The Efficacy of Dry Beans (*Phaseolus vulgaris* L.) for Improving Vascular Function: Exploring Seed Coat Colour as an Indicator of Functionality

by

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Abstract

Consuming pulses over several weeks improves vascular function and decreases cardiovascular disease risk; however, it is unknown whether pulses can positively modulate postprandial vascular responses, a delimiting factor in projecting vascular health. Bioactive compounds within pulses are the suggested effectors; thus, dry beans (*Phaseolus vulgaris* L.) were investigated due to their contrasting seed coat colours, indicative of distinct phytochemical profiles.

A randomized crossover study investigated the effects of beans with different seed coat colours (white, tan, red, and black) on postprandial vascular and metabolic responses in healthy adults. Darker-coloured beans induced positive postprandial vascular responses, as evidenced by blood vessel relaxation within six hours of consumption. Additionally, the different beans elicited distinct responses for various vascular and metabolic parameters depending on seed coat colour. Thus, bioactive compounds unique to each bean type are likely responsible for the different biological effects. To investigate potential vasorelaxation mechanisms, serum samples were analyzed by untargetted metabolomics to identify novel endogenous metabolites that changed following consumption of the different bean types. Darker-coloured beans altered 47 metabolites within six hours that were unchanged by lighter-coloured beans. Circulating acylcarnitines were tentatively identified as compounds of interest because they are linked to improved blood flow, but absolute identities were not confirmed.

The chronic effects of navy and black beans on vascular structure and function were investigated in spontaneously hypertensive rats, a model of essential hypertension. Black beans, but not navy beans, improved vascular compliance of mesenteric resistance arteries, but this was

reversed when bean consumption ceased for 4 weeks. These findings indicate that regular consumption of black beans was required to retain the positive effects on the vasculature.

In summary, darker-coloured beans improved vascular function in healthy individuals and hypertensive animals. The findings indicate that bean seed coat colour may be a visual marker for the vascular effects of beans. Although a mechanism of action was not elucidated, darker-coloured beans modulated the endogenous metabolome and this provides opportunity for further exploration of mechanism. Overall, this research provides evidence for including darker-coloured beans regularly in the diet as a strategy to maintain blood vessel health.

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"*I was just guessing at numbers and figures*" - The Scientist by Coldplay

Dedication

This dissertation is dedicated to my dad (Jim Clark), my cat (Mia Moo), and "that cute baby" (Lawson).

Dad, you are gone but never forgotten. Thank you for sharing your love of science and academia. I know you were looking forward to celebrating this accomplishment with me, and I hope in your final resting place you can take comfort knowing I completed this goal close to both of our hearts.
I love you and I miss you.

Mia, thank you for being so soft and fuzzy during times of stress over these past years. You are a source of emotional support and never fail to bring a smile to my face, except when you do bad things.

Lawson, you have been the greatest joy in this past year. Your smiles and laughs are the best medicine and always make me happy. I hope I can inspire you to follow your future passions and conquer your challenges just as others did for me.
Happy 1st birthday, Baby Laws.
Love, Auntie Jam.

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List of Abbreviations

ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end-product
AIx	Augmentation index
AKG	Alpha-ketoglutarate
ANOVA	Analysis of variance
AP	Augmentation pressure
BB	Black bean
BCAA	Branched-chain amino acid
BCAT	Branched-chain amino acid transferase
BP	Blood pressure
CO	Cardiac output
CSA	Cross-sectional area
CTRL	Control
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ECM	Extracellular matrix
EDCF	Endothelium-dependent contraction factor
EDRF	Endothelium-dependent relaxation factor
eNOS	Endothelial nitric oxide synthase
ESI	Electrospray ionization
HDL-C	High density lipoprotein-cholesterol
HMDB	Human Metabolome Database
HPLC	High performance liquid chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH	Krebs-Henseleit
LDL-C	Low density lipoprotein-cholesterol
MAP	Mean arterial pressure
MFE	Molecular Feature Extraction
MHQ	MassHunter Qualitative
MMP	Matrix metalloproteinase
MPP	Mass Profiler Professional
MS	Mass spectrometry
NB	Navy bean
NO	Nitric oxide
NOx	Nitrate/nitrite
PB	Pinto bean
PP	Pulse pressure

PWV	Pulse wave velocity
QC	Quality control
QTOF	Quadrupole-time-of-flight
RAAS	Renin-angiotensin-aldosterone system
RKB	Red kidney bean
RM	Reflection magnitude
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rat
SMC	Smooth muscle cell
TC	Total cholesterol
TG	Triglyceride
TVR	Total vascular resistance
VSMC	Vascular smooth muscle cell
WKY	Wistar Kyoto

List of Equations

Contribution Statement

The results presented in this thesis are arranged in manuscript format (Chapters 4-6).

The contributions of each author for the respective manuscripts are described below.

Chapter 4

I, Jaime Clark, made the following contributions to this manuscript:

- Assisted with the study design
- Prepared food samples (food articles) for participants
- Randomized participant visits and food articles according to the randomization code prepared by a statistician
- Supervised consumption of food articles by participants
- Processed biological samples
- Performed nitric oxide assay
- Performed statistical analyses for all experiments
- Interpreted results and wrote the manuscript

I, Jaime Clark, would like to acknowledge the contributions of Dr. Carla Taylor and Dr. Peter Zahradka to this manuscript:

- Generated the study design
- Edited the manuscript

Chapter 5:

I, Jaime Clark, made the following contributions to this manuscript:

- Assisted with the study design
- Prepared food articles for participants
- Randomized participant visits and food articles according to the randomization code prepared by a statistician
- Supervised consumption of food articles by participants
- Processed biological samples
- Prepared sample extracts for metabolomics analysis
- Interpreted results and wrote the manuscript

I, Jaime Clark, would like to acknowledge Dr. Michel Aliani for his contribution to this manuscript:

- Data processing through MPP and MHQ
- Statistical analysis through MPP

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- Generated the study design
- Edited the manuscript

Chapter 6

I, Jaime Clark, made the following contributions to this manuscript:

Phase I:

- Assisted with the study design & diet preparation
- Assisted with all animal handling and *in vivo* measurements (feed intake, fasting blood collection, body weights, blood pressure, body composition)
- Performed *ex vivo* pressure myography
- Analyzed and interpreted blood pressure, pulse wave velocity, pressure myography data, and morphology data
- Performed statistical analysis for blood pressure, pulse wave velocity, pressure myography, morphology data
- Generated all figures
- Wrote 95% of this manuscript section

Phase 2:

- Assisted with study design & diet preparation
- Performed all animal handling and *in vivo* measurements (feed intake, fasting blood collection, body weights, blood pressure, body composition)
- Performed *ex vivo* pressure myography
- Analyzed pulse wave velocity and pressure myography data
- Performed staining and morphometry analysis of aorta sections
- Performed statistical analyses for all experiments
- Generated all figures

- Interpreted results and wrote 100% of this manuscript section

I, Jaime Clark, would like to acknowledge the contribution of Tara Loader to the Phase I portion of this manuscript:

- Assisted with study design & diet preparation
- Assisted with all animal handling and *in vivo* measurements (feed intake, fasting blood collection, body weights, blood pressure, body composition)
- Performed sectioning and staining of aorta samples, and imaged stained aorta samples
- Performed statistical analyses for the above-mentioned results
- Generated figures for the above-mentioned results
- Interpreted the above-mentioned results
- Wrote 5% of Phase 1 manuscript section

I, Jaime Clark, would like to acknowledge the contribution of Dr. Hope Anderson to this manuscript:

- Provided pressure myography equipment and equations required for calculations of vascular compliance and related parameters

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- Generated the study design
- Edited the manuscript

Chapter 1: Overall Introduction

1.1 Introduction

According to the World Health Organization, cardiovascular disease (CVD) is projected to be the cause of more than 23 million deaths over the next 15 years, making it and its accompanying risk factors global public health concerns (Clark *et al* 2015). Hypertension (high blood pressure (BP)) and arteriosclerosis (thickening and stiffening of the arterial wall (Brown 2011; Xu 2004)) are traditional risk factors for CVD (Ecobici & Stoicescu 2017). Previous studies in our lab have demonstrated that pulses, such as dried beans, are effective for reducing BP and improving vascular health in subjects (human and animal) with hypertension and arterial stiffness (Hanson *et al* 2014; Zahradka *et al* 2013). While this evidence demonstrates pulses can improve vascular structure and function with chronic consumption, it is uncertain whether pulses can have an effect on vascular health before arterial disease is established.

The postprandial response is the earliest point of intervention for maintaining vascular health, as how blood vessels respond in the postprandial state is a determining factor in their propensity for vascular disease. Evidence is emerging that certain foods, particularly high-fat meals characteristic of the Western diet, can impair postprandial vascular responses (Jackson 2000; Jakulj *et al* 2007; Rudolph *et al* 2007; Vogel *et al* 1997). These impaired postprandial vascular responses, when repeated every few days or multiple times per day, can promote arterial remodelling and arterial stiffness (Intengan & Schiffrin 2001; Li *et al* 2013; Staiculescu *et al* 2013), known factors in the development of CVD (van Varik *et al* 2012). On the other hand, foods that induce a positive postprandial vascular response, such as those rich in polyphenols (Alqurashi *et al* 2016; Habauzit & Morand 2012; Rendeiro *et al* 2016), would be expected to

promote a healthy vascular environment, and thus reduce the risk of developing CVD (Habauzit & Morand 2012).

Interestingly, in both key background studies (Hanson *et al* 2014; Zahradka *et al* 2013), there was no correlation between reduced serum cholesterol and blood vessel function, thus indicating a factor other than fibre is responsible for the observed vascular improvements. Rather, it is likely that bioactive phytochemical compounds within the pulses are directly acting on the vessel walls. Dried beans are the most commonly produced and consumed of the four pulses (Zhu *et al* 2012), and have a variety of contrasting seed coat colours amongst their different types. Typically, flavonoids and other phenolic compounds are stored in the testa (seed coat), and thus, seed coat colour is considered reflective of phytochemical content (Pitura & Arntfield 2019). Certain polyphenolic compounds are only present in beans with brown/tan, red, or black seed coat colours, such as flavonols (e.g. myricetin, kaempferol, quercetin), flavones (e.g. luteolin), flavanones (e.g. naringenin), and anthocyanins (e.g. delphinidin, cyanidin), to name a few (Clark *et al* 2015; Ombra *et al* 2016; Pitura & Arntfield 2019). In contrast, beans with a light-coloured seed coat, such as white navy beans, do not contain anthocyanins or flavonols (McClean *et al* 2015; Pitura & Arntfield 2019), but do contain phenolic acids (Ombra *et al* 2016).

Overall, pulses (mixed and lentils) have proved effective at improving vascular structure and function in subjects with overt hypertension and/or arterial stiffness when consumed long-term (Hanson *et al* 2016; Zahradka *et al* 2013), likely due to the phytochemical compounds within the seed coat. However, their efficacy for inducing positive postprandial vascular responses, a contributor toward vascular health, has not been determined. Additionally, seed coat colour, which is indicative of phytochemical profile, has not been evaluated as a marker for the potential

of different bean types to elicit positive vascular functionality. Furthermore, it is unknown if beans can modify the structure and function of blood vessels in a hypertensive model, as has been reported previously for lentils (Hanson *et al* 2016).

Therefore, the overall aims of this thesis were to determine (1) if the seed coat colour of beans is an indicator of vascular functionality *in vivo;* (2) whether different bean varieties can modify postprandial vascular and metabolic responses in healthy adults; and (3) the long-term effects of black and navy beans in improving hypertension-induced vascular dysfunction and structural changes in a rodent model.

A randomized, controlled crossover study was designed to determine the ability of beans to modify postprandial vascular and metabolic responses in healthy adults. An untargetted metabolomics approach was utilized to profile changing levels of circulating endogenous metabolites in response to bean consumption. A pre-clinical animal study using spontaneously hypertensive rats (SHR), a model of essential hypertension, was designed to determine the effects of black and navy beans on vascular structure and function. The research in this dissertation not only investigates the short- and long-term vascular effects of bean consumption, but also explores the potential differences in vascular functionality related to bean seed coat colour. The following chapter will review pertinent literature to provide context for the rationale of the studies contained in this dissertation.

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Chapter 2: Literature Review

2.1 Arterial Network

Types and Functions of Arteries

Arteries comprise one of three major types of blood vessels in the cardiovascular system (Marieb & Hoehn 2007). The primary function of arteries is to carry oxygen-rich blood from the heart to the periphery, thus supplying essential substances such as nutrients and oxygen to the cells (Marieb & Hoehn 2007; Thomas & Suman 2016). As blood is ejected from the left ventricle of the heart, it enters a network of arterial branches, starting with the aorta which is the main trunk of the network (Klabunde 2012). From the aorta, the arterial network branches off into successively smaller vessels: large and medium arteries, small arteries, and arterioles (Marieb & Hoehn 2007; Klabunde 2012).

The arteries within this network can be classified according to their size and function (**Table 2.1**). The largest artery is the aorta (25 mm luminal diameter in a human adult). Its high elasticity allows it to absorb the blood being ejected from the left ventricle of the heart, thus lessening the pulsatile pressure before the blood is distributed to successive arterial branches (Klabunde 2012; Levick 1995). The first-order of branching arteries (e.g. left common carotid, brachiocephalic, subclavian, renal, celiac, superior and inferior mesenteric, common iliac arteries) range between large (10-15 mm) or medium (1-10 mm) in size and they function to conduct blood to the second-order branches (Borg *et al* 2014; Levick 1995; Marieb & Hoehn 2007). The second-order arteries (e.g. right common carotid, splenic, hepatic) distribute the blood flow to even smaller arteries (Marieb & Hoehn 2007). These smaller arteries (0.1-1.0 mm in diameter) continue to help with blood flow distribution, but some also may be classified according to their function as resistance arteries (Klabunde 2012; Levick 1995). Resistance

arteries include those small arteries and arterioles (≤300 μm), that form the microcirculation and that generate resistance to blood flow (i.e. peripheral or vascular resistance) by changing their lumen diameter. In this way, they regulate blood pressure (BP) and flow (Hadoke *et al* 2000; Klabunde 2012; London & Pannier 2010; Mulvany 2002; Schiffrin 1996).

Small arterioles lead directly to the capillaries, the smallest blood vessels in the body. Capillaries enable the exchange of gases, hormones, nutrients, and other materials within the tissues and organs (Klabunde 2012; Marieb & Hoehn 2007). It is important to note that while arterioles feed into the capillary bed, capillaries represent a class of vessels distinct from arteries (Marieb & Hoehn 2007); thus, they will not be the focus of discussion from this point forward.

Table 2.1. Function and relative composition of arteries.

