthmatic and non-asthmatic participants (He et al., 2019). Additionally, collection of whole blood for transcriptomic analysis has the added benefits of maintained RNA stability at room temperature and requires no processing steps before the samples are stored in the freezer (Rainen et al., 2002). Therefore, this approach provides a promising technique for clinical settings.

In the current study, we did not observe any significantly over- or under-expressed genes in the High compared to Low group. Nonetheless, GO enrichment analyses yielded numerous increased and decreased biological processes. Increased processes included vascular smooth muscle changes that are influenced by hypertensive stimuli and promote cardiovascular disease (Najjar et al., 2005). We also observed increased positive regulation of epinephrine secretion, which supports the well-established role of enhanced sympathetic nervous system activity in

hypertension (Floras, 1992; Grassi, Mark, & Esler, 2015). Additionally, GO enrichment showed a decrease in chemokine-mediated signaling pathways. Interestingly, we also saw a nonsignificant decrease in circulating IP-10, consistent with the RNA enrichment analysis. These findings contrast previous studies that have shown IP-10 to be elevated in hypertension (Antonelli et al., 2012, 2008; Stumpf et al., 2011). It is unclear why our results disagree with these reports; however, comorbidities and other cardiovascular risk factors in some participants in these studies may have enhanced IP-10 concentrations.

Despite the modest group-by-group differences described above, we did find several gene modules/clusters that correlated (modestly) with a number of inflammatory markers in our network analysis. Indeed, the relationship between patterns of inflammatory gene expression (red module) and chemokines (eotaxin, MIP-1α, TARC) in High participants paralleled the trending differences in circulating chemokines between groups. The network analysis also revealed a moderate relationship between the red module and vascular inflammatory marker vascular adhesion molecule 1 (VCAM-1). A strong body of literature supports both an increase in inflammation (marked by increased expression pro-inflammatory proteins like adhesion molecules) and blood pressure with age (Assar et al., 2016; Buford, 2016; Norlander et al., 2018). Our data are consistent with these reports and provide new evidence to support the idea that changes in immune function at the transcriptome level accompany elevated blood pressure in a group of older adults, and that these changes correspond with inflammatory proteins linked with SBP. However, we did not find a strong, direct relationship between these transcriptome changes in immune function and SBP, suggesting (again) that the contribution of immune dysregulation/inflammation to elevated SBP in older adults is complex.

Experimental Considerations

Numerous studies suggest that hypertension pathogenesis involves immune and transcriptomic dysregulation in a variety of tissues (Basu et al., 2017; Marques et al., 2011; Norlander et al., 2018). The discovery of immune-mediated increases in sympathetic nervous system demonstrate how remote inflammation can trigger hypertensive stimuli (Oke & Tracey, 2009). Indeed, it is possible that local tissue inflammation (e.g., vascular, adrenal, renal, adipose, etc.) that we could not detect from blood samples contributes to increased blood pressure in healthy older adults. Similarly, gene expression is not uniform throughout the body. Recently, whole blood transcriptomics was used to predict gene expression patterns in more than 30 different tissues (Basu, Wang, Ruppin, & Hannenhalli, 2021). This study found whole blood transcriptomics could predict 60% of genes in various tissues including arterial, adrenal, and brain tissue. While these are exciting findings, these results reflect substantial heterogeneity in local gene expression patterns. Therefore, our analysis of whole blood transcriptomics may have missed transcriptomic dysregulation in multiple tissue types (Basu et al., 2017).

Perspectives

The process of aging itself is associated with both inflammation and immunosenescence (reduced immune function). This may have created a challenge when comparing the extent of inflammation between older High and Low participants. Indeed, we did not find a group difference in circulating inflammatory proteins CRP and SAA. Interestingly, both High and Low groups had CRP concentrations that represent intermediate risk of cardiovascular disease (Ansar & Ghosh, 2013). In other words, both groups had a basal level of inflammation. These data support the notion of age-related inflammation independent of hypertensive status. Previous studies that have examined age-related differences in chemokines such as eotaxin have found

that concentrations are elevated in middle-aged compared to younger adults (Hoefer et al., 2017). We did observe a nonsignificant trend for elevated eotaxin in the High vs Low group, a positive correlation between eotaxin and SBP, and our network analysis showed increased gene expression related to eotaxin in the High group. However, the high basal level of eotaxin in both groups may have minimized our ability to detect group differences in the chemokine. Nonetheless, it is possible that there is an interaction between aging and circulating eoxtaxin that influences hypertension pathogenesis older adults.

Increased inflammation accompanies aging and has been extensively studied as a contributor to experimental hypertension (Assar et al., 2016; Norlander et al., 2018). Furthermore, most older adults have hypertension (Benjamin et al., 2018). Transcriptomic dysregulation has also been observed in the context of increased inflammation and hypertension (Basu et al., 2017; Huan et al., 2015; Lin et al., 2017; Marques et al., 2011; Zeller et al., 2017). Therefore, there is little doubt that both immune and transcriptomic data contribute to hypertension. However, collectively, our data do not support the notion that inflammation *per se* drives hypertension in healthy older adults. Rather, our data suggest that inflammation may play a modest role, and that other hypertensive stimuli may precede widespread circulating immune and transcriptomic dysregulation that further increases blood pressure in hypertensive older adults.