Artery (Type)	Function	Luminal Diameter	Relative Composition
			Endothelium, Smooth muscle, Elastic tissue, Collagenous tissue
Aorta (Elastic)	• Pulsatile pressure dampening • Blood flow distribution	15-25 mm	
Large arteries (Elastic)	• Blood flow distribution	10-15 mm	
Medium (Muscular)	• Blood flow distribution	1-10 mm	
Small arteries (Muscular)	• Blood flow distribution • Resistance	0.3-1.0 mm	
Arterioles (Muscular)	• Resistance	10 µm-300 µm	
Capillaries	• Material exchange between vessels and tissues/ organs	6-10 µm	

Table adapted from Levick 1995; Marieb & Hoehn 2007.

Structural Arrangement of Arteries

Arteries are comprised of three layers (in order from inside to outside): the tunica intima, tunica media, and tunica adventitia (**Figure 2.1)** (Klabunde 2012; Marieb & Hoehn 2007). The tunica intima is the innermost layer of the vessel wall that surrounds the lumen (Marieb & Hoehn 2007; Wagenseil & Mecham 2009). The intima consists of a single layer of longitudinally arranged endothelial cells (Mulvany 2002). In larger arteries, the endothelium is attached to a subendothelial layer consisting of basal lamina supported by the internal elastic lamina (Marieb & Hoehn 2007; Wagenseil & Mecham 2009). The tunica media is the middle layer of the vessel wall and consists of circumferentially arranged smooth muscle cells (SMCs), collagen fibres, and proteoglycan-rich extracellular matrix (ECM) between sheets of elastin, known as lamellae (Mulvany 2002; Wagenseil & Mecham 2009). The adventitia is the outermost layer of the vessel wall and consists primarily of collagen-rich ECM (for structural support) and fibroblasts (Marieb & Hoehn 2007; Wagenseil & Mecham 2009; Wang *et al* 2017).

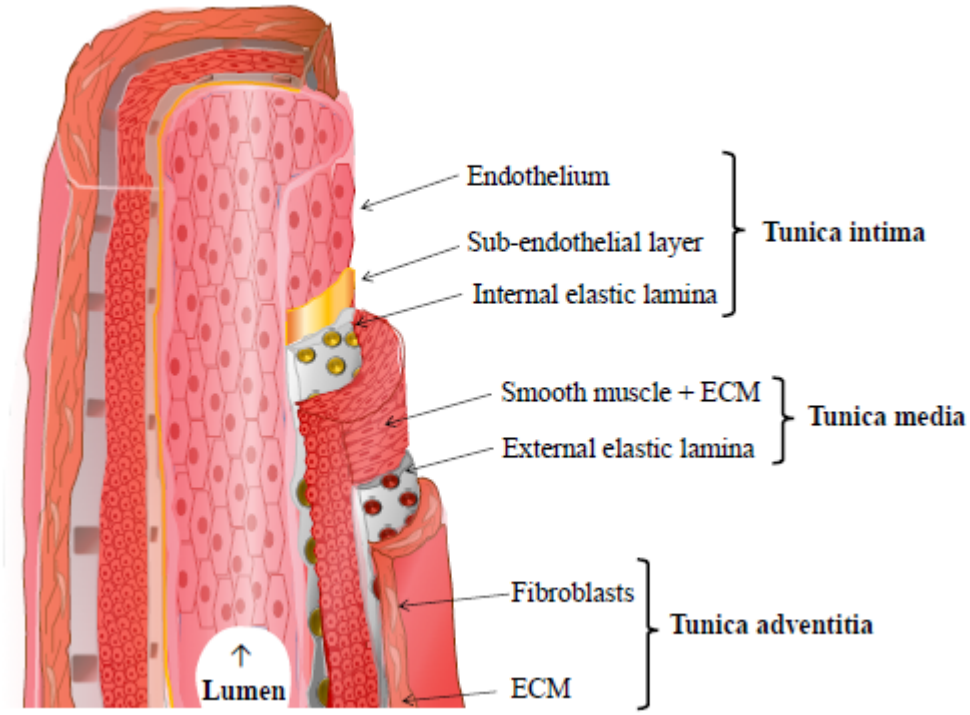

Figure 2.1. Structural layers of arteries.

Artistic representation of the large-artery structural layers. Figure was adapted from Almeida 2013 and designed using Microsoft PowerPoint. *Abbreviations*: ECM, extracellular matrix.

Heterogeneity exists within the structural arrangement of the arteries which has a corresponding role in their functions (Table 2.1). The large arteries near the heart are elastic, meaning they contain more elastin in comparison to the other arteries (Marieb & Hoehn 2007). Elastin provides stretch and flexibility, referred to as elasticity. This elasticity aids in the function of these arteries as pressure reservoirs, allowing for the expansion and recoil required to maintain consistent blood flow to the periphery with little change in pressure (Marieb & Hoehn 2007; Wagenseil & Mecham 2009). The medium-sized, and some of the smaller, arteries are muscular (e.g. brachial, radial, femoral), meaning they proportionately have the thickest tunica media with more SMCs and less elastin (Marieb & Hoehn 2007; Tucker & Mahajan 2018). The smallest arteries are the arterioles which lead into the capillary beds. Larger arterioles have all three structural layers, while the smaller arterioles connecting with the capillary beds have only a single layer of SMCs surrounding the intima (Marieb & Hoehn 2007).

The geometry, or structural properties, of arteries can be determined by measuring lumen diameter, media thickness, wall thickness, media:lumen ratio, and wall:lumen ratio when the smooth muscle cells are relaxed and the vessel is exposed to a constant intraluminal pressure (Mulvany 2002).

Cellular Wall Components

The primary cell types within the arterial wall are endothelial cells and vascular SMCs (VSMCs) (Roostalu & Wong 2018).

As mentioned previously, the tunica intima consists of a single layer of endothelial cells, known as the endothelium, which serves as the primary interface between the blood and tissues (Klabunde 2012; Khaddaj Mallat *et al* 2017). Endothelial cells are elongated, flat, single-nucleated, simple squamous epithelial cells (Klabunde 2012; Marieb & Hoehn 2007). The flat

nature of endothelial cells allows for a smooth surface for blood flow (Marieb & Hoehn 2007). Endothelial cells are connected by tight junctions which aid in their barrier function as mediators in the exchange of fluid, gases, molecules, and cells (Klabunde 2012). They produce vasoactive substances such as endothelial-dependent relaxation factors (EDRFs; e.g., nitric oxide (NO) and prostacyclin) and endothelial-dependent contraction factors (EDCFs; e.g. endothelin-1) (Du *et al* 2018; Klabunde 2012; Lüscher & Tanner 1993). The EDRFs are important for modulating platelet aggregation and leukocyte adhesion, along with endothelial-expressed surface adhesion molecules, while both EDRFs and EDCFs are important for regulating SMC function within the tunica media (Klabunde 2012). Thus, endothelial cells are important regulators of vascular tone (Loscalzo 1995).

VSMCs are present in the arterial media and regulate the size of the vessel lumen by contraction or relaxation, resulting in vasoconstriction (lumen narrowing) or vasodilation (lumen widening), respectively (Brozovich *et al* 2016; Klabunde 2012; Marieb & Hoehn 2007). In the homeostatic state, VSMCs are partially contracted, forming the basal vascular tone of the vessel (Holzapfel & Ogden 2018). This tone determines the lumen diameters that strongly affects vascular resistance in resistance arteries and arterioles (Touyz *et al* 2018). VSMC activity is regulated by various stimuli: sympathetic vasomotor nerve fibres of the autonomic nervous system, electrical stimuli via changes in ion concentration (e.g. calcium), hormonal stimuli (e.g., norepinephrine, angiotensin II, endothelin-1), and at times, mechanical stimuli (e.g. shear stress) (Klabunde 2012; Marieb & Hoehn 2007).

In the homeostatic, mature arterial wall, both endothelial cells and VSMCs exist in the quiescent state (Rajendran *et al* 2013; Yamin & Morgan 2012). For endothelial cells, their quiescence maintains the homeostasis of the tightly regulated vascular environment, ensuring a

balance between pro- and anti-oxidants, vasodilators and vasoconstrictors, pro- and anti-inflammatory mediators, and pro- and anti-thrombotic signals (Donato *et al* 2015). For VSMCs, this quiescence results in the contractile phenotype, whereby the cells can contract and relax, but not proliferate or migrate (Yamin & Morgan 2012; Louis & Zahradka 2010). The phenotype of endothelial cells and VSMCs can be affected by changes in the vascular environment (Rajendran *et al* 2013; Yamin & Morgan 2012). For example, inflammation and high shear stress can activate endothelial cells to create a prothrombotic vascular environment (Rajendran *et al* 2013), while inflammatory mediators and growth factors can induce VSMCs to become proliferative, contributing to the development of vascular disease (Yamin & Morgan 2012). The pathophysiological consequences of the phenotype switch will be discussed in Section 2.2 of this Chapter.

Structural Wall Proteins

Surrounding the cells within the arterial wall is a dynamic structure of proteins, enzymes, and other components collectively composing the ECM (Duca *et al* 2016). However, there are two proteins that remain fundamental to maintaining the structural integrity and mechanical properties of the vessel wall: elastin and collagen (Wagenseil & Mecham 2009). Elastin and collagen in the arterial media are produced primarily by VSMCs, while adventitial collagen is primarily produced by fibroblasts (Xu & Shi 2014). Elastin is the dominant ECM protein in the arterial wall, arranged in a three-dimensional network of fenestrated sheets (laminae) along with VSMCs, which determines the elasticity of the vessel (Duca *et al* 2016; Wagenseil & Mecham 2009). Elastin consists of fibrillin-rich microfibrils which act as a structural scaffold to guide elastin deposition and assembly within the vessel wall (Xu & Shi 2014). Mature elastin fibres

also play a fundamental role in maintaining the quiescent contractile phenotype of VSMCs in the arterial wall by providing a physical barrier against cellular migration (Xu & Shi 2014).

Between the elastin lamellar layers in the media are bundles of collagen fibres, primarily types I and III, that become circumferentially aligned as pressure increases (Karimi & Milewicz 2016; Wagenseil & Mecham 2009). In the adventitia, the collagen fibres are wavy and oriented in the axial direction (Holzapfel & Ogden 2018). While elastin provides the flexibility to the vessel wall, collagen is the stiff, load-bearing protein that supports the structural arrangement of the artery (Holzapfel 2008; Karimi & Milewicz 2016). Collagen fibres become engaged upon stretching of the artery to support tension, and the larger bundles in the adventitia aid in preventing arterial rupture at higher pressures (Holzapfel & Ogden 2018).

Mechanical Properties of Arteries

Elastin, collagen, and SMCs all contribute toward governing the mechanical properties of the arterial network (Holzapfel & Ogden 2018). These arterial mechanics determine the propagation of energy from the heart to the periphery of the body (Peterson *et al* 1960) and can be expressed as arterial compliance or its inverse, stiffness, to describe the elasticity of the arterial wall (Ebrahimi 2009; Hayashi & Hirama 2017). Arterial compliance refers to the ability of the vessel to buffer changes in pressure (Intengan *et al* 1999) and can be determined by measuring medial stress, medial strain, and elastic modulus in smaller resistance arteries, and pulse wave velocity (PWV) in larger muscular arteries (Akhtar *et al* 2011; Hanson *et al* 2016; Hayashi & Hirayama 2017).

Stress, Strain, and Elastic Modulus

Medial stress, also known as circumferential stress, reflects the tension in the vessel wall that develops to protect the wall against pressure-induced distension, such as that occurring with

high BP (Behbahani *et al* 2010; Kamenskiy *et al* 2013). Medial strain, or circumferential strain, reflects the relative change in lumen diameter induced by pressure (Behbahani *et al* 2010). Circumferential strain is also referred to as the degree of stretch exhibited by the vessel wall due to increasing pressure (Lu & Kassab 2011a).

Stress and strain can be calculated as follows (Behbahani *et al* 2010):

$$Stress\ (\sigma) = (PD)/(2WT) \qquad \textbf{(Equation 2.1)}$$

Where: P, intraluminal pressure (converted to 1 mmHg = 1.334×10^3 dynes/cm^2); D, lumen diameter (μm); WT, wall thickness (μm).

$$Strain\ (\varepsilon) = (D_i - D_o)/D_o \qquad \textbf{(Equation 2.2)}$$

Where: D_i, observed lumen diameter for a given P; D_o, baseline lumen diameter at P = 3 mmHg.

The non-linear nature of the stress-strain relationship is a result of the separate engagement of elastin and collagen that occurs at different physiological pressures (Ebrahimi 2009; Wagenseil & Mecham 2009). Thus, the stress-strain curve can be divided into two moduli (**Figure 2.2a**): the lower modulus and the upper modulus (Ebrahimi 2009). Within the stress-strain relationship, the lower modulus is the region of the stress-strain line lying almost parallel to the x-axis (Aziz *et al* 2016; Peterson *et al* 1960). The lower modulus is engaged during lower pressures where there is small strain and elastin is the predominate protein influencing distensibility (elasticity) within the arterial wall (Ebrahimi 2009; Khamdaeng *et al* 2012). As pressure increases, collagen fibres are recruited to support tension and restrict distention of the arterial wall, resulting in higher strain (Khamdaeng *et al* 2012; Wagenseil & Mecham 2009). At this point, known as the transition point, the stress-strain line curves upward, now entering the upper modulus where it begins to straighten almost parallel to the y-axis (Ebrahimi 2009;

Khamdaeng *et al* 2012). Overall, the stress-strain relationship is displayed as a J-shaped curve illustrating the changes in arterial elasticity, or compliance, as pressure increases (Khamdaeng *et al* 2012; Wagenseil & Mecham 2009). Therefore, the stress-strain curve will be shifted leftward for stiff, less compliant vessels where collagen fibres are engaged more quickly, or shifted rightward for more compliant vessels where elastin remains predominant at higher pressures (**Figure 2.2b**) (Nichols *et al* 2011).

Changes in the mechanical properties of arterial walls may occur due to changes in the elastic properties of the vessel wall components, independent of vessel geometry (Laurant *et al* 2004). Elastic modulus represents the intrinsic elastic properties of the wall materials and is related to the structural composition of the vessel wall (Hayashi & Hirayama 2017). Incremental elastic modulus is determined from the stress-strain relationship, and the slope of incremental elastic modulus *vs* stress can be used to represent the wall component stiffness independent of vessel geometry (Intengan *et al* 1999; Laurant *et al* 2004).

Elastic modulus can be calculated by fitting the stress-strain data from each vessel to an exponential curve as follows (Behbahani *et al* 2010; Park & Schiffrin 2001):

$$Elastic\ modulus = \sigma_o\, e^{\beta\varepsilon} \qquad \textbf{(Equation 2.3)}$$

Where: σ_o, stress at D_o (baseline diameter); β, constant related to increase of the stress-strain curve; ε, strain.

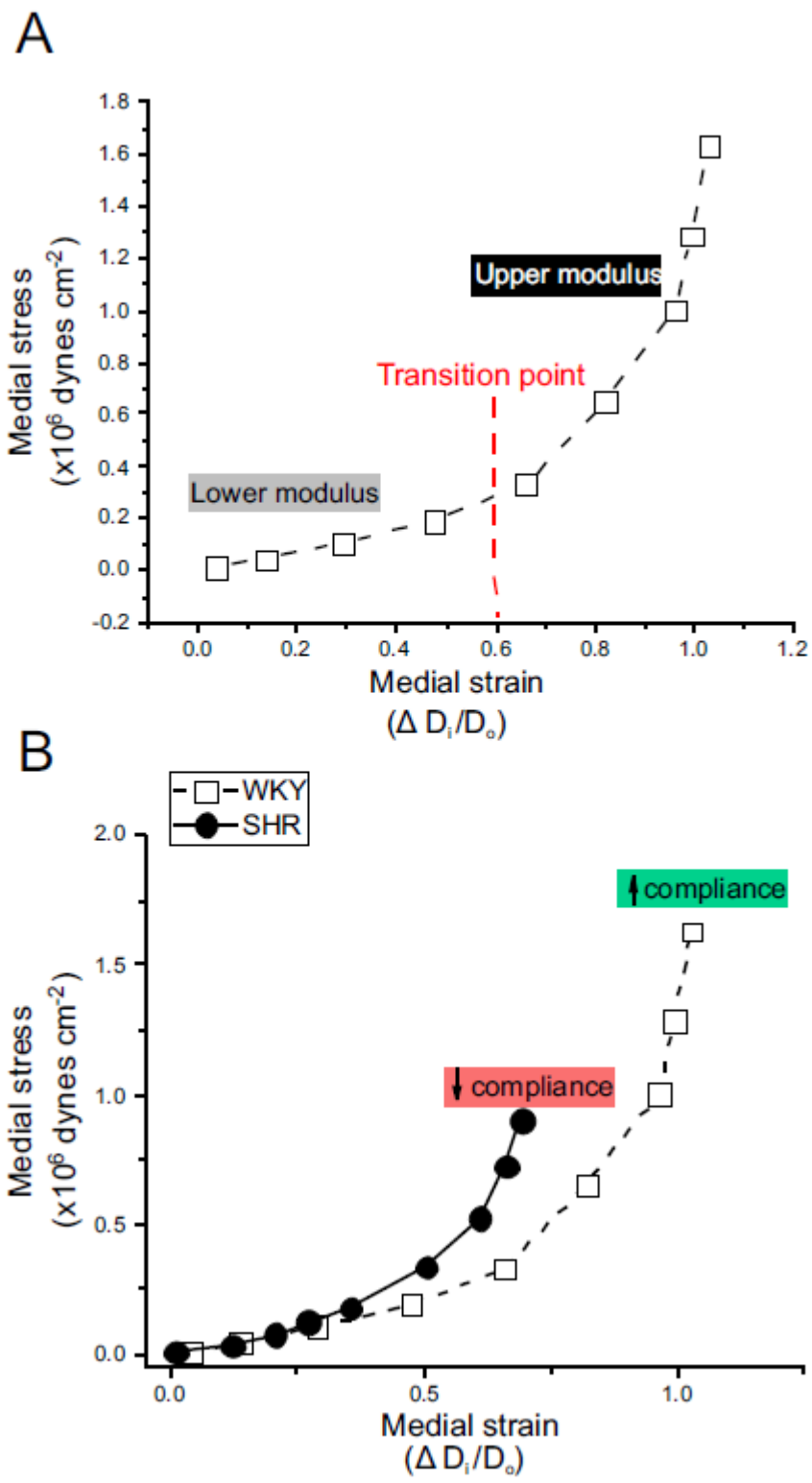
A
Medial stress
(x10⁶ dynes cm⁻²)
Upper modulus
Transition point
Lower modulus
Medial strain
(Δ Dᵢ/Dₒ)
B
WKY
SHR
compliance
compliance
Medial stress
(x10⁶ dynes cm⁻²)
Medial strain
(Δ Dᵢ/Dₒ)

Figure 2.2 Example of a non-linear stress-strain curve.

(A) Lower and upper moduli with point of curve transition; **(B)** Stress-strain curves representing arterial compliance of hypertensive SHR and normotensive WKY rats. *Abbreviations*: ↓, decreased; ↑, increased; D_i, lumen diameter at a given pressure (P); D_o, baseline lumen diameter at P = 3 mmHg; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Pressure Myography

One of the techniques used to measure the mechanical properties of blood vessels, particularly for small resistance arteries, is pressure myography (Akhtar *et al* 2011). Pressure myography provides *ex vivo* measurements of small arteries or arterioles (≤300 μm) (Akhtar *et al* 2011) obtained through biopsy (e.g. human subcutaneous gluteal resistance arteries) (Intengan *et al* 1999) or excision of the whole vascular bed (e.g. rodent mesenteric resistance arteries) (Hanson *et al* 2016). The arteries are mounted between two glass cannulae and pressurized in the myograph chamber. The pressurized preparation more closely mimics the vascular physiology *in vivo,* without causing damage to the endothelium (Lu & Kassab 2011b). The pressure myograph shows changes in vessel diameter and wall thickness under isobaric conditions (Lu & Kassab 2011b; Dunn & Gardiner 1995), while preserving the vessel's cylindrical shape (Gündüz *et al* 2009). However, given the invasive nature of this procedure, it is not an ideal technique for clinical practice.

Pulse Wave Velocity

PWV is more frequently used in clinical practice to assess arterial stiffness rather than elastic modulus or the stress-strain curve (Hayashi *et al* 2015), as it is a non-invasive procedure that can estimate the elastic properties of vessels *in vivo* (Akhtar *et al* 2011; Fitch *et al* 2001). PWV is a measure of the velocity of the pulse pressure wave, generated during systolic contraction, as it propagates along the arterial tree (Pereira *et al* 2015). It is determined as a ratio of the distance separating two locations and the transit time needed for the wave to cover that distance (Dogui *et al* 2011). PWV is affected by changes in mechanical properties along the arterial network (Pereira *et al* 2015). Higher PWV corresponds to vessels with lower compliance and elasticity, and higher stiffness (Pereira *et al* 2015; Williams *et al* 2007). Typical sites for

measuring PWV are those where pulsation is easily felt, such as in the carotid, brachial, radial, or femoral arteries (Pereira *et al* 2015). Doppler ultrasound is commonly used to measure PWV, and when compared against other gold standard techniques, it produces similar reliable and reproducible results (Calabia *et al* 2011).

Arterial Pulse Wave Propagation

When the left ventricle contracts, it generates a pulse wave which travels along the arterial network (Greenwald 2002) propagated by BP and flow pulsations (van de Vosse & Stergiopulos 2011). The resultant pulse pressure wave interacts with the arterial walls as it propagates; thus, indices can be derived from the pulse wave to provide insight into the elasticity of those arterial walls (Alastruey *et al* 2012; van de Vosse & Stergiopulos 2011).

As the pulse wave propagates away from the heart toward the periphery, it is said to be traveling forward (O'Rourke *et al* 2002). Arterial pulse waves travel forward until they encounter sites of impedance, such as bifurcations, tapering, stenoses, aneurysms, and curves (van de Vosse & Stergiopulos 2011; Westerhof *et al* 1972). At these sites, a portion of the forward wave is reflected back towards the heart and is termed the reflected wave (O'Rourke *et al* 2002; van de Vosse & Stergiopulos 2011). The measured reflected wave is a summation of the various reflected waves arising at bifurcations near the heart and those arriving from the periphery (Westerhof *et al* 1972). The amplitude, or magnitude, of the reflected wave can be expressed as the amplitude ratio of the backward and forward pressure waves (Westerhof & Westerhof 2012). In stiff or tense vessels, this reflection magnitude is larger (Nichols *et al* 2008; Westerhof *et al* 1972). The reflected wave combines with forward-moving waves to produce a composite pulse pressure wave, which travels along the arterial tree (Ahuja *et al* 2009). A boost of systolic pressure follows from the reflected wave summating with the forward wave, referred

to as systolic augmentation pressure (AP) (Jockel-Schneider *et al* 2014). Augmentation index (AIx; determined as the ratio of AP and pulse pressure) is another wave parameter to assess arterial stiffness (Westerhof & Westerhof 2012), and is sometimes used as a proxy of wave reflection (Hughes *et al* 2013). However, AIx is influenced by other factors such as body height, age, heart rate, pulse wave shape, etc.; therefore, while considered to be a sensitive parameter for arterial stiffness (Westerhof & Westerhof 2012), its usefulness to assess wave reflection is limited (Hughes *et al* 2013).

2.2 Hypertension and Arteriosclerotic Disease: Processes and Interventions

Hypertension

Hypertension can be classified into two stages: Stage 1 is clinically defined as having a resting systolic BP (SBP) of ≥130 mmHg, and/or a diastolic BP (DBP) of ≥80 mmHg; Stage 2 is clinically defined as having a resting SBP of ≥140 mmHg, and/or a DBP of ≥90 mmHg (Whelton *et al* 2017). SBP is the pressure generated during ventricular contraction when blood is pumped out of the heart (systole), and DBP is the pressure occurring when the left ventricle is resting between beats (diastole) (American Heart Association 2019). Increased BP can arise from increased cardiac output (CO) and/or total vascular resistance (TVR) (Beevers *et al* 2001; Rahimmanesh *et al* 2012). TVR is modulated by arterial lumen diameter, where small changes in lumen diameter will have a profound effect on resistance, as per Poiseuille's law (Mayet & Hughes 2003). CO is the volume of blood ejected by the left ventricle over the course of one minute (Levick 1995), which is affected by factors such as venous return, ventricular contraction, and afterload (the pressure the left ventricle ejects against) (Klabunde 2017; Mayet & Hughes 2003). Most hypertension cases (95%) have no known cause or cure (known as essential hypertension) (Clark *et al* 2015). Factors that increase the risk for essential hypertension include

age, poor diet, physical inactivity, alcohol, overweight and obesity, stress, and smoking (Clark *et al* 2015; Whelton *et al* 2017). When hypertension remains uncontrolled or prolonged, pathological changes to target organs (cardiovascular system, brain, and kidneys) can occur, thereby increasing the risk for coronary artery disease, myocardial infarction, stroke, and renal failure (Bakris 2018).

Spontaneously Hypertensive Rat

The spontaneously hypertensive rat (SHR) is the most commonly used animal model for essential hypertension research (Doggrell & Brown 1998). The SHR strain originated from the mating of a spontaneously hypertensive Wistar Kyoto (WKY) male rat and a female WKY rat with elevated BP, followed by inbreeding of the hypertensive siblings over several generations (Doggrell & Brown 1998; Sarikonda *et al* 2009). The WKY rat is used as the normotensive control to its SHR counterpart (Doggrell & Brown 1998).

Hypertension in the SHR progresses similarly to that of humans, beginning with elevated BP followed by sustained hypertensive phases, each lasting several weeks for SHR (Doggrell & Brown 1998). A rise in BP appears within the first 5-6 weeks of life for SHRs, and then steadily increases over the next 12-14 weeks (Doggrell & Brown 1998) and reaches a SBP of approximately 180-200 mmHg (Pinto *et al* 1998). At 15 weeks of age, there are established differences in the BP levels of SHR and WKY rats. As well, hypertension is more prevalent in males than age-matched females (equivalent to premenopausal women in humans) in both SHR and humans (Doggrell & Brown 1998; Everett & Zajacova 2015). For these reasons, 15-week old male SHRs have been used consistently in our studies investigating the effects of diet to manage hypertension (Hanson *et al* 2014; Hanson *et al* 2016).

Arteriosclerosis

Arteriosclerosis is a slowly developing, systemic disease of the arteries characterized by pathological processes that result in the thickening and stiffening of the arterial wall (Brown 2011; Xu 2004). Arteriosclerosis and atherosclerosis are terms often used synonymously; however, this use of terminology is incorrect. Atherosclerosis is an arteriosclerotic disease characterized by chronic inflammation, lipid retention, cell death, fibrosis, and ultimately luminal occlusion and thrombosis (Hansson & Libby 2006; Insull 2009). Therefore, arteriosclerosis can include, but is not exclusive to, atherosclerosis.

For the purposes of this dissertation, the pathophysiological development of non-atherosclerotic arteriosclerosis, i.e. arterial wall thickening and stiffening, will be described below.

Increased Vascular Tone

The formation and modulation of vascular tone is controlled by the contractility of VSMCs (Tykocki *et al* 2017); thus, vascular tone is regulated by signals imparted from the endothelium or other stimuli for vasodilation or vasoconstriction (Klabunde 2012; Lüscher & Tanner 1993; Marieb & Hoehn 2007). Vascular tone represents the rapid and acute adaptative response of vessel diameter due to vasoconstriction (Touyz *et al* 2018), and can be considered representative of short-term changes in endothelial cell or VSMC properties (Raffetto & Khalil 2008). Increased vascular tone can be promoted by injury or dysfunction of the endothelium, due to the increased production of vasoconstrictor substances (Loscalzo 1995).

The contracted state of vascular tone can have a substantial impact on cardiovascular health outcomes. For example, increased vascular resistance and prolonged vasoconstriction, both associated with increased vascular tone, are the main stimuli that induce structural changes

within the arterial wall due to hypertension (Staiculescu *et al* 2013) and arterial stiffness (Blacher *et al* 1999). Staiculescu *et al* (2013) showed that prolonged vasoconstriction induces polymerization of actin filaments in the cytoskeleton of VSMCs, which in turn contributes to the role of VSMCs in remodelling the arterial wall. This actin polymerization occurs in a time-dependent manner, such that polymerization will increase as vasoconstriction persists over time (Staiculescu *et al* 2013).

Endothelial Dysfunction

Alterations in endothelial cell function precede the development of arteriosclerotic changes (Nogueira *et al* 2012). Endothelial dysfunction is implicated in the pathophysiology of arteriosclerosis (Endenmann & Schiffrin 2004) and hypertension (Böhm & Pernow 2007) due to the endothelium having lost its ability to prevent prolonged vasoconstriction and inhibit platelet aggregation and SMC proliferation (Anderson *et al* 1995). Endothelial dysfunction is defined as the imbalance between the production and bioavailability of EDRFs and EDCFs and is associated with increased production of reactive oxygen species (ROS) and the presence of oxidative stress (Silva *et al* 2012). Attenuation of endothelial nitric oxide synthase (eNOS), responsible for the majority of vascular NO production through the conversion of L-arginine to L-citrulline (Govers & Rabelink 2001; Wu & Yen 1999), can reduce NO availability and may contribute to endothelial dysfunction (Förstermann & Münzel 2006; Teixeira *et al* 2014). Furthermore, the reduced bioavailability of NO and release of EDCFs contributes to increased stiffness of the arteries (Kurtel *et al* 2013) possibly by promoting vascular wall remodelling (Fernández-Varo *et al* 2003; Gil-Ortega *et al* 2016).