CHAPTER 5 – PERSPECTIVES & CONCLUSIONS

The work highlighted in this dissertation provides novel insights into contributors of elevated blood pressure in older adults. In Study 1, we demonstrate, for the first time, that systemic Rho-kinase inhibition leads to *sustained* decreases in blood pressure in older but not young adults. This reduction in blood pressure was concomitant with reduced vascular resistance in older adults. Interestingly, we did not find that Rho-kinase inhibition altered endothelial function in older adults. These data indicate that enhanced Rho-kinase activity may be present in the vascular smooth muscle of older adults, promoting increased vascular tone and elevated blood pressure. In Study 2, we investigated *unestablished* mechanisms that contribute to high blood pressure in older adults and found a unique gene expression pattern in peripheral blood cells of individuals with high blood pressure. This pattern was remarkably similar in two different data sets. We also found that the differential gene expression in participants with high SBP was related to increased and decreased relevant biological functions that impact vascular regulation and immune function. In Study 3, we further investigated the role of altered immune function in healthy older adults with high blood pressure. The results of this study demonstrated that several chemokines were positively correlated with SBP in older adults in the absence of other chronic diseases. We also found a positive relationship between genes that influence immune function and circulating chemokines in older adults. Overall, these studies indicate that some known contributors to *experimental* hypertension may have a causal role in elevating blood pressure in healthy older adults (Rho-kinase), while other novel factors (circulating RNA and immune function) have a less clearly defined impact on blood pressure in this population.

Impact on human health

The risk of developing hypertension over the lifespan is 90% for middle-aged adults (Vasan et al., 2002). This risk emphasizes the need to understand mechanisms that contribute to age-associated hypertension and to find biomarkers to predicted rises in blood pressure. Previous work has demonstrated the efficacy of fasudil in improving coronary artery dilation in patients with angina and endothelial function in patients with coronary artery disease (Nohria et al., 2006; Otsuka et al., 2008). Additionally, treatment with fasuidl has also been shown to improve neurological outcomes in acute stroke patients (Shibuya et al., 2005). Considering hypertension leads to elevated risk of cardio- and cerebrovascular diseases, chronic fasudil treatment in healthy older adults may promote reductions in blood pressure and cardiovascular morbidity and mortality. Large-scale clinical trials are needed to determine the efficacy and dosage of fasudil for hypertension management in older adults.

Transcriptomics using RNA-seq is an exciting new technology for identifying novel therapeutic targets and biomarkers of hypertension. However, some obvious considerations are the relative ease of measuring blood pressure itself compared to RNA-seq and the cost associated with sequencing. Thus, it would be more reasonable to evaluate blood pressure directly than to assess gene expression to diagnose hypertension. In recent years, the cost of RNA-seq has dramatically declined. Therefore, this technology is becoming more affordable to implement in in clinical studies. Future research should utilize RNA-seq to determine whether transcriptomic dysregulation precedes hypertension in older adults. Circulating transcripts in this context represent potential biomarkers for future hypertension.

Deterioration of immune function is a major focus in understanding age-related diseases. However, the common link of inflammation among many diseases creates challenges for

determining a causal role in hypertension in presence of other chronic diseases. The scientific literature is filled with examples of inflammation and hypertension corresponding with age, but these studies also tend to include individuals with comorbidities (Antonelli et al., 2012; Rabkin et al., 2013; Ridker et al., 2017). Currently, irrefutable evidence showing inflammation causes hypertension in humans is lacking (Buford, 2016). The work reported in this dissertation supports a modest involvement of inflammation (specifically chemokines) in healthy older adults with *established* hypertension. Nonetheless, future studies will need to investigate how changes in immune function affect the initiation of hypertensive processes. Overall, these findings provide the basis for future novel treatments of hypertension in older adults.

REFERENCES

Ansar, W., & Ghosh, S. (2013). C-reactive protein and the biology of disease. *Immunologic Research*, *56*(1), 131–142. https://doi.org/10.1007/s12026-013-8384-0

Antonelli, A., Fallahi, P., Ferrari, S. M., Ghiadoni, L., Virdis, A., Mancusi, C., … Ferrannini, E. (2012). High serum levels of CXC (CXCL10) and CC (CCL2) chemokines in untreated essential hypertension. *International Journal of Immunopathology and Pharmacology*, *25*(2), 387–395. https://doi.org/10.1177/039463201202500208

Antonelli, A., Fallahi, P., Rotondi, M., Ferrari, S. M., Romagnani, P., Ghiadoni, L., … Ferrannini, E. (2008). High serum levels of CXC chemokine ligand 10 in untreated essential hypertension. *Journal of Human Hypertension*, *22*(8), 579–581. https://doi.org/10.1038/jhh.2008.15

Assar, M. El, Angulo, J., & Rodríguez-Mañas, L. (2016). Diabetes and ageing-induced vascular inflammation. *Journal of Physiology*, *594*(8), 2125–2146. https://doi.org/10.1113/JP270841

Ba, D., Takeichi, N., Kodama, T., & Kobayashi, H. (1982). Restoration of T cell depression and suppression of blood pressure in spontaneously hypertensive rats (SHR) by thymus grafts or thymus extracts. *Journal of Immunology*, *128*(3), 1211–1216. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6120194

Basu, M., Sharmin, M., Das, A., Nair, N. U., Wang, K., Lee, J. S., … Hannenhalli, S. (2017). Prediction and Subtyping of Hypertension from Pan-Tissue Transcriptomic and Genetic Analyses. *Gentics*, *207*(November), 1121–1134. https://doi.org/10.1534/genetics.117.300280/-/DC1.1

Basu, M., Wang, K., Ruppin, E., & Hannenhalli, S. (2021). Predicting tissue-specific gene expression from whole blood transcriptome. *Science Advances*, *7*(14), 1–8. https://doi.org/10.1126/sciadv.abd6991