Vascular Remodelling and Arterial Stiffness

With age and conditions such as prolonged vasoconstriction and hypertension (Staiculescu *et al* 2013), structural changes within the vascular wall occur, known as remodelling, that diminish the natural elasticity of arteries, resulting in increased arterial stiffness (Blacher *et al* 1999).

Baumbach & Heistad first defined the term 'remodeling' as changes in resistance vessel structure without changes in volume (Mulvany 1999). Mulvany (1999) later refined vascular remodelling into six precise classes (**Table 2.2**).

Table 2.2 The six classes of vascular remodelling.

	Hypertrophic	**Eutrophic**	**Hypotrophic**
Inward	Decrease in lumen diameter + Increase of cross-sectional area (CSA)	Decrease in lumen diameter + No change in CSA	Decrease in lumen diameter + Decrease of CSA
Outward	Increase in lumen diameter + Increases of CSA	Increase in lumen diameter + No change in CSA	Increase in lumen diameter + Decrease of CSA

Vascular remodelling involves lasting structural changes that represent the dynamic response of the vascular wall components to chronic haemodynamic changes (Raffetto & Khalil 2008; Touyz *et al* 2018). The vascular remodelling that occurs during essential hypertension in resistance arteries is often inward eutrophic (Table 2.2), meaning that there is an increase in the wall:lumen ratio (decreased lumen diameter + media thickness) with no change in CSA

(Mulvany 1999). This form of remodelling is exhibited in patients with mild essential hypertension and SHRs (Intengan & Schiffrin 2001).

During aging or pathological conditions, matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases, are activated (Wang *et al* 2015). Active MMPs selectively degrade ECM components (Castro *et al* 2011; Intengan & Schiffrin 2000), and certain MMPs are also associated with phenotype switching of endothelial and VSMCs (Wang *et al* 2015). For example, MMP-1 has been shown to enhance endothelial cell senescence (cell growth arrest) (Rossman *et al* 2017)), while MMP-2 has been shown to initiate endothelial cell death (apoptosis or necrosis), diminish NO production, promote platelet aggregation and thrombus formation, and increase the permeability of the endothelium (Wang *et al* 2015). MMP-2 has also been shown to cleave the differentiation marker, calponin-1, from VSMCs, thus enabling the cell to re-enter the cell cycle and switch from the differentiated contractile phenotype to the de-differentiated synthetic phenotype (Touyz *et al* 2018; Wang *et al* 2015). MMP-14 (also known as membrane-type 1-MMP) activates MMP-2 in response to vascular injury (Zahradka *et al* 2004) and facilitates cell migration by creating a path while degrading ECM (Itoh 2006). Synthetic SMCs continue to proliferate and migrate, accumulating in the media of the vascular wall, leading to wall thickening (Zain *et al* 2019). Additionally, one of the actions of the synthetic phenotype is secretion of high levels of signaling molecules associated with cell growth, migration, fibrosis, and inflammation, which further promote the activity of MMPs (Touyz *et al* 2018; Zain *et al* 2019).

MMPs also modulate the collagen:elastin ratio within the arterial wall by inducing excessive synthesis of collagen and degradation of elastin (Castro *et al* 2011; McNulty *et al* 2006; Wang *et al* 2015). For example, MMP-2 and MMP-9, often activated during hypertension,

digest elastin fibers, while MMP-2 indirectly enhances VSMC production of collagen fibers and fibronectin by enabling transition to the synthetic phenotype, contributing to diminished elasticity and increased stiffness of the arteries (Wang *et al* 2015).

Arterial stiffness is a major risk factor for CVD (Blacher *et al* 1999) and is an independent predictor of cardiovascular mortality (Dogui *et al* 2011). The loss of elasticity with arterial stiffness reduces the buffering capacity of the arteries, thereby adversely modulating pulsatile pressure and wave propagation, which in turn affects wave velocity and reflection throughout the arterial network (Aviolo 2013). Consequently, with arterial stiffness there is increased PWV, earlier arrival of the reflected wave leading to increased AP (associated with the reflected wave summating with the forward wave (Wilson 2006)), increased pulse pressure, and increased wave reflection magnitude (RM) (Aviolo 2013; Nichols *et al* 2008; Westerhof 1972; Westerhof & Westerhof 2012; Wilson 2006). Increased PWV and pulse pressure are both independent predictors and highly significant contributors to cardiovascular morbidity and mortality (Aviolo 2013). Other detrimental haemodynamic changes in response to arterial stiffness include low DBP, high SBP, and increased left ventricular workload, and these may contribute to the development of myocardial ischemia, fibrosis, and heart failure (Shirwany & Zou 2010; Wilkinson et al 2009).

Interventions

Pharmacological

Anti-hypertensive medications aim to manage arterial pressure through reducing CO and/or TVR (Jackson & Bellamy 2015). Inhibitors and antagonists of the renin-angiotensin-aldosterone system (RAAS) appear to be the most effective anti-hypertensive drugs through their ability to reduce both CO and TVR (Jackson & Bellamy 2015). The RAAS drugs include

angiotensin-converting-enzyme (ACE) inhibitors, angiotensin receptor blockers, and renin inhibitors (Jackson & Bellamy 2015). ACE inhibitors have been shown to inhibit the VSMC proliferative phenotype and VSMC proliferation (Kawahara *et al* 2005; Uehara *et al* 1993). Non-RAAS medications work by increasing water and sodium excretion (e.g. diuretics) to reduce CO, or by vasodilation and the resultant decrease in vascular tone and TVR (e.g. calcium channel blockers, vasodilators). Alternative anti-hypertensive medications, such as ß-blockers, are used when hypertension occurs concomitantly with other cardiovascular morbidities such as myocardial infarction (Jackson & Bellamy 2015).

Due to the multi-factorial pathophysiology of arterial stiffness, a multitude of pharmacological therapies have been investigated for their potential to reduce arterial stiffness (Upala *et al* 2017). Medications include, but are not limited to, anti-hypertensive agents, anti-diabetic agents, statins, and advanced glycation end-product (AGE) cross-link breakers (Janíc *et al* 2014; Topouchian *et al* 2017; Upala *et al* 2017; Wu *et al* 2015); however, anti-diabetic agents are only effective for patients with diabetes. ACE inhibitors have shown to be the most effective of the anti-hypertensive agents for long-term reduction of systemic arterial stiffness (Janíc *et al* 2014; Topouchian *et al* 2007), whereas calcium channel blockers have only been effective for decreasing aortic stiffness, but not peripheral stiffness (Topouchian *et al* 2007). In a meta-analysis, Upala *et al* (2017) concluded that short-term statin therapy (two weeks to six months) was favourable for improving aortic stiffness measured by PWV in six randomized controlled trials. The mechanism of action for statins is suggested to be increased eNOS activity, anti-inflammatory activity, and anti-oxidant activity, as well as suppressed sympathetic neural activity and ROS production (Janíc *et al* 2014; Upala *et al* 2017). AGE cross-link breakers function to block the formation of AGEs or break the cross-links between AGEs and collagen,

which are a source of stiffening that occur with age, diabetes, and/or hypertension (Janíc *et al* 2014).

While pharmacological interventions are largely utilized to manage hypertension and arterial stiffness, they are not without adverse effects (Golomb & Evans 2008; Tedla & Bautista 2016). Approximately 20-97% (depends on type of medication) of patients who take anti-hypertensive medications experience undesirable side effects, and it has been determined that there is an independent association between drug-related side effects and lower compliance to medication (Tedla & Bautista 2016). For most patients and practitioners, the benefits of these pharmacological interventions outweigh the adverse effects (Golomb & Evans 2008); however, therapies that can provide the benefits without the risks are highly sought after.

Nonpharmacological

Nonpharmacological therapies are utilized as the first-line approach for intervention against cardiovascular risk factors and are especially useful for preventative measures (Tanaka & Safar 2005; Whelton *et al* 2017). Nonpharmacological interventions can be accomplished through behavioural strategies aimed at lifestyle changes and diet modifications (Whelton *et al* 2017). Lifestyle changes include weight loss for overweight or obese individuals, increased physical activity (particularly aerobic or endurance exercise), and limited daily alcohol consumption (Tanaka & Safar 2005; Whelton *et al* 2017; Wu *et al* 2015). Dietary modifications for hypertension, as recommended by government organizations, include reduced intakes of sodium, saturated and trans fat, and increased intakes of fruits and vegetables, potassium, whole grains, and dietary fibre (Clark *et al* 2015; Whelton *et al* 2017). Currently, government dietary guidelines for reducing arterial stiffness are not available; however, intervention studies have presented dietary suggestions based on scientific evidence. These suggestions include reduced

sodium intake, or increased ingestion of omega-3 fatty acid supplements, anti-oxidant vitamins (ascorbic acid and α-tocopherol), and polyphenols (bioactive plant compounds) (Sacre *et al* 2014; Tanaka & Safar 2005). Additionally, daily consumption of pulses (dried beans, dried peas, chickpeas, lentils) over eight weeks has been shown to improve arterial stiffness in individuals with peripheral artery disease (Zahradka *et al* 2013).

2.3 The Common Bean

Of the four pulses that are global staple foods, dried beans are the most widely produced and consumed non-processed legume (Zhu *et al* 2012). The common dry bean was first cultivated in Peru and Mexico around 8000 years ago and is now cultivated worldwide (Ganesan & Xu 2017). Beans are grown for their green leaves, green pods, and immature and/or dry seeds (Ganesan & Xu 2017); however, only the dry edible seeds are 'pulses' (Clark *et al* 2018). Canada produces a wide variety of dried beans, with production mainly in Manitoba, Quebec, and Ontario (Pulse Canada 2019). In 2018, Manitoba farmers seeded over 120,000 acres of edible beans (Government of Manitoba 2018).

Nutritional Content of Beans

Carbohydrates are the predominant macronutrient in dried beans (approximately 64% w/w (**Table S4.1**)), present primarily as starch which contributes 60-65% of the energy content (Hutchins *et al* 2012). All bean varieties contain a higher ratio of slowly digestible:readily digestible starch compared to other starchy foods. Beans also contain a large amount of resistant starch, which can be considered as dietary fibre. Accordingly, beans are an excellent source of fibre, providing 3-9 grams of soluble and insoluble fibre per half-cup serving (Hutchins *et al* 2012). They contain a high amount of protein, approximately 15-35% w/w (depending on the cultivar; average composition is 24% w/w according to Table S4.1), with a high protein

digestibility of around 80% (Ganesan & Xu 2017); for example, 100 grams of beans providing 25 grams of protein contributes approximately 20% of energy from protein (Ganesan & Xu 2017). They are rich in essential amino acids such as lysine, but lack methionine and tryptophan (Anderson *et al* 1999), making beans a great complementary plant food with grains (cereals, corn, rice) (American Society for Nutrition 2011; Ganesan & Xu 2017). Beans contain little fat, generally less than 3% of the energy content (average of 2% w/w (Table S4.1)), but they do provide some monounsaturated and polyunsaturated fatty acids (Anderson *et al* 1999).

In terms of micronutrients, dry beans are high in folate, iron, magnesium, potassium, zinc, phosphorus, calcium, and copper, but are low in sodium (Anderson *et al* 1999; Hutchins *et al* 2012).

Non-Nutritive Bioactive Compounds in Beans

Dried beans also contain several non-nutritive bioactive compounds that confer negative and positive outcomes (Messina 2014; Pedrosa *et al* 2015). These bioactive compounds, formerly referred to as anti-nutritional factors (Pedrosa *et al* 2015), can be broadly termed as types of "phytochemicals" (Lampe & Chang 2007) and include trypsin inhibitors, phytates, tannins, alpha-galactosides and oligosaccharides (Sampathkumar 2011), hemaglutinnins (Hernández-Infante *et al* 1998; Tsopmo & Muir 2010), and polyphenols (Pedrosa *et al* 2015). Heat processing of the raw dry bean seeds generally eliminates a majority of these compounds; however, not all the compounds are left inactivated by cooking (Aguilera *et al* 2009; Hernández-Infante *et al* 1998). Therefore, it is important to understand the balance between the positive and negative effects of these bioactive compounds to determine the overall functionality of cooked beans.

Trypsin inhibitors, in large quantities (Chung *et al* 1998), often receive negative attention due to their ability to bind endopeptidases, thus forming protein complexes that inactivate these enzymes and inhibit the digestion and absorption of protein (Sampathkumar 2011). Trypsin inhibitors can also have beneficial actions, such as inhibiting human cancer cell growth *in vitro* and promoting the release of cholecystokinin which is associated with feelings of fullness and satiety (Pedrosa *et al* 2015).

Alpha-galactosides and oligosaccharides are responsible for flatulence from bean consumption (Sampathkumar 2011). The presence of small amounts of these carbohydrates in the diet facilitates an increase in the bifidobacteria population in the colon by acting as prebiotics (Pedrosa *et al* 2015).

Phytates in beans can form insoluble complexes with minerals, such as zinc and iron (Thavarajah *et al* 2009), thereby lowering their bioavailability (Sampathkumar 2011). However, low doses of phytates can reduce blood glucose levels, exert an anti-oxidant effect, and act as a prebiotic (Pedrosa *et al* 2015).

Hemagglutinins cause red blood cells to clump together in a mass (Goddard & Mendel 1929).

Polyphenols are another contributor of bioactivity within beans. Excessive amounts of polyphenols may inhibit the bioavailability of iron and protein by forming insoluble complexes (Singh & Basu 2012). However, polyphenols have shown benefits through anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-adipogenic, and anti-diabetic activities (Cardona *et al* 2013; Singh & Basu 2012). The polyphenolic compounds that make up the different seed coat pigments generally show the greatest variation between bean types. The colour of the seed coat is based on the presence, or absence, of certain polyphenols, such as anthocyanins, and condensed

tannins (Ganesan & Xu 2017). Darker-coloured beans, such as red kidney and black beans, normally have the highest anthocyanin content (Singh & Basu 2012), whereas lighter colours such as yellow and pink, are generally based on the presence of condensed tannins (Ganesan & Xu 2017).

Contrary to their name, navy beans are not blue in colour, but rather are light-coloured white beans. The 'navy' component of their name derives from having been a source of sustenance in the diet of the U.S. Navy (Ganesan & Xu 2017). Researchers have recently ascertained that the lack of red/black pigmentation in navy beans is due to a recessive phenotype for the *Pigment (P)* gene (McClean *et al* 2018), where *P* encodes a specific transcription factor necessary for the activation of the anthocyanin pathway (McClean *et al* 2018). Therefore, navy beans do not contain anthocyanins.