Bautista, L. E., Vera, L. M., Arenas, I. A., & Gamarra, G. (2005). Independent association between inflammatory markers (C-reactive protein, interleukin-6, and TNF-α) and essential hypertension. *Journal of Human Hypertension*, *19*(2), 149–154. https://doi.org/10.1038/sj.jhh.1001785

Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., … Muntner, P. (2018). Heart disease and stroke statistics - 2018 update: A report from the American Heart Association. In *Circulation* (Vol. 137). https://doi.org/10.1161/CIR.0000000000000558

Bisoendial, R. J., Boekholdt, S. M., Vergeer, M., Stroes, E. S. G., & Kastelein, J. J. P. (2010). C-reactive protein is a mediator of cardiovascular disease. *European Heart Journal*, *31*(17), 2087–2095. https://doi.org/10.1093/eurheartj/ehq238

Boutouyrie, P., Chowienczyk, P., Humphrey, J. D., & Mitchell, G. F. (2021). Arterial Stiffness and Cardiovascular Risk in Hypertension. *Circulation Research*, 864–886. https://doi.org/10.1161/CIRCRESAHA.121.318061

Brown, I. A. M., Diederich, L., Good, M. E., DeLalio, L. J., Murphy, S. A., Cortese-Krott, M. M., … Isakson, B. E. (2018). Vascular smooth muscle remodeling in conductive and resistance arteries in hypertension. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *38*(9), 1969–1985. https://doi.org/10.1161/ATVBAHA.118.311229

Buford, T. W. (2016). Hypertension and aging. *Ageing Research Reviews*, *26*, 96–111. https://doi.org/10.1016/j.arr.2016.01.007

Büssemaker, E., Pistrosch, F., Förster, S., Herbrig, K., Gross, P., Passauer, J., & Brandes, R. P. (2007). Rho kinase contributes to basal vascular tone in humans: Role of endothelium-derived nitric oxide. *American Journal of Physiology - Heart and Circulatory Physiology*, *293*(1), 541–547. https://doi.org/10.1152/ajpheart.00770.2006

Calhoun, D. A., Jones, D., Textor, S., Goff, D. C., Murphy, T. P., Toto, R. D., … Carey, R. M. (2008). Resistant hypertension: Siagnosis, evaluation, and treatment a scientific statement from the american heart association professional education committee of the council for high blood pressure research. *Hypertension*, *51*(6), 1403–1419. https://doi.org/10.1161/HYPERTENSIONAHA.108.189141

Celermajer, D. S., Sorensen, K. E., Spiegelhalter, D. J., Georgakopoulos, D., Robinson, J., & Deanfield, J. E. (1994). Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *Journal of the American College of Cardiology*, *24*(2), 471–476. https://doi.org/10.1016/0735-1097(94)90305-0

Chen, C., Wu, N., Duan, Q., Yang, H., Wang, X., Yang, P., … Zheng, Y. (2018). C10orf99 contributes to the development of psoriasis by promoting the proliferation of keratinocytes. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-26996-z

Chen, X. H., Ruan, C. C., Ge, Q., Ma, Y., Xu, J. Z., Zhang, Z. B., … Gao, P. J. (2018). Deficiency of Complement C3a and C5a Receptors Prevents Angiotensin II-Induced Hypertension via Regulatory T Cells. *Circulation Research*, *122*(7), 970–983. https://doi.org/10.1161/CIRCRESAHA.117.312153

Cohuet, G., & Struijker-Boudier, H. (2006). Mechanisms of target organ damage caused by hypertension: Therapeutic potential. *Pharmacology and Therapeutics*, *111*(1), 81–98. https://doi.org/10.1016/j.pharmthera.2005.09.002

Covic, A., Goldsmith, D. J. A., Panaghiu, L., Covic, M., & Sedor, J. (2000). Analysis of the effect of hemodialysis on peripheral and central arterial pressure waveforms. *Kidney International*, *57*(6), 2634–2643. https://doi.org/10.1046/j.1523-1755.2000.00124.x

Donato, A. J., Gano, L. B., Eskurza, I., Silver, A. E., Gates, P. E., Jablonski, K., & Seals, D. R. (2009). Vascular endothelial dysfunction with aging : endothelin-1 and endothelial nitric oxide synthase. *American Journal of Physiology - Heart and Circulatory Physiology*, *297*(1), H425–H432. https://doi.org/10.1152/ajpheart.00689.2008.

Eden, E., Navon, R., Steinfeld, I., Lipson, D., & Yakhini, Z. (2009). GOrilla: A tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics*, *10*, 1–7. https://doi.org/10.1186/1471-2105-10-48

Elsner, J., Escher, S. E., & Forssmann, U. (2004). Chemokine receptor antagonists: A novel therapeutic approach in allergic diseases. *Allergy: European Journal of Allergy and Clinical Immunology*, *59*(12), 1243–1258. https://doi.org/10.1111/j.1398-9995.2004.00710.x

Emanuele, E., Falcone, C., D'Angelo, A., Minoretti, P., Buzzi, M. P., Bertona, M., & Geroldi, D. (2006). Association of plasma eotaxin levels with the presence and extent of angiographic coronary artery disease. *Atherosclerosis*, *186*(1), 140–145. https://doi.org/10.1016/j.atherosclerosis.2005.07.002

Floras, J. S. (1992). Epinephrine and the genesis of hypertension. *Hypertension*, *20*(4), 583. https://doi.org/10.1161/01.HYP.20.4.583

Friese, R. S., Altshuler, A. E., Zhang, K., Miramontes-Gonzalez, J. P., Hightower, C. M., Jirout, M. L., … O'Connor, D. T. (2013). MicroRNA-22 and promoter motif polymorphisms at the Chga locus in genetic hypertension: Functional and therapeutic implications for gene expression and the pathogenesis of hypertension. *Human Molecular Genetics*, *22*(18),

3624–3640. https://doi.org/10.1093/hmg/ddt213
Fukui, S., Fukumoto, Y., Suzuki, J., Saji, K., Nawata, J., Tawara, S., … Shimokawa, H. (2008). Long-term inhibition of Rho-kinase ameliorates diastolic heart failure in hypertensive rats. *Journal of Cardiovascular Pharmacology*, *51*(3), 317–326. https://doi.org/10.1097/FJC.0b013e31816533b7
Grassi, G., Mark, A., & Esler, M. (2015). The Sympathetic Nervous System Alterations in Human Hypertension. *Circulation Research*, *116*(6), 976–990. https://doi.org/10.1161/CIRCRESAHA.116.303604
Green, D. J., Dawson, E. A., Groenewoud, H. M. M., Jones, H., & Thijssen, D. H. J. (2014). Is flow-mediated dilation nitric oxide mediated?: A meta-analysis. *Hypertension*, *63*(2), 376–382. https://doi.org/10.1161/HYPERTENSIONAHA.113.02044
Guo, P., Luo, Y., Mai, G., Zhang, M., Wang, G., Zhao, M., … Zhou, F. (2014). Gene expression profile based classification models of psoriasis. *Genomics*, *103*(1), 48–55. https://doi.org/10.1016/j.ygeno.2013.11.001
Guzik, T. J., Hoch, N. E., Brown, K. A., McCann, L. A., Rahman, A., Dikalov, S., … Harrison, D. G. (2007). Role of the T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. *Journal of Experimental Medicine*, *204*(10), 2449–2460. https://doi.org/10.1084/jem.20070657
Harrison, D. G., Coffman, T. M., & Wilcox, C. S. (2021). Pathophysiology of Hypertension: The Mosaic Theory and beyond. *Circulation Research*, 847–863. https://doi.org/10.1161/CIRCRESAHA.121.318082
He, D., Yang, C. X., Sahin, B., Singh, A., Shannon, C. P., Oliveria, J. P., … Tebbutt, S. J. (2019). Whole blood vs PBMC: Compartmental differences in gene expression profiling exemplified in asthma. *Allergy, Asthma and Clinical Immunology*, *15*(1), 1–10. https://doi.org/10.1186/s13223-019-0382-x
Hoefer, J., Luger, M., Dal-Pont, C., Culig, Z., Schennach, H., & Jochberger, S. (2017). The "aging factor" eotaxin-1 (CCL11) is detectable in transfusion blood products and increases with the donor's age. *Frontiers in Aging Neuroscience*, *9*(DEC), 1–8. https://doi.org/10.3389/fnagi.2017.00402
Hotamisligil, G. S. (2017). Foundations of Immunometabolism and Implications for Metabolic Health and Disease. *Immunity*, *47*(3), 406–420. https://doi.org/10.1016/j.immuni.2017.08.009
Huan, T., Chen, G., Liu, C., Bhattacharya, A., Rong, J., Chen, B. H., … Levy, D. (2018). Age-associated microRNA expression in human peripheral blood is associated with all-cause mortality and age-related traits. *Aging Cell*, *17*(1), 1–10. https://doi.org/10.1111/acel.12687
Huan, T., Esko, T., Peters, M. J., Pilling, L. C., Schramm, K., Schurmann, C., … Boerwinkle, E. (2015). A Meta-analysis of Gene Expression Signatures of Blood Pressure and Hypertension. *PLoS Genetics*, *11*(3), 1–29. https://doi.org/10.1371/journal.pgen.1005035
Huang, L., Wang, A., Hao, Y., Li, W., Liu, C., Yang, Z., … Zhou, M. S. (2018). Macrophage depletion lowered blood pressure and attenuated hypertensive renal injury and fibrosis. *Frontiers in Physiology*, *9*(MAY), 1–11. https://doi.org/10.3389/fphys.2018.00473
Hughes, C. E., & Nibbs, R. J. B. (2018). A guide to chemokines and their receptors. *FEBS Journal*, *285*(16), 2944–2971. https://doi.org/10.1111/febs.14466
Hwang, M. H., Yoo, J. K., Kim, H. K., Hwang, C. L., Mackay, K., Hemstreet, O., … Christou, D. D. (2014). Validity and reliability of aortic pulse wave velocity and augmentation index determined by the new cuff-based SphygmoCor Xcel. *Journal of Human Hypertension*,

28(8), 475–481. https://doi.org/10.1038/jhh.2013.144

Ito, K., Hirooka, Y., Sakai, K., Kishi, T., Kaibuchi, K., Shimokawa, H., & Takeshita, A. (2003). Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: Possible involvement in neural mechanisms of hypertension. *Circulation Research*, *92*(12), 1337–1343. https://doi.org/10.1161/01.RES.0000079941.59846.D4

Jhuang, Y. H., Kao, T. W., Peng, T. C., Chen, W. L., Li, Y. W., Chang, P. K., & Wu, L. W. (2019). Neutrophil to lymphocyte ratio as predictor for incident hypertension: a 9-year cohort study in Taiwan. *Hypertension Research*, *42*(8), 1209–1214. https://doi.org/10.1038/s41440-019-0245-3

Julius, S., Nesbitt, S. D., Egan, B. M., Weber, M. A., Michelson, E. L., Kaciroti, N., … Schork, M. A. (2006). Feasibility of Treating Prehypertension with an Angiotensin-Receptor Blocker. *New England Journal of Medicine*, *354*(16), 1685–1697. https://doi.org/10.1056/nejmoa060838