2.4 Phytochemicals

While the previously termed 'anti-nutritional factors' fall under the category of phytochemicals, 'phytochemicals' is a much broader term defining secondary metabolites produced by plants that are non-nutritive bioactive compounds (Carnauba *et al* 2016; Xiao *et al* 2017). Phytochemicals include more than a dozen subclasses, such as polyphenolic compounds, terpenoids, organosulfur compounds, phytosterols and phytostanols, saponins, alkaloids, tocotrienols, and nondigestible carbohydrates (Carnauba *et al* 2016; Upadhyay & Dixit 2015; Xiao *et al* 2016).

Polyphenols are the most abundant and studied phytochemicals and constitute a largely diverse group of water-soluble compounds (Bohn *et al* 2015; Lampe & Chang 2007). Their total dietary intake can be as high as 1 gram/day, placing polyphenols above all other classes of phytochemicals in terms of intake (Scalbert *et al* 2005). Polyphenols can be divided into five

subclasses: flavonoids, stilbenes, phenolic acids, coumarins, and tannins (Lampe & Chang 2007). Flavonoids and phenolic acids comprise most of the polyphenolic compounds found in dry beans (Hart *et al* 2015; Ranilla *et al* 2007).

Digestion, Absorption, Metabolism, and Bioavailability

Phytochemicals are present in plants as glucosides or other conjugates that need to be hydrolyzed for absorption (Lampe & Chang 2007). Following oral ingestion, phytochemicals are hydrolyzed to produce aglycones by β-glucosidases at the intestinal brush border membrane, or by bacterial β-glucosidases in the lower small intestine and colon (Lampe & Chang 2007). Since phytochemicals are recognized by the body as xenobiotics, they undergo phase I and/or phase II metabolism after absorption (Lampe & Chang 2007; Xiao *et al* 2016). However, not all phytochemicals undergo Phase I metabolism; lignans, isoflavones, some flavonoids, and isothiocyanates are examples of compounds that undergo Phase I reactions such as oxidation and hydroxylation in the liver (Bohn *et al* 2015; Lampe & Chang 2007). Phase II metabolism consists of conjugation with glutathione, glucuronic acid, or sulfate by transferases in the liver or gut epithelium (Bohn *et al* 2015; Lampe & Chang 2007). Polyphenols are mostly metabolized by colonic microbiota, and therefore the colon may be a major site for polyphenol uptake (Bohn *et al* 2015). Many phytochemical metabolites are formed after these different metabolism phases, and they can be released into the systemic circulation and be taken up by the cells of various tissues (Bohn *et al* 2015; Lampe & Chang 2007; Selby-Pham *et al* 2017). Metabolites formed during Phase II metabolism can also be excreted in urine or bile, or recycled back to the small intestine through bile to be deconjugated by microbial glucuronidases and undergo enterohepatic recycling (Bohn *et al* 2015; Lampe & Chang 2007).

Bioavailability refers to the proportion of an ingested compound in food that is absorbed and available for normal metabolic and physiological functions or storage (Gibson *et al* 2006). Most phytochemicals, including polyphenols, exhibit low bioavailability due to a number of factors, such as solubility, stability, and metabolic processing *in vivo* (Upadhyay & Dixit 2015). However, even with their low bioavailability, phytochemicals have demonstrated favourable effects as a result of high potency against chronic diseases, including effects on the pathogenesis of CVDs (Upadhyay & Dixit 2015). Given the quantity and diversity of phytochemicals, the mechanisms by which they exert their biological activities in the body are vast and complex, and thus, not fully understood. However, phytochemicals have demonstrated their ability to modulate endogenous compounds in the body, such as vasodilators, pro-inflammatory molecules, anti-oxidant enzymes, and cell growth markers, which may be responsible for the observed biological actions (Upadhyay & Dixit 2015).

Metabolomics

Metabolomics employs techniques aimed at measuring low molecular weight molecules (metabolites) in a cell, tissue, or organism, and can be divided into two approaches: targetted and untargetted (Roberts *et al* 2012). Targetted metabolomics involves predefining lists of analytes to be measured in the desired sample (Roberts *et al* 2012). In contrast, untargetted metabolomics involves a comprehensive analysis of all measurable analytes in a sample, including those that are unknown (Roberts *et al* 2012). This approach provides an opportunity to identify novel metabolites by measuring any molecule that ionizes within a specific range of mass values (Mayengbam *et al* 2015; Vinayavekhin & Saghatelian 2010); however, identifying unknown compounds is a labourious process for which high-throughput approaches do not exist (Hamdalla *et al* 2013).

There are many analytical techniques utilized in the field of metabolomics; however, mass spectrometry with liquid or gas chromatography, and nuclear magnetic resonance spectroscopy are currently the major techniques used to profile large numbers of metabolites (Hamdalla *et al* 2013). The human metabolome consists of two primary fractions: (1) the endogenous metabolome of all metabolites from the host, including chemicals needed for, or extracted from, cellular metabolism; and (2) the food metabolome of chemicals derived from foods after digestion and metabolism (Scalbert *et al* 2014). Fortunately, with the assistance of various databases, such as Human Metabolome Database (HMDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG), many compounds related to human endogenous biochemical compounds have been identified (Hamdalla *et al* 2013).

Therefore, metabolomics offers multiple avenues for exploring metabolites within the context of food and health, including: (1) measuring metabolites within food samples (plant- or animal-specific metabolomes); (2) measuring food metabolites in the human body (food metabolome); and (3) measuring changes to human endogenous compounds in response to foods (endogenous metabolome).

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Chapter 3: Rationale, Hypotheses, and Objectives

3.1 Rationale

Hypertension and arteriosclerosis are traditional risk factors for CVD (Ecobici & Stoicescu 2017). It is projected that 90% of adults will develop hypertension in their lifetime (Whelton *et al* 2017), and both hypertension and arterial stiffness (arteriosclerosis) are increased with age (Sun 2015). The development of hypertension and arterial stiffness has both dynamic and stable components, due to the constituents of the vascular wall. These dynamic and stable actions reflect acute and chronic changes within the vasculature. For instance, fluctuations in vascular function can be observed over the short-term due to recurring modulation of the interactions between cells via humoral factors (e.g. vascular tone), while long-term adaptations can be observed as the ECM is altered (e.g. vascular remodelling) (Alvim *et al* 2017). Studies have shown that eating pulses (dried seeds of beans, peas, lentils, and chickpeas) over many weeks improves blood vessel structure (remodelling) and function in rodents and humans with arterial stiffness and hypertension (Hanson *et al* 2014; Hanson *et al* 2016; Zahradka *et al* 2013). However, the acute postprandial effects of pulses on vascular responses, i.e. vascular tone, have not been investigated. This is an important knowledge gap to be addressed, as poor postprandial vascular responses, when habitually repeated, promote the development of arteriosclerosis (Intengan & Schiffrin 2001; Li *et al* 2013; Staiculescu *et al* 2013). Therefore, a question to be addressed is whether pulses can maintain blood vessel health before arterial disease is established in humans.

The vascular benefits of pulses, such as dried beans, are often attributed to their soluble fibre content and the subsequent effects on cholesterol metabolism (Gunness & Gidley 2010; Zhu *et al* 2012). However, key studies in humans (Zahradka *et al* 2013) and animals (Hanson *et*

al 2014) have shown that vascular improvements produced after eating pulses occur independent of reduced serum cholesterol. These findings indicate that soluble fibre is not the factor responsible for the observed improvements in vascular structure and function, and this is supported by a recently published review (Ravnskov *et al* 2018). Rather, it is likely that phytochemical compounds present in these seeds must act through either a direct or indirect mechanism on the vessel wall. However, the multitude of phytochemicals in pulses makes it difficult to determine which one(s) are the most biologically active with respect to vascular structure and function, and the mechanism(s) by which they operate.

Changes occurring in the body can be reflected by profiling metabolites of the human metabolome (Takahashi *et al* 2018). Metabolomics provides opportunities to explore changes in endogenous metabolites thus providing insight into the molecules and/or pathways modulated in response to the consumption of certain foods, such as pulses. Studies have shown that plant compounds can produce vascular relaxation through many endogenous mechanisms, such as inhibition or activation of certain pathway activators (e.g., prostaglandins, NO, cyclic adenosine monophosphate), blocking or releasing ions (K^+, Ca^{2+}), blocking or activating ion channels, and many others (Luna-Vázquez *et al* 2013). Therefore, an untargetted approach to profile the changes in endogenous serum metabolites may provide insight into the mechanism(s) of action responsible for vascular outcomes occurring in response to pulse consumption.

Differences in biological functionality have been reported for lentils with different seed coat colours. Hanson *et al* (2016) determined that while green lentils and red lentils improved vascular compliance in SHR compared to the SHR-control group, green lentils had a greater magnitude of effect compared to red lentils as evidenced by green lentils improving vascular compliance such that it was similar to normotensive WKY rats. While green lentils were the

most efficacious lentil for vascular function, red lentils were the most effective for reducing serum LDL- and HDL-cholesterol levels compared to SHR fed the control diet. These different biological responses were attributed to the differing phytochemical profiles associated with the seed coats of green versus red lentils. Typically, seed coat colour is used as a phenotypic marker of traits concerning seed quality and consumer acceptability (Chen 2016); however, given the differences between lentils with different seed coat colours on vascular function, seed coat colour may also be a phenotypic marker for vascular functionality among pulses. Therefore, to address this knowledge gap, the research described in this dissertation was focused on examining the effect of dry beans on vascular function, as they have a variety of contrasting seed coat colours (red, tan/brown, white, and black) and thus a range of distinct phytochemicals (Ganesan & Xu 2017). Additionally, while lentils previously proved effective for improving vascular structure and function in SHR (Hanson *et al* 2016), it is unknown if individual bean types can modify the structure and function of blood vessels during hypertension. Beans are the most widely produced and consumed pulse crop in the world (Ganesan & Xu 2017; Pedrosa *et al* 2015; Pitura & Arntfield 2019). Therefore, given that 90% of adults will develop hypertension in their lifetime (Whelton *et al* 2017), beans may represent a nutrient-rich, inexpensive, widely available dietary approach for improving vascular health (Pulses.org 2019; Ganesan & Xu 2017).

As previously stated, key studies have shown long-term consumption of pulses (4-8 weeks) can improve vascular function in subjects with hypertension and/or arterial stiffness (Hanson *et al* 2014; Hanson *et al* 2016; Zahradka *et al* 2013). At the same time, it is unknown if pulses, such as dried beans, need to be habitually consumed to maintain their beneficial effects on the vasculature, or if their biological effects are retained after long-term consumption is halted. Therefore, to address this knowledge gap, vascular structure and function should be

assessed at time intervals after stopping chronic bean consumption to ascertain how long the vascular effects of beans are maintained once their intake is discontinued.

To address the knowledge gaps surrounding the vascular functionality of beans, the studies reported in this thesis utilized a clinical trial (Chapters 4 & 5) and an animal study (Chapter 6) for the following reasons:

1. The use of human participants in the clinical trial (Chapter 4) allowed noninvasive *in vivo* measurements of postprandial vascular responses at multiple time points. In comparison, in animal study designs, vascular tone responses are often measured *ex vivo* on vascular tissue as an end-point outcome after animal sacrifice (Caniffi *et al* 2016; Di Pietro *et al* 2018; Spijkers *et al* 2012; Tatchum-Talom *et al* 2011). Thus, the use of a clinical study provides an alternative approach for obtaining acute vascular response measurements that avoids unnecessary use of animals, thereby complying with the Three Rs of animal welfare (Replacement, Reduction, and Refinement) (Canadian Council on Animal Care 2019). Furthermore, having the research completed in humans means that the findings are immediately translatable.
2. The crossover design of the clinical study in Chapter 4 was an economical use of resources as it required fewer participants than would a parallel clinical study or an animal study. This is due to each participant being used several times in the crossover design rather than having separate groups of participants for treatment comparisons, as would be required for the two latter study designs (Velengtas *et al* 2012). The crossover design also allows each participant to act as his or her own control, thereby eliminating between-participant variability, and this design permits within-participant and between-participant comparisons (Mills *et al* 2009).

3. The metabolomics analysis (Chapter 5) required 350 μL of serum at each time point (baseline, 2 hours, and 6 hours) for profiling endogenous metabolites in response to consuming the four bean types or white rice. Approximately 875 μL of blood would be required for obtaining 350 μL of serum (Quest Diagnostics Inc 2019). The Canadian Council on Animal Care instructs no more than 7.5% total blood volume collected over 24 hours per rodent (Gourdon 2016). Therefore, for a 330g rat (approximate average body weight of WKY and SHR at baseline (Chapter 6)), 525 μL is the maximum blood volume that could be collected per draw, which would be insufficient for metabolomics analysis during a time course study.
4. The investigation of how beans affect remodelling and mechanics of hypertensive arteries (Chapter 6) requires the removal of vascular tissues from the animals to obtain structural (aortic remodelling) and functional (small-artery mechanics) measurements. Thus, the invasive nature of this study, particularly the removal of the aorta, was not appropriate for living human participants.
5. The SHR is the most commonly used animal model for essential hypertension research (Doggrell & Brown 1998), and has been used previously in key studies investigating the effects of pulses on hypertension-induced vascular remodelling and dysfunction (Hanson *et al* 2014; Hanson *et al* 2016). Therefore, the SHR and its normotensive control, the WKY rat, were appropriate animal models to investigate the effects of different coloured beans on hypertension-induced vascular remodelling and dysfunction (Chapter 6).

Overall, this research project was divided into three main chapters to investigate the novel effects of different colored dry beans to determine their potential for improving vascular health.

This was achieved by assessing the efficacy of several bean varieties with distinct seed coat colours on: 1) acute vascular tone in a randomized crossover controlled clinical study (Chapter 4); 2) postprandial modulation of endogenous serum metabolites utilizing untargetted metabolomics for insight into potential mechanism(s) contributing to postprandial vascular responses (Chapter 5); and 3) hypertension-induced structural and mechanical changes in SHR (Chapter 6).

3.2 Hypotheses

The overall hypothesis for this research project was:

Black beans with dark seed coat colour, an indicator of greater variety and/or amount of phytochemicals, will be the most effective bean type for improving postprandial and chronic vascular function.

The overall hypothesis was tested by separating its various components into the following sub-hypotheses:

1. Black beans, with a greater variety and/or quantity of phytochemicals, as evidenced by their dark seed coat colour, will yield greater improvements in postprandial vascular outcomes compared to rice as the control (Chapter 4).