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., … Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho- kinase). *Science*, *273*(5272), 245–248. https://doi.org/10.1126/science.273.5272.245

Koch, J. C., Tatenhorst, L., Roser, A. E., Saal, K. A., Tönges, L., & Lingor, P. (2018). ROCK inhibition in models of neurodegeneration and its potential for clinical translation. *Pharmacology and Therapeutics*, *189*, 1–21. https://doi.org/10.1016/j.pharmthera.2018.03.008

Korkor, M. T., Meng, F. B., Xing, S. Y., Zhang, M. C., Guo, J. R., Zhu, X. X., & Yang, P. (2011). Microarray analysis of differential gene expression profile in peripheral blood cells of patients with human essential hypertension. *International Journal of Medical Sciences*, *8*(2), 168–179. https://doi.org/10.7150/ijms.8.168

Krum, H., Viskoper, R., Lacourciere, Y., Budde, M., & Charlon, V. (1998). The effect of an enodthelin-receptor antagonist, Bosentan, on blood pressure in patients with essential hypertension. *New England Journal of Medicine*, *388*(12), 784–790.

Kureishi, Y., Kobayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., … Ito, M. (1997). Rho-associated Kinase Directly Induces Smooth Muscle Contraction through Myosin Light Chain Phosphorylation. *The Journal of Biological Chemistry*, *272*(19), 12257–12260.

Lakatta, E. G., & Levy, D. (2003). Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises: Part I: Aging arteries: A “set up” for vascular disease. *Circulation*, *107*(1), 139–146. https://doi.org/10.1161/01.CIR.0000048892.83521.58

Landmesser, U., Hornig, B., & Drexler, H. (2004). Endothelial Function: A Critical Determinant in Atherosclerosis? *Circulation*, *109*(21_suppl_1), II-27-II–33. https://doi.org/10.1161/01.cir.0000129501.88485.1f

LaRocca, T. J., Mariani, A., Watkins, L. R., & Link, C. D. (2019). TDP-43 knockdown causes innate immune activation via protein kinase R in astrocytes. *Neurobiology of Disease*, *132*(January), 104514. https://doi.org/10.1016/j.nbd.2019.104514

Limberg, J. K., Casey, D. P., Trinity, J. D., Nicholson, W. T., Wray, D. W., Tschakovsky, M. E., … Padilla, J. (2020). Assessment of resistance vessel function in human skeletal muscle: guidelines for experimental design, Doppler ultrasound, and pharmacology. *American Journal of Physiology. Heart and Circulatory Physiology*, *318*(2), H301–H325. https://doi.org/10.1152/ajpheart.00649.2019

Lin, H., Lunetta, K. L., Zhao, Q., Rong, J., Benjamin, E. J., Mendelson, M. M., … Murabito, J. M. (2017). Transcriptome-wide association study of inflammatory biologic age. *Aging*, *9*(11), 2288–2301. https://doi.org/10.18632/aging.101321

Lowe, R., Shirley, N., Bleackley, M., Dolan, S., & Shafee, T. (2017). Transcriptomics technologies. *PLoS Computational Biology*, *13*(5), 1–23. https://doi.org/10.1371/journal.pcbi.1005457

Marques, F. Z., Booth, S. A., & Charchar, F. J. (2015). The emerging role of non-coding RNA in essential hypertension and blood pressure regulation. *Journal of Human Hypertension*, *29*(8), 459–467. https://doi.org/10.1038/jhh.2014.99

Marques, F. Z., Campain, A. E., Tomaszewski, M., Zukowska-Szczechowska, E., Yang, Y. H. J., Charchar, F. J., & Morris, B. J. (2011). Gene expression profiling reveals renin mRNA overexpression in human hypertensive kidneys and a role for microRNAs. *Hypertension*, *58*(6), 1093–1098. https://doi.org/10.1161/HYPERTENSIONAHA.111.180729

Masumoto, A., Hirooka, Y., Shimokawa, H., Hironaga, K., Setoguchi, S., & Takeshita, A. (2001). Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension*, *38*(6), 1307–1310. https://doi.org/10.1161/hy1201.096541

Matthews, A. N., Friend, D. S., Zimmermann, N., Sarafi, M. N., Luster, A. D., Pearlman, E., … Rothenberg, M. E. (1998). Eotaxin is required for the baseline level of tissue eosinophils. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11), 6273–6278. https://doi.org/10.1073/pnas.95.11.6273

McEniery, C. M., Cockcroft, J. R., Roman, M. J., Franklin, S. S., & Wilkinson, I. B. (2014). Central blood pressure: Current evidence and clinical importance. *European Heart Journal*, *35*(26), 1719–1725. https://doi.org/10.1093/eurheartj/eht565

McEniery, C. M., Yasmin, McDonnell, B., Munnery, M., Wallace, S. M., Rowe, C. V., … Wilkinson, I. B. (2008). Central pressure: Variability and impact of cardiovascular risk factors the anglo-cardiff collaborative trial II. *Hypertension*, *51*(6), 1476–1482. https://doi.org/10.1161/HYPERTENSIONAHA.107.105445

Mikolajczyk, T. P., Szczepaniak, P., Vidler, F., Maffia, P., Graham, G. J., & Guzik, T. J. (2021). Role of inflammatory chemokines in hypertension. *Pharmacology and Therapeutics*, *223*. https://doi.org/10.1016/j.pharmthera.2020.107799

Ming, X.-F., Viswambharan, H., Barandier, C., Ruffieux, J., Kaibuchi, K., Rusconi, S., & Yang, Z. (2002). Rho GTPase/Rho Kinase Negatively Regulates Endothelial Nitric Oxide Synthase Phosphorylation through the Inhibition of Protein Kinase B/Akt in Human Endothelial Cells. *Molecular and Cellular Biology*, *22*(24), 8467–8477. https://doi.org/10.1128/mcb.22.24.8467-8477.2002

Moore, J. P., Vinh, A., Tuck, K. L., Sakkal, S., Krishnan, S. M., Chan, C. T., … Drummond, G. R. (2015). M2 macrophage accumulation in the aortic wall during angiotensin ii infusion in mice is associated with fibrosis, elastin loss, and elevated blood pressure. *American Journal of Physiology - Heart and Circulatory Physiology*, *309*(5), H906–H917. https://doi.org/10.1152/ajpheart.00821.2014

Mukai, Y., Shimokawa, H., Matoba, T., Kandabashi, T., Satoh, S., Hiroki, J., … Takeshita, A. (2001). Involvement of Rho-kinase in hypertensive vascular disease--a novel therapeutic target in hypertension. *The FASEB Journal*, *15*(6). https://doi.org/10.1096/fsb2fj000735fje

Najjar, S. S., Scuteri, A., & Lakatta, E. G. (2005). Arterial aging: Is it an immutable cardiovascular risk factor? *Hypertension*, *46*(3), 454–462. https://doi.org/10.1161/01.HYP.0000177474.06749.98

Nieves-Cintrón, M., Syed, A. U., Nystoriak, M. A., & Navedo, M. F. (2018). Regulation of voltage-gated potassium channels in vascular smooth muscle during hypertension and metabolic disorders. *Microcirculation*, *25*(1), 1–9. https://doi.org/10.1111/micc.12423

Nohria, A., Grunert, M. E., Rikitake, Y., Noma, K., Prsic, A., Ganz, P., … Creager, M. A. (2006). Rho kinase inhibition improves endothelial function in human subjects with coronary artery disease. *Circulation Research*, *99*(12), 1426–1432. https://doi.org/10.1161/01.RES.0000251668.39526.c7

Noma, K., Goto, C., Nishioka, K., Jitsuiki, D., Umemura, T., Ueda, K., … Higashi, Y. (2007). Roles of Rho-Associated Kinase and Oxidative Stress in the Pathogenesis of Aortic Stiffness. *Journal of the American College of Cardiology*, *49*(6), 698–705. https://doi.org/10.1016/j.jacc.2006.06.082

Norlander, A. E., Madhur, M. S., & Harrison, D. G. (2018). The immunology of hypertension. *Journal of Experimental Medicine*, *215*(2), 719. https://doi.org/10.1084/jem.20171773010220l8c

Nosalski, R., McGinnigle, E., Siedlinski, M., & Guzik, T. J. (2017). Novel Immune Mechanisms in Hypertension and Cardiovascular Risk. *Current Cardiovascular Risk Reports*, *11*(4). https://doi.org/10.1007/s12170-017-0537-6

Oke, S. L., & Tracey, K. J. (2009). The inflammatory reflex and the role of complementary and alternative medical therapies. *Annals of the New York Academy of Sciences*, *1172*, 172–180. https://doi.org/10.1196/annals.1393.013

Oparil, S., Zaman, M. A., & Calhoun, D. A. (2003). Pathogenesis of hypertension. *Annals of Internal Medicine*, *139*(9), 761–776. https://doi.org/10.1016/0002-9343(51)90241-0

Otsuka, T., Ibuki, C., Suzuki, T., Ishii, K., Yoshida, H., Kodani, E., … Mizuno, K. (2008). Administration of the Rho-kinase inhibitor, fasudil, following nitroglycerin additionally dilates the site of coronary spasm in patients with vasospastic angina. *Coronary Artery Disease*, *19*(2), 105–110. https://doi.org/10.1097/MCA.0b013e3282f3420c

Park, C. M., Korolkova, O., Davies, J. E., Parker, K. H., Siggers, J. H., March, K., … Hughes, A. D. (2014). Arterial pressure: Agreement between a brachial cuff-based device and radial tonometry. *Journal of Hypertension*, *32*(4), 865–872. https://doi.org/10.1097/HJH.0000000000000082

Parker, B. A., Trehearn, T. L., & Meendering, J. R. (2009). Pick your Poiseuille: normalizing the shear stimulus in studies of flow-mediated dilation. *Journal of Applied Physiology (Bethesda, Md. : 1985)*, *107*(4), 1357–1359. https://doi.org/10.1152/japplphysiol.00769.2009

Picone, D. S., Schultz, M. G., Otahal, P., Aakhus, S., Al-Jumaily, A. M., Black, J. A., … Sharman, J. E. (2017). Accuracy of Cuff-Measured Blood Pressure: Systematic Reviews and Meta-Analyses. *Journal of the American College of Cardiology*, *70*(5), 572–586. https://doi.org/10.1016/j.jacc.2017.05.064

Pierce, G. L., Lesniewski, L. A., Lawson, B. R., Beske, S. D., & Seals, D. R. (2009). Nuclear factor-κB activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle-aged and older humans. *Circulation*, *119*(9), 1284–1292. https://doi.org/10.1161/CIRCULATIONAHA.108.804294

Prospective Studies Collaboration. (2002). Age-specific relevance of blood pressure to cause-specific mortality is best assessed by collaborative meta-analysis of individual participant data from the separate prospective studies. *Lancet*, *360*, 1903–1913. Retrieved from www.thelancet.com1903