2. Consumption of darker-coloured beans (red kidney or black beans) will differentially modulate endogenous compounds related to acute vascular function, either directly or indirectly, compared to consumption of lighter-coloured beans (navy or pinto beans) (Chapter 5).

3. Consumption of black beans, which have a more extensive phytochemical composition (amount and/or variety) as indicated by the darker seed-coat colour, will produce greater improvements in blood vessel structure and mechanics in hypertensive rats compared to consumption of lighter-coloured navy beans (Chapter 6).

3.3 Objectives

1. Compare the postprandial vascular and metabolic responses of different bean varieties (black, navy, pinto, and red kidney) in healthy adults on:

    a. Haemodynamic parameters (blood pressure and total vascular resistance assessed by a Mobil-O-Graph)

    b. Vascular tone parameters (pulse wave velocity, wave reflection magnitude, augmentation pressure, and augmentation index assessed by a Mobil-O-Graph)

    c. Serum biochemistry (glucose, cholesterol, triglycerides).

This objective is addressed in Chapter 4.

2. Determine if the consumption of different bean varieties (black, navy, pinto, and red kidney) alters the presence of endogenous compounds in blood at 2 and 6 hours postprandially in healthy adults using an untargetted metabolomics approach employing liquid chromatography-quadrupole time-of-flight-mass spectrometry (LC-QTOF-MS).

This objective is addressed in Chapter 5.

3. Investigate the effects of dietary intervention with black or navy beans (*vs* bean-free control diet) for 8 weeks in SHR on:

a. Arterial stiffness (pulse wave velocity);

b. Blood pressure (tail cuff plethysmography);

c. Vascular compliance of mesenteric resistance arteries (pressure myography);

d. Vascular remodelling of aorta (histology and morphometry);

e. Metabolic parameters (body composition and serum lipids (cholesterol and triglycerides)).

This objective is addressed in Chapter 6.

4. Determine if the improved vascular function in SHR observed after 8 weeks of bean consumption is maintained at 2 weeks and 4 weeks after bean consumption is discontinued by assessing:

a. Arterial stiffness (pulse wave velocity);

b. Blood pressure (tail cuff plethysmography);

c. Vascular compliance of mesenteric resistance arteries (pressure myography);

d. Vascular remodelling of aorta (histology and morphometry).

This objective is addressed in Chapter 6.

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Chapter 4: Darker-Coloured Beans Induce Positive Postprandial Vascular Responses in Healthy Adults: A Randomized Crossover Study

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4.1 Abstract

Consuming pulses (dried beans, dried peas, chickpeas, lentils) over several weeks can improve vascular function and decrease CVD risk; however, it is unknown whether pulses can positively modulate postprandial vascular responses. The objective of this study was to compare different bean varieties (black (BB), navy (NB), pinto (PB), red kidney (RKB)) and white rice for their acute postprandial effects on vascular and metabolic responses in healthy individuals. The study was designed as a single-blinded, randomized crossover trial with a minimum 6 days between consumption of the food articles. Vascular (primary and secondary endpoints) and serum biochemistry (tertiary endpoints) parameters were measured in 8 healthy adults before and at 1, 2, and 6 hours after eating ¾ cup of beans or rice. BP and PWV were lower at 2 hours following RKB and PB consumption compared to rice and NB, respectively ($p<0.05$). There was greater vasorelaxation 6 hours following consumption of darker-coloured beans, as shown by decreased vascular tone: PWV was lower after consuming BB compared to PB, augmentation pressure was lower after consuming BB compared to rice and PB, and wave reflection magnitude was lower after consuming RKB and BB compared to rice, NB, and PB ($p<0.05$). LDL-cholesterol concentrations were lower 6 hours after BB consumption compared to rice ($p<0.05$). Overall, darker-coloured beans elicited a positive effect on the tensile properties of blood vessels, and this acute response may provide insight for how pulses modify vascular function.

4.2 Introduction

Atherosclerosis is a CVD that progresses silently over several decades until manifesting to a life-threatening clinical event (Karunakaran *et al* 2016; Kavurma *et al* 2017). The stealth of its manifestation prevents early recognition and intervention, leading to atherosclerosis being accountable for approximately 30% of deaths worldwide (Roquer & Ois 2010).

Over the last few years, studies have shown that eating pulses (dried beans, dried peas, lentils, chickpeas) for several weeks can improve the function and structure of arteries. Peripheral artery disease (PAD) is characterized by a decrease in blood flow to the limbs due to the presence of atherosclerotic plaque. Zahradka *et al* (2013) reported that consumption of ½ cup of cooked mixed pulses daily for 8 weeks by individuals with PAD significantly improved the ankle-brachial index, indicating improved blood flow to the legs. Additionally, consumption of lentils (30% w/w) attenuated arterial wall thickening within 4 weeks (Hanson *et al* 2014) and improved arterial compliance over 8 weeks (Hanson *et al* 2016) in SHR. While this evidence demonstrates the ability of pulses to improve arterial structure and function with chronic consumption, whether pulses can affect factors that occur at earlier stages before arterial disease becomes established remains unexplored.

Vascular tone is the slightly contracted basal state of the arteries required to modulate blood pressure and blood flow that is achieved by a fine-tuned balance between vasodilation and vasoconstriction (Jackson 2000; Orshal & Khalil 2004; Wong *et al* 2010). People spend the majority of the day in the postprandial state (Delgado-Lista *et al* 2011; McManus *et al* 2016), and evidence is emerging that certain foods, particularly high-fat meals characteristic of the Western diet, can impair vascular tone (Jackson 2000; Jakulj *et al* 2007; Rudolph *et al* 2007; Vogel *et al* 1997). This suppression of postprandial vascular tone responses, when repeated

every few days or multiple times per day, can promote arterial remodelling and arterial stiffness (Intengan & Schiffrin 2001; Li *et al* 2013; Staiculescu *et al* 2013), known factors in the development of atherosclerosis (van Varik *et al* 2012). Therefore, given the influence of diet on vascular tone, the importance of vascular tone for long-term cardiovascular health, and the evidence that long-term consumption of pulses improves vascular function, our objective was to determine if pulses, such as dried beans, can improve postprandial vascular responses.

This unique study compared the postprandial effects of dried bean varieties with contrasting seed coat colours [navy (white), pinto (tan/brown), red kidney, black beans] to represent differing phytochemical profiles, and white rice, on vascular and metabolic responses in healthy individuals. We hypothesized that black beans, with a greater variety and/or quantity of phytochemicals, as evidenced by their dark seed coat colour, would yield greater improvements in postprandial vascular outcomes compared to rice as the control.

4.3 Materials and Methods

Participants

Eight healthy individuals, four men and four women, were recruited via advertisement through various media from Winnipeg, Canada, between April 2015 and January 2016. Eligibility criteria were males or females ≥18 to ≤50 years of age; a normal blood lipid profile (total cholesterol <4.6 mmol/L, high-density lipoprotein (HDL)-cholesterol >1.1 mmol/L, low-density lipoprotein (LDL)-cholesterol <3.0 mmol/L, triglycerides <1.7 mmol/L), creatinine ≥35 to ≤97 μmol/L, alanine aminotransferase <30 U/L for men and <25 U/L for women, and glycated hemoglobin <6%; systolic blood pressure <140 mmHg, diastolic blood pressure <90 mmHg; body mass index ≥20 to ≤30 kg/m^2; a stable regimen for the past 3 months if taking vitamin and mineral/dietary/herbal supplements; and willingness to comply with the protocol requirements

and provide informed consent. The exclusion criteria included allergies to beans, bean flour, bean products, or rice; the presence of a clinically diagnosed disease affecting the circulatory, respiratory, immune, skeletal, urinary, muscular, endocrine, digestive, nervous, or reproductive systems that required medical treatment; use of prescribed medications within 3 months prior to study enrolment, or supplements that affect gastrointestinal function within 3 months prior to study enrolment; weight loss ≥3 kg within the 6 months prior to study enrolment; a bacterial, viral, or fungal infection within 30 days prior to study enrolment; regular intake of over-the-counter medications; and women who were pregnant or lactating.

Study Design

The study was a single-blinded, crossover, randomized controlled trial. Each participant completed six visits separated by a minimum of six days. At each visit the participant arrived in the fasted state and consumed ¾ cup of cooked beans (navy, pinto, red kidney, black) or rice with 100 mL of water. The order of the food samples (food articles) was randomized by a statistician. The research nurse responsible for all study measurements and biological sample collections during this trial was blinded to the identity of the food articles. This study was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee, and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study protocol was registered on ClinicalTrials.gov (NCT02342340). All participants provided written informed consent consistent with guidelines for the protection of human research participants.

Participants were enrolled in the study by the study coordinator and were instructed to maintain their usual diet except to refrain from consuming pulses (dry beans, dry peas, chickpeas, lentils) and pulse products, including isoflavone supplements.

Vascular tone parameters were the primary endpoints. Secondary endpoints were other determinants of vascular function such as BP and TVR. Tertiary endpoints were serum concentrations of glucose, blood lipids (total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and triglycerides (TG)) and nitric oxide (NO) products.

Food Article Preparation

All food articles were prepared by a Manitoba Health Certified Food Handler in a research/teaching laboratory kitchen at the University of Manitoba. The dry beans were sourced by Pulse Canada and prepared by the traditional stove-top boiling method following the instructions outlined by Pulse Canada (2019) and that were used previously by Zahradka *et al* (2013). In brief, the dry beans were soaked in water (1:3 wt/wt) overnight at 4ºC, rinsed the next day, and then cooked in commercially available low-sodium chicken broth (Campbell's; 1:3 wt/wt) for 60 minutes. Chicken broth was used as a cooking medium to improve the flavour and palatability of the food articles. The cooked beans were then rinsed, portioned into ¾ cup samples, and kept frozen at -20ºC until used. Samples of the cooked beans and rice underwent proximate analysis to determine composition of macronutrients and energy (**Table S4.1**). White basmati rice (Uncle Ben's) was cooked in low-sodium chicken broth (1:1.75 wt/wt) for 12 minutes. Once cooked, individual ¾ cup samples were portioned and frozen at -20ºC until used. At each study visit, the bean or rice samples were reheated in a microwave and served to the participant with 100 mL water for drinking. Consumption of the food articles was observed by a study coordinator and the time required for consumption was recorded.

Blood Sampling and Analytical Methods

Blood samples were collected using an intravenous catheter. Blood samples were allowed to sit for 30 minutes on ice and were then centrifuged for 10 minutes at 1800 *g*. Serum was prepared from blood samples collected at baseline, 1, 2, and 6 hours post consumption of the food articles.

Serum glucose, TC, HDL-C, LDL-C, and TG were determined using the automated Cobas c 111 Analyzer (Roche Diagnostics). Nitrates and nitrites are used clinically as markers of nitric oxide synthase activity and NO production, and an indication of vascular function (Derosa *et al* 2010). Serum nitrate/nitrite (NOx) were determined using a commercially available colorimetric assay kit (Cayman Chemical Co.).

Vascular Measurements

Peripheral and central vascular measurements were obtained using the Mobil-O-Graph monitor (I.E.M. GmbH) at each visit for three time-points: baseline, 2, and 6 hours. Outputs included SBP, DBP, mean arterial pressure (MAP), pulse pressure (PP), heart rate, CO, TVR, AP, AIx (normalized to a heart rate of 75 bpm), RM, and PWV.

Sample Size

The sample size was estimated based on studies where acute wave reflection (Klop *et al* 2014) or arterial elasticity (Grassi *et al* 2015; Sanchez-Aguadero *et al* 2017) was assessed following the consumption of a nutritional item, which indicated a sample size of 6-10 participants would be sufficient to detect postprandial changes in reflection parameters (two-sided, alpha = 0.05, and power = 0.8). RM and related vascular tone parameters were defined as the primary endpoints.

Statistical Analyses

The data were analyzed using a mixed methods linear model (PROC MIXED) and baseline as a covariate to identify significant differences among bean types and rice using SAS 9.3 software (SAS Institute Inc.). The Tukey-Kramer test was used to determine differences between least-squares means. Shapiro-Wilk and Levene's tests were used to verify normality and homogeneity of the data, respectively. Results were considered statistically significant at $p<0.05$.

4.4 Results

Participant Characteristics

A total of eight participants completed the study (**Figure 4.1**), and their characteristics are summarized in **Table 4.1**. Participants (4 males, 4 females) were 37 ± 11 years old (mean ± SD) and were considered healthy based on their body mass index, blood pressure and serum biochemistry at recruitment. All participants received each of the five food articles and were analyzed for the primary endpoints.

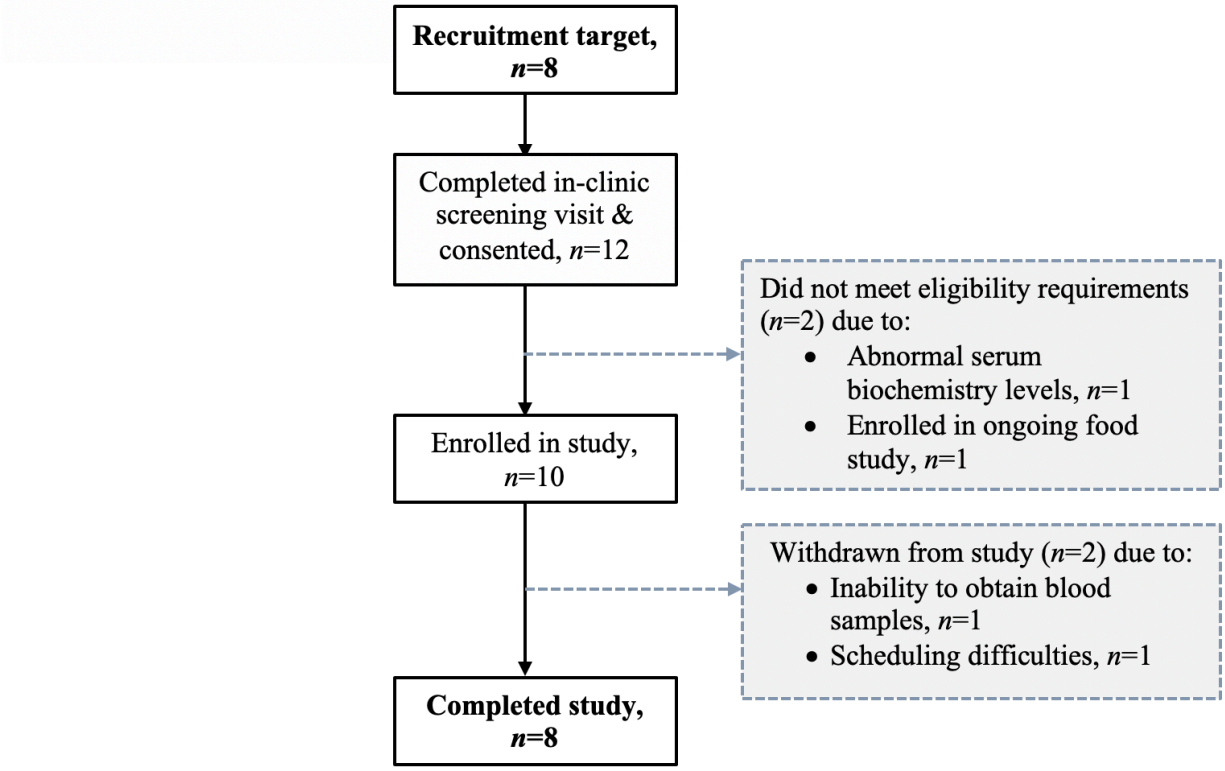

Figure 4.1 Participant recruitment and reasons for exclusion.

Table 4.1. Participant characteristics[a]

Characteristic	Mean ± SD (range)
Sex, male/female	4/4
Age (years)	37 ± 11 (21 to 50)
Height (cm)	170 ± 17 (152 to 192)
Weight (kg)	71.1 ± 15.1 (51.9 to 94.7)
Body mass index (kg/m^2)	24 ± 3 (21 to 27)
Waist circumference (cm)	83.2 ± 9.9 (71.0 to 95.5)
Systolic blood pressure (mmHg)	112 ± 8.61 (100 to 126)
Diastolic blood pressure (mmHg)	75 ± 6.45 (65 to 86)
Creatinine (µmol/L)[b]	75 ± 20 (53 to 97)
Alanine transaminase (U/L)[b]	15 ± 4 (10 to 21)
Total cholesterol (mmol/L)[b]	4.3 ± 0.8 (2.6 to 5.0)
HDL-cholesterol (mmol/L)[b]	1.4 ± 0.3 (1.0 to 1.9)
LDL-cholesterol (mmol/L)[b]	2.4 ± 0.6 (1.2 to 3.2)
Total cholesterol: HDL-cholesterol [b]	3.1 ± 0.6 (2.4 to 4.1)
LDL-cholesterol: HDL-cholesterol[b]	1.7 ± 0.5 (1.1 to 2.4)
Triglycerides (mmol/L)[b]	1.0 ± 0.4 (0.7 to 1.6)
HbA1c (%)[b]	5.4 ± 0.4 (4.6 to 5.8)

[a]Values are expressed as mean ± SD (minimum to maximum value), *n*=8.
[b]Fasting plasma concentrations.

Serum Biochemistry

Serum TG was higher after 6 hours compared to 1 and/or 2 hours following the consumption of rice, pinto beans, or red kidney beans (**Figure 4.2a**), and serum TC was higher after 6 hours compared to 1 and 2 hours following the consumption of white rice (**Figure 4.2b**). Serum glucose was lower after 6 hours compared to 1 and/or 2 hours following the consumption of white rice, red kidney beans, or black beans (**Figure 4.2c**). LDL-C was lower at 6 hours following the consumption of black beans compared to white rice (**Figure 4.2d**). There were no differences between the white rice or beans at 2 or 6 hours for serum glucose, TG, TC, or HDL-C (**Figure 4.2e**).

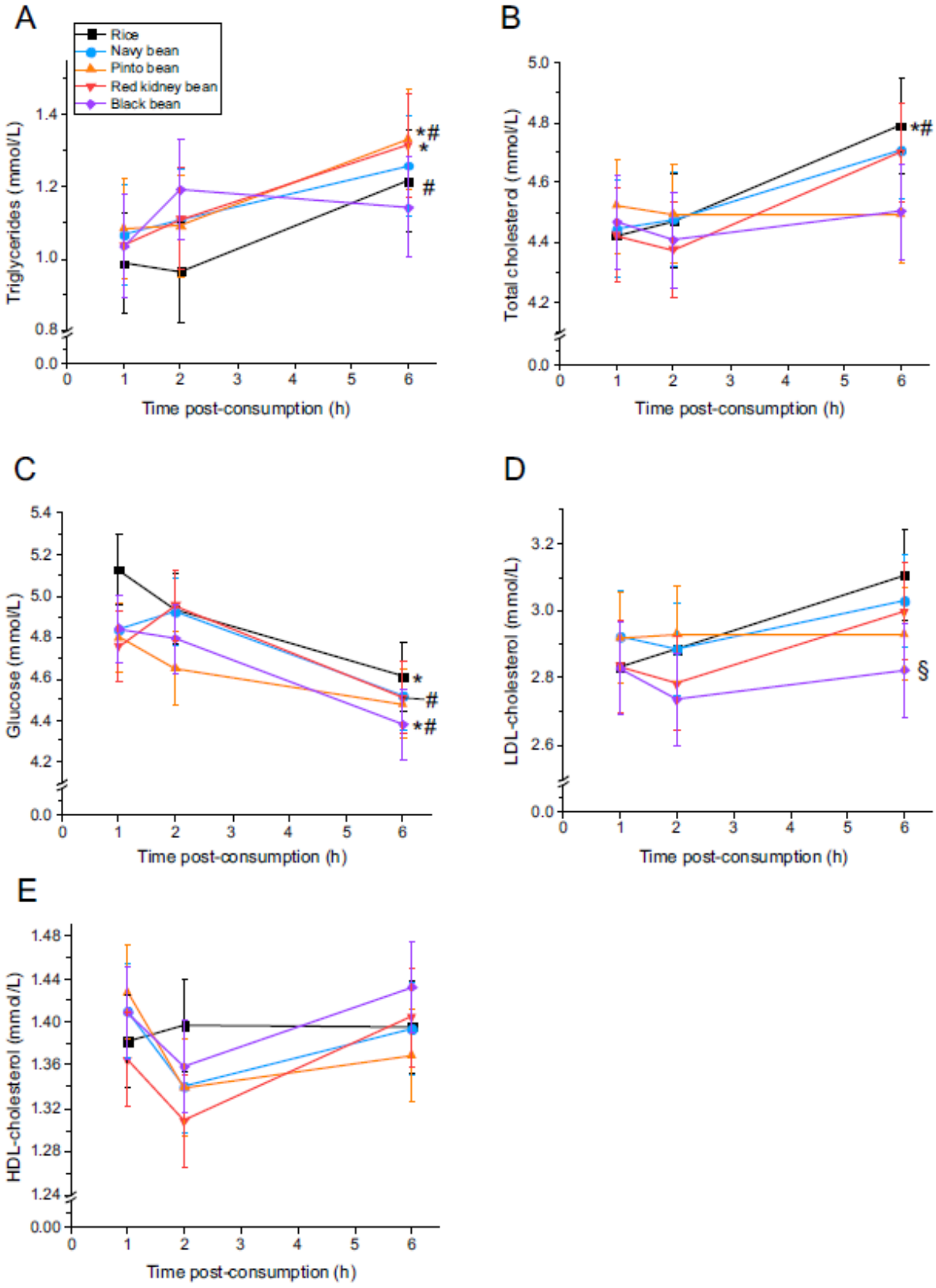
A
Rice
Navy bean
Pinto bean
Red kidney bean
Black bean
Triglycerides (mmol/L)
Time post-consumption (h)
B
Total cholesterol (mmol/L)
Time post-consumption (h)
C
Glucose (mmol/L)
Time post-consumption (h)
D
LDL-cholesterol (mmol/L)
Time post-consumption (h)
E
HDL-cholesterol (mmol/L)
Time post-consumption (h)

Figure 4.2 Postprandial metabolic responses.

(A) Serum triglyceride; **(B)** Serum total cholesterol; **(C)** Serum glucose; **(D)** Serum LDL-cholesterol; **(E)** Serum HDL-cholesterol. Results are expressed as least-squares means ± SEM, *n*=8. Data were analyzed using a mixed methods linear model (PROC MIXED; SAS version 9.3) with baseline as a covariate. *, significantly different ($p<0.05$) compared to 1-hour; #, significantly different ($p<0.05$) compared to 2-hours; §, significantly different ($p<0.05$) compared to rice control at the same time point.

Haemodynamics

SBP and MAP were lower at 2 hours following the consumption of pinto beans and red kidney beans, compared to the rice control (**Table 4.2**). SBP was also lower at 2 hours after consumption of red kidney beans compared to navy beans. There was a postprandial decrease in SBP and MAP at 6 hours compared to 2 hours following the consumption of pinto beans and red kidney beans. There were no differences in DBP between food articles or time points. PP was lower at 6 hours following the consumption of black beans compared to rice.

Pinto beans lowered TVR at 2 hours compared to navy beans and red kidney beans (Table 4.2). HR was higher at 6 hours after consumption of red kidney beans compared to rice, navy beans, and pinto beans. There was no effect of the food articles on HR at 2 hours or on CO at 2 hours or 6 hours.

Table 4.2 Haemodynamic measurements[1]

Parameter	Time (h)	Rice	Navy bean	Pinto bean	Red kidney bean	Black bean
Systolic blood pressure (mmHg)	2	120 ± 4	117 ± 4	114 ± 4§	111 ± 4§†	116 ± 5
	6	121 ± 4	121 ± 4	120 ± 4#	120 ± 5#	117 ± 4
Diastolic blood pressure (mmHg)	2	79 ± 3	78 ± 3	76 ± 3	76 ± 3	79 ± 3
	6	78 ± 3	81 ± 3	80 ± 3	82 ± 4	81 ± 3
Mean arterial pressure (mmHg)	2	98 ± 3	96 ± 3	93 ± 3§†	92 ± 3§†	95 ± 3
	6	98 ± 3	99 ± 3	98 ± 3#	99 ± 3#	98 ± 3
Pulse pressure (mmHg)	2	40 ± 3	39 ± 3	38 ± 3	35 ± 3	39 ± 3
	6	44 ± 3	39 ± 3	39 ± 3	38 ± 3	35 ± 3§
Total vascular resistance (s × mmHg/mL)	2	1.20 ± 0.06	1.25 ± 0.06‡	1.13 ± 0.06	1.24 ± 0.06‡	1.19 ± 0.06
	6	1.27 ± 0.06	1.25 ± 0.06	1.23 ± 0.06	1.37 ± 0.06	1.24 ± 0.06
Heart rate (bpm)	2	68 ± 3	68 ± 2	67 ± 2	70 ± 3	68 ± 3
	6	66 ± 2	66 ± 2	68 ± 2	73 ± 3§†‡	69 ± 3
Cardiac output (L/min)	2	4.9 ± 0.3	4.6 ± 0.3	5.1 ± 0.3	4.5 ± 0.3	4.9 ± 0.3
	6	4.7 ± 0.3	4.8 ± 0.3	4.9 ± 0.3	4.5 ± 0.3	4.8 ± 0.3

[1] Results are expressed as least-squares means ± SEM, n=8. Data were analyzed using a mixed methods linear model (PROC MIXED; SAS 9.3) and controlled for baseline as a covariate. #Compared to 2-hours (p<0.05); §Compared to rice at the same time point (p<0.05); †Compared to navy beans at the same time point (p<0.05); ‡Compared to pinto beans at the same time point (p<0.05).

Vascular Tone

Changes in vascular tone were ascertained by measuring parameters related to the arterial pulse wave as it propagates along the arterial tree. Specifically, we evaluated PWV (the velocity at which the pulse wave propagates along the arterial tree following left ventricular ejection), RM (the height of the reflected wave), AP (the boost in systolic pressure that follows wave reflection), and AIx (the proportion of AP in relation to PP) (Haun *et al* 2016; Jockel-Schneider *et al* 2014; Lillie *et al* 2015; Nichols *et al* 2008; Pereira *et al* 2015).

PWV was lower at 2 hours after consumption of red kidney beans and pinto beans compared to navy beans (**Figure 4.3a**), and at 6 hours after consumption of black beans compared to pinto beans. These lower PWV values are indicative of vessel relaxation since PWV is lower when vessels are more relaxed. There were no differences in AIx between food articles or time points (**Figure 4.3b**). AP was lower at 6 hours following consumption of black beans compared to rice and pinto beans (**Figure 4.3c**), revealing there was less systolic pressure following from the reflected waves, a further indication of vessel relaxation. Pulse wave RM was lower at 2 and 6 hours after red kidney bean consumption compared to rice (**Figure 4.3d).** Additionally, RM was lower at 6 hours after consumption of red kidney beans and black beans compared to navy and pinto beans. A lower magnitude of wave reflection (i.e. RM) indicates the vessels are more relaxed. RM was also lower at 6 hours compared to 2 hours post-consumption for pinto beans. The above reductions in postprandial PWV, AP, and RM indicate acute vessel relaxation, i.e. lower vascular tone.

Serum NOx reflect the amount of endothelium-derived NO available for vasodilation (Vrancken *et al* 2016), and thus was explored as a potential mechanism responsible for the

changes in vascular tone. There were no differences in NOx between the food articles or time points (**Figure 4.3e**).