Rabkin, S. W., Langer, A., Ur, E., Calciu, C. D., & Leiter, L. A. (2013). Inflammatory biomarkers CRP, MCP-1, serum amyloid alpha and interleukin-18 in patients with HTN and dyslipidemia: Impact of diabetes mellitus on metabolic syndrome and the effect of statin therapy. *Hypertension Research*, *36*(6), 550–558. https://doi.org/10.1038/hr.2012.214

Rainen, L., Oelmueller, U., Jurgensen, S., Wyrich, R., Ballas, C., Schram, J., … Tryon, V. (2002). Stabilization of mRNA expression in whole blood samples. *Clinical Chemistry*, *48*(11), 1883–1890. https://doi.org/10.1093/clinchem/48.11.1883

Ran, L., Zhao, W., Tan, X., Wang, H., Mizuno, K., Takagi, K., … Bu, H. (2020). Association between Serum Vitamin C and the Blood Pressure: A Systematic Review and Meta-Analysis of Observational Studies. *Cardiovascular Therapeutics*, *2020*. https://doi.org/10.1016/j.ctim.2020.102579

Ridker, P. M., Everett, B. M., Thuren, T., MacFadyen, J. G., Chang, W. H., Ballantyne, C., … Glynn, R. J. (2017). Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *New England Journal of Medicine*, *377*(12), 1119–1131. https://doi.org/10.1056/nejmoa1707914

Roman, M. J., Devereux, R. B., Kizer, J. R., Lee, E. T., Galloway, J. M., Ali, T., … Howard, B. V. (2007). Central pressure more strongly relates to vascular disease and outcome than does brachial pressure: The strong heart study. *Hypertension*, *50*(1), 197–203. https://doi.org/10.1161/HYPERTENSIONAHA.107.089078

Rossman, M. J., Santos-Parker, J. R., Steward, C. A. C., Bispham, N. Z., Cuevas, L. M., Rosenberg, H. L., … Seals, D. R. (2018). Chronic Supplementation With a Mitochondrial Antioxidant (MitoQ) Improves Vascular Function in Healthy Older Adults. *Hypertension*, *71*(6), 1056–1063. https://doi.org/10.1161/hypertensionaha.117.10787

Sarver, A. L., Sarver, A. E., Yuan, C., & Subramanian, S. (2018). OMCD: OncomiR Cancer Database. *BMC Cancer*, *18*(1), 1–6. https://doi.org/10.1186/s12885-018-5085-z

Schultz, M. G., Picone, D. S., Armstrong, M. K., Black, J. A., Dwyer, N., Roberts-Thomson, P., & Sharman, J. E. (2020). Validation Study to Determine the Accuracy of Central Blood Pressure Measurement Using the Sphygmocor Xcel Cuff Device. *Hypertension*, *76*(1), 244–250. https://doi.org/10.1161/HYPERTENSIONAHA.120.14916

Seretis, A., Cividini, S., Markozannes, G., Tseretopoulou, X., Lopez, D. S., Ntzani, E. E., & Tsilidis, K. K. (2019). Association between blood pressure and risk of cancer development: a systematic review and meta-analysis of observational studies. *Scientific Reports*, *9*(1), 1–12. https://doi.org/10.1038/s41598-019-45014-4

Shah, K. H., Shi, P., Giani, J. F., Janjulia, T., Bernstein, E. A., Li, Y., … Shen, X. Z. (2015). Myeloid Suppressor Cells Accumulate and Regulate Blood Pressure in Hypertension. *Circulation Research*, *117*(10), 858–869. https://doi.org/10.1161/CIRCRESAHA.115.306539

Shechter, M., Shechter, A., Koren-Morag, N., Feinberg, M. S., & Hiersch, L. (2014). Usefulness of brachial artery flow-mediated dilation to predict long-term cardiovascular events in subjects without heart disease. *American Journal of Cardiology*, *113*(1), 162–167. https://doi.org/10.1016/j.amjcard.2013.08.051

Shibuya, M., Hirai, S., Seto, M., Satoh, S. I., & Ohtomo, E. (2005). Effects of fasudil in acute ischemic stroke: Results of a prospective placebo-controlled double-blind trial. *Journal of the Neurological Sciences*, *238*(1–2), 31–39. https://doi.org/10.1016/j.jns.2005.06.003

Shimokawa, H., Sunamura, S., & Satoh, K. (2016). RhoA/Rho-Kinase in the Cardiovascular System. *Circulation Research*, *118*(2), 352–366.