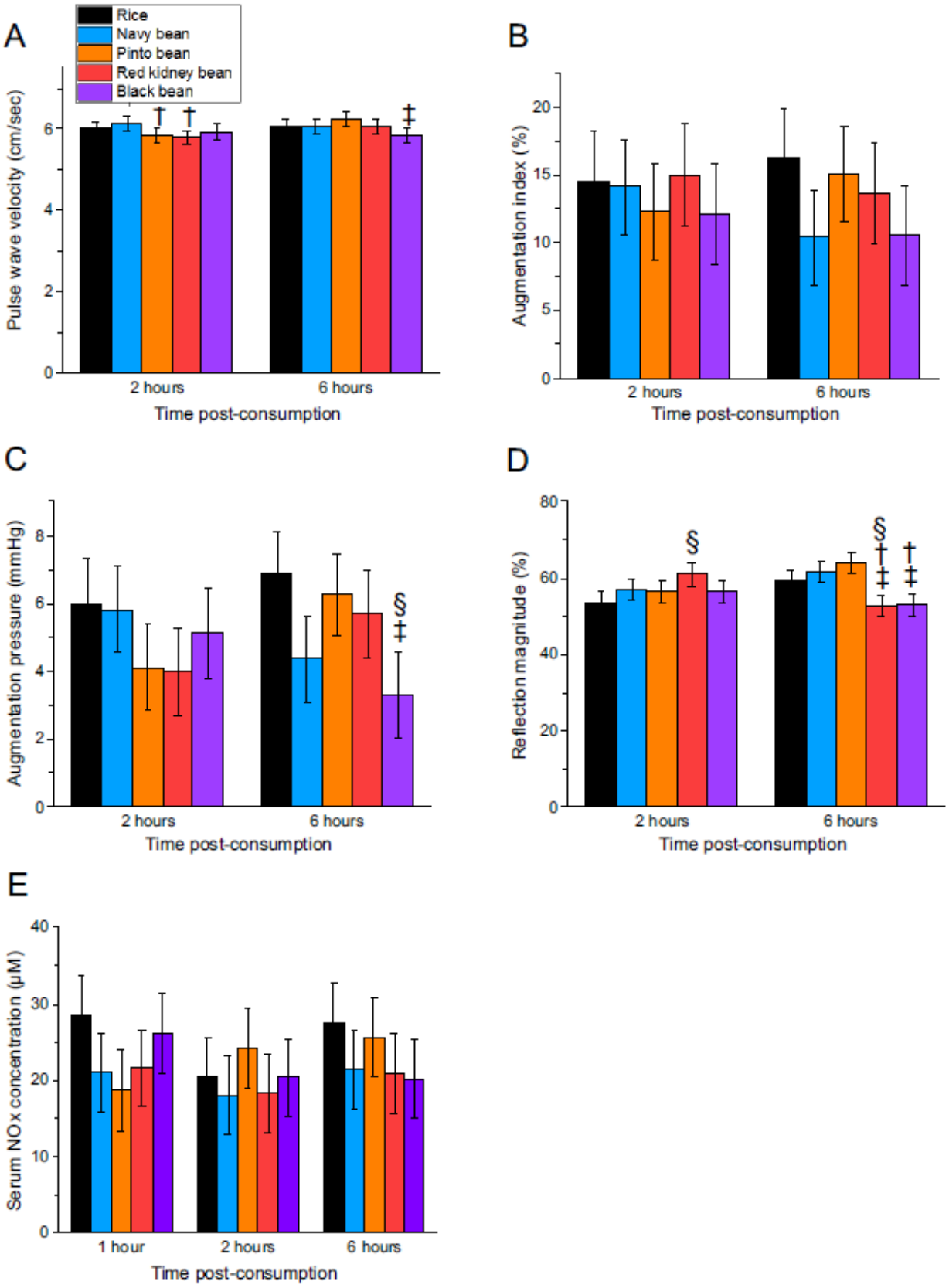
A
Rice
Navy bean
Pinto bean
Red kidney bean
Black bean
Pulse wave velocity (cm/sec)
0
2
4
6
† †
‡
2 hours
6 hours
Time post-consumption
B
Augmentation index (%)
0
5
10
15
20
2 hours
6 hours
Time post-consumption
C
Augmentation pressure (mmHg)
0
2
4
6
8
§
‡
2 hours
6 hours
Time post-consumption
D
Reflection magnitude (%)
0
20
40
60
80
§
§
†
‡
†
‡
2 hours
6 hours
Time post-consumption
E
Serum NOx concentration (μM)
0
10
20
30
40
1 hour
2 hours
6 hours
Time post-consumption

Figure 4.3 Postprandial vascular tone responses.

(A) Pulse wave velocity; **(B)** Augmentation index; **(C)** Augmentation pressure; **(D)** Pulse wave reflection magnitude; **(E)** Serum NOx. Results are expressed as least-squares means ± SEM, *n*=8. Data were analyzed using a mixed methods linear model (PROC MIXED; SAS 9.3) with baseline as a covariate. #, significantly different ($p<0.05$) compared to 2-hours; **§**, significantly different ($p<0.05$) compared to rice control at the same time point**;** †, significantly different ($p<0.05$) compared to navy beans at the same time point; ‡, significantly different ($p<0.05$) compared to pinto beans at the same time point. *Abbreviations:* NOx, nitrate/nitrite

4.5 Discussion

In the present study, we aimed to determine if cooked dry beans could improve postprandial metabolic and vascular responses in healthy individuals. Our principal finding was that darker-coloured beans induced the relaxation of blood vessels within a few hours of consumption as evidenced by lower values for parameters related to vascular tone (PWV, AP, and RM), compared to the lighter-coloured beans. This is the first randomized controlled trial to demonstrate the novel postprandial vasorelaxation capability of beans. Our second original discovery was that distinct responses were obtained for certain vascular (BP, TVR, PWV, AP, RM) and metabolic (serum LDL-C) parameters among the four types of coloured beans. To our knowledge, this is the first study to directly compare four different bean types and report different biological responses with respect to either vascular or metabolic parameters. Our data reveal that darker-coloured beans induce postprandial vasorelaxation by modulating vascular tone, which may supply long-term benefits given how repeated exposure to diets that acutely impair endothelial function and increase vascular tone can promote the development of atherosclerosis (Intengan & Schiffrin 2001; Vogel *et al* 1997). Thus, these observations support the inclusion of darker-coloured beans in the diet to improve vascular health and potentially help prevent the development of atherosclerosis.

We found that the postprandial biological responses measured in this study differed depending on the type of bean consumed. This is of considerable interest since to date the biological, and thus health effects, of beans have been generalized to include the *Phaseolus vulgaris* species as a whole, rather than comprehending that individual varieties may elicit distinct biological responses. For example, it is recognized that beans are able to lower cholesterol (Messina 2014; Surampudi *et al* 2016); however, among the four bean types

investigated in our study, only black beans produced lower serum LDL-C compared to rice at 6 hours, despite all bean types having similar amounts of fibre (Table S4.1). These findings indicate that the LDL-C-lowering obtained with black beans is most likely due to a specific component(s) that amplifies the known cholesterol-lowering effect of fibre. Further examples of the individual biological responses from the different bean varieties include lower AP and PP by black beans and lower TVR by pinto beans. Interestingly, the white-pigmented navy bean was the only bean type that did not induce a positive biological response compared to the other bean types or white rice. These results suggest seed coat colour, indicative of varying phytochemical profiles, may be a factor in the functionality of the different bean types.

The ability to positively modulate the postprandial vascular response has significant implications for improving vascular health (Bond *et al* 2015). We showed that consuming ¾ cup of darker-coloured beans can induce positive postprandial vascular responses, notably lowering BP and vascular tone. The lower SBP and MAP values at 2 hours following the consumption of red kidney beans and pinto beans, but none of the other food articles, indicates there is a potential BP-lowering effect specific to these two bean types. It is unknown if the differential effects among the four bean types on BP would be present with chronic consumption, or if long-term consumption would eventually induce a BP-lowering response by all four bean types. A meta-analysis revealed that a median intake of 162 g/day of cooked pulses for 10 weeks reduces SBP and MAP; however, none of the studies investigated beans separately from the other pulse types (Jayalath *et al* 2014). Hanson *et al* (2014) reported that chronic intake of a mixed bean diet (navy, pinto, red kidney, and black beans) had an intermediate BP-lowering effect in SHR compared to other pulse types. Based on the data obtained in the current study, it is possible that the navy and black beans blunted the response of the red kidney beans or pinto beans in the SHR

(Hanson *et al* 2014); however, only by testing the effect of the combination will it be possible to determine if this is the case.

The lower TVR values following consumption of pinto beans mirrors the lower MAP values at 2 hours. Since MAP is influenced by TVR and/or CO, MAP should be lower if TVR or CO decline (Siddiqui 2011). However, the reductions in TVR and MAP by pinto beans were different when compared to navy beans and rice, respectively, thus, the lower MAP is not associated with a lower TVR. The lower MAP values are most likely related to the lower SBP values observed for pinto beans, as well as red kidney beans, since SBP factors in the calculation of MAP.

Darker-coloured beans such as red kidney and black beans showed novel effects on vascular tone after 6 hours. This is the first study to demonstrate not only that beans have a postprandial vascular effect but also that darker-coloured beans induce postprandial vasorelaxation. NO was investigated as a potential mechanism by which vasorelaxation was induced; however, there were no observed changes in NOx among the darker-coloured beans. This lack of response suggests that the observed vasorelaxation is due to a signalling pathway other than NO, such as adrenergic receptors (Fok *et al* 2012; Leblanc & Tabrizchi 2018) or changes in specific vasoactive compounds (Djurica *et al* 2016; Schinzari *et al* 2018). These pathways may be stimulated by bioactive plant compounds within the darker-coloured beans.

Dry beans are rich in nutrients, such as slowly digestible carbohydrates, fibre, protein, B vitamins, iron, zinc, magnesium and potassium, that are suggested to impart cardiometabolic health benefits (Afshin *et al* 2014; Jenkins *et al* 2012; Mudryj *et al* 2014). However, these nutrients are not likely responsible for the observed changes in vascular properties, based on proximate analysis reporting minimal differences in macronutrient content among the bean types

examined in this study (Table S4.1). The most likely effectors are phytochemicals unique to each bean. For example, anthocyanins, which are responsible for the dark red, blue, and purple pigments in plants such as beans (Clark *et al* 2018), reportedly induce endothelium-dependent vasodilation within an acute timeframe (Zhu *et al* 2011). Therefore, further analysis intended to identify the individual phytochemicals within the bean types responsible for the observations we have reported is warranted as a means of determining the relationships between the bioactive plant compounds and the vascular effects that were detected.

The cardiovascular benefits of beans are often attributed to their hypocholesterolemic effects (Messina 2014). However, human (Zahradka *et al* 2013) and animal studies (Hanson *et al* 2014) have shown that improvements in the structure and function of blood vessels after consumption of pulses occur independently of changes in serum cholesterol. Furthermore, in the current investigation, a reduction in cholesterol was not observed in all cases where improvements in vascular parameters were obtained. Therefore, based on this evidence, we believe the postprandial changes in serum cholesterol in our study are not coupled to the observed changes in vasorelaxation. Interestingly, only black beans demonstrated a serum LDL-C lowering effect at 6 hours, suggesting that phytochemicals unique to black beans are responsible since there were similar macronutrient amounts among the bean types (Table S4.1). Anthocyanin intake over many weeks reportedly reduces serum LDL-C in individuals at risk for CVD, possibly via inhibition of cholesteryl ester transfer protein (Shah & Shah 2018). Whether this response is true in a healthy population within an acute timeframe is unknown; however, the presence of anthocyanins in black beans (Ganesan & Xu 2017) along with the LDL-C lowering effect observed in the current study warrants further investigation into this mechanism.

Confounding factors such as background diets and second meal effects are noted as limitations with respect to postprandial studies (Winham *et al* 2017). However, the randomized, controlled, crossover study design and minimum one-week separation between study visits of the current investigation reduces such confounders (Winham *et al* 2017).

Overall, our principal finding demonstrates that within hours of consuming darker-coloured beans, blood vessels relax. This novel conclusion suggests that phytochemicals, particularly those unique to the darker pigments in the bean seed coat, may be responsible for the observed positive postprandial changes in the tensile properties of the blood vessels. Our study, therefore, supports the inclusion of darker-coloured beans in the diet as a means of improving vascular health, and suggests that a mixture of different bean types will be more advantageous since this would broaden the potential benefits that can ensue.

Acknowledgements

A special thanks to Danielle Perera (study coordinator), Angela Wilson (research nurse), and the individuals who volunteered to participate in the study.

4.6 Supplementary Materials

Table S4.1 Proximate composition of food articles.

Proximate analysis (g/100 g DW)	Analytical method	White rice	Navy bean	Pinto bean	Red kidney bean	Black bean
Protein	AOAC 990.13*	8.58	24.18	23.13	24.36	23.15
Fat	AOCS Am 5-04	0.22	2.03	2.11	1.51	1.72
Available carbohydrate	n/a	87.57	62.52	63.60	62.79	65.24
Fibre	AOCS Ba6a-05	0.63	5.12	4.90	4.36	4.46
Total digestible nutrients	n/a	88.37	80.73	80.84	80.66	82.11
Total ash	AOAC 942.05	2.65	5.60	5.81	6.28	5.01

Determinations based on dry weight (DW) of cooked food samples. Proximate analysis was conducted by Central Testing Labs Ltd (Winnipeg, MB). *Modified from original AOAC method. Proximates without an available analytical method were determined by calculations performed by Central Testing Labs Ltd. *Abbreviations:* AOAC, Association of Official Analytical Chemists; AOCS, American Oil Chemist Society; DW, dry weight.

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Transition Statement One

Objective 1 of this thesis was to determine if beans induced positive postprandial vascular responses, thus indicating an acute effect on vascular function, and if the postprandial responses varied based on seed coat colour. The preceding chapter determined that beans with darker-coloured seed coats (red kidney beans and black beans) induced postprandial vasorelaxation in healthy adults 6 hours after consumption. Beans with lighter-coloured seed coats (navy beans and pinto beans) did not produce vasorelaxant effects. These findings indicate that bioactive compounds specific to the darker-coloured beans may be responsible for the observed vascular relaxation. However, neither the bioactive compounds nor their potential mechanisms of action were investigated in Chapter 4. Certain bioactive compounds, such as anthocyanins, can be difficult to detect in serum and food as they are sensitive to degradation during the extraction process. In lieu of this challenge, an alternative method for investigating the effects of food bioactives *in vivo* is to analyze circulating endogenous metabolites of the host organism. These metabolites reflect the changes in molecules and/or pathways occurring in the body, such as those changing in response to consuming beans. Therefore, the direction of change for circulating endogenous metabolites (reflective of molecules and/or pathways) in combination with observed biological responses (e.g. vasorelaxation) may provide insight into potential mechanisms of action for the biological response. Thus, the next chapter (Chapter 5) used an untargetted metabolomics approach to explore the effects of darker-coloured beans on circulating endogenous metabolites at time points relevant to the postprandial vasorelaxation observed in Chapter 4.

Chapter 5: The Effect of Darker-Coloured Bean Consumption on Endogenous Serum Metabolites in Healthy Individuals: Application of an Untargetted Metabolomics Approach

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5.1 Abstract

Chapter 5 describes the endogenous metabolite profiles of serum samples obtained from the study described in Chapter 4 which showed darker-coloured beans induced acute vasorelaxation in healthy adults. Specifically, untargetted metabolomics was performed on serum samples collected at baseline, 2 hours, and 6 hours after the consumption of darker-coloured beans (red kidney or black), lighter-coloured beans (navy or pinto), or white rice. The study was designed as a single-blinded, randomized crossover trial with a minimum 6 days between consumption of the food articles. Serum samples were extracted using acetonitrile and analyzed by high-performance liquid chromatography-quadrupole-time-of-flight-mass spectrometry (HPLC-QTOF-MS). Eighty-eight endogenous serum metabolites that may be relevant to the postprandial vascular responses observed in Chapter 4 were significantly increased or decreased among the samples obtained at baseline, 2 hours, or 6 hours. Out of these 88 compounds, 47 compounds were increased or decreased among the three time points after consumption of the darker-coloured beans. Only 5 compounds had identities corroborated by the Human Metabolome Database based on *m/z* ratios. Of these 5 compounds (absolute identities not confirmed), acylcarnitine levels, which may have links to vascular function, were lower after consuming black beans. In summary, consumption of darker-coloured beans induced unique changes in the endogenous metabolome that did not occur with lighter-coloured beans or white rice. However, most of the compounds showed conflicting database results and their identities could not be verified. Therefore, further investigation