https://doi.org/10.1161/CIRCRESAHA.115.306532
Soga, J., Noma, K., Hata, T., Hidaka, T., Fujii, Y., Idei, N., … Higashi, Y. (2011). Rho-associated kinase activity, endothelial function, and cardiovascular risk factors. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(10), 2353–2359. https://doi.org/10.1161/ATVBAHA.111.227892
Stewart, A. D., Millasseau, S. C., Kearney, M. T., Ritter, J. M., & Chowienczyk, P. J. (2003). Effects of Inhibition of Basal Nitric Oxide Synthesis on Carotid-Femoral Pulse Wave Velocity and Augmentation Index in Humans. *Hypertension*, *42*(5), 915–918. https://doi.org/10.1161/01.HYP.0000092882.65699.19
Stumpf, C., Auer, C., Yilmaz, A., Lewczuk, P., Klinghammer, L., Schneider, M., … Garlichs, C. D. (2011). Serum levels of the Th1 chemoattractant interferon-gamma-inducible protein (IP) 10 are elevated in patients with essential hypertension. *Hypertension Research*, *34*(4), 484–488. https://doi.org/10.1038/hr.2010.258
Sutton-Tyrrell, K., Najjar, S. S., Boudreau, R. M., Venkitachalam, L., Kupelian, V., Simonsick, E. M., … Newman, A. (2005). Elevated aortic pulse wave velocity, a marker of arterial stiffness, predicts cardiovascular events in well-functioning older adults. *Circulation*, *111*(25), 3384–3390. https://doi.org/10.1161/CIRCULATIONAHA.104.483628
Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A., & Salvetti, A. (2001). Age-Related Reduction of NO Availability and Oxidative Stress in Humans. *Hypertension*, *38*(2), 274–279. https://doi.org/10.1161/01.hyp.38.2.274
Taube, A., Schlich, R., Sell, H., Eckardt, K., & Eckel, J. (2012). Inflammation and metabolic dysfunction: Links to cardiovascular diseases. *American Journal of Physiology - Heart and Circulatory Physiology*, *302*(11), 2148–2165. https://doi.org/10.1152/ajpheart.00907.2011
Terwoord, J. D., Hearon, C. M., Racine, M. L., Ketelhut, N. B., Luckasen, G. J., Richards, J. C., & Dinenno, F. A. (2020). KIR channel activation links local vasodilatation with muscle fibre recruitment during exercise in humans. *Journal of Physiology*, *598*(13), 2621–2636. https://doi.org/10.1113/JP279682
Thijssen, D. H. J., Black, M. A., Pyke, K. E., Padilla, J., Atkinson, G., Harris, R. A., … Green, D. J. (2011). Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *AJP: Heart and Circulatory Physiology*, *300*(1), H2–H12. https://doi.org/10.1152/ajpheart.00471.2010
Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., … Shuh, N. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*, *389*(October).
Vahebi, S., Kobayashi, T., Warren, C. M., De Tombe, P. P., & Solaro, R. J. (2005). Functional effects of Rho-kinase-dependent phosphorylation of specific sites on cardiac troponin. *Circulation Research*, *96*(7), 740–747. https://doi.org/10.1161/01.RES.0000162457.56568.7d
Van Der Sijde, F., Li, Y., Schraauwen, R., De Koning, W., Van Eijck, C. H. J., & Mustafa, D. A. M. (2020). RNA from stabilized whole blood enables more comprehensive immune gene expression profiling compared to RNA from peripheral blood mononuclear cells. *PLoS ONE*, *15*(6 June), 1–12. https://doi.org/10.1371/journal.pone.0235413
Vasan, R. S., Beiser, A., Seshadri, S., Larson, M. G., Kannel, W. B., D'Agostino, R. B., & Levy, D. (2002). Residual lifetime risk for developing hypertension in middle-aged women and men: The Framingham Heart Study. *Journal of the American Medical Association*, *287*(8), 1003–1010. https://doi.org/10.1001/jama.287.8.1003

Wahl, D., Cavalier, A. N., Smith, M., Seals, D. R., & LarRocca, T. J. (2021). Healthy Aging Interventions Reduce Repetitive Element Transcripts. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, *76*(5), 805–810. https://doi.org/10.1093/gerona/glaa302

Whelton, P. K., Carey, R. M., Aronow, W. S., Casey, D. E., Collins, K. J., Dennison Himmelfarb, C., … Wright, J. T. (2018). 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Pr. *Journal of the American College of Cardiology*, *71*(19), e127–e248. https://doi.org/10.1016/j.jacc.2017.11.006

Williams, B., Lacy, P. S., Thom, S. M., Cruickshank, K., Stanton, A., Collier, D., … O'Rourke, M. (2006). Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: Principal results of the Conduit Artery Function Evaluation (CAFE) study. *Circulation*, *113*(9), 1213–1225. https://doi.org/10.1161/CIRCULATIONAHA.105.595496

Witte, D. R., Westerink, J., De Koning, E. J., Van Der Graaf, Y., Grobbee, D. E., & Bots, M. L. (2005). Is the association between flow-mediated dilation and cardiovascular risk limited to low-risk populations? *Journal of the American College of Cardiology*, *45*(12), 1987–1993. https://doi.org/10.1016/j.jacc.2005.02.073

Wolf-Maier, K., Cooper, R. S., Banegas, J. R., Giampaoli, S., Hense, H. W., Joffres, M., … Vescio, F. (2003). Hypertension Prevalence and Blood Pressure Levels in 6 European Countries, Canada, and the United States. *Journal of the American Medical Association*, *289*(18), 2363–2369. https://doi.org/10.1001/jama.289.18.2363

Wu, J., Kraja, A. T., Oberman, A., Lewis, C. E., Ellison, R. C., Arnett, D. K., … Rao, D. C. (2005). A summary of the effects of antihypertensive medications on measured blood pressure. *American Journal of Hypertension*, *18*(7), 935–942. https://doi.org/10.1016/j.amjhyper.2005.01.011

Wu, K. I. S., & Schmid-Schönbein, G. W. (2011). Nuclear factor kappa b and matrix metalloproteinase induced receptor cleavage in the spontaneously hypertensive rat. *Hypertension*, *57*(2), 261–268. https://doi.org/10.1161/HYPERTENSIONAHA.110.158709

Youn, J. C., Yu, H. T., Lim, B. J., Koh, M. J., Lee, J., Chang, D. Y., … Park, S. (2013). Immunosenescent CD8+ T clls and C-X-C cemokine receptor type 3 chemokines are increased in human hypertension. *Hypertension*, *62*(1), 126–133. https://doi.org/10.1161/HYPERTENSIONAHA.113.00689

Zeller, T., Schurmann, C., Schramm, K., Müller, C., Kwon, S., Wild, P. S., … Homuth, G. (2017). Transcriptome-wide analysis identifies novel associations with blood pressure. *Hypertension*, *70*(4), 743–750. https://doi.org/10.1161/HYPERTENSIONAHA.117.09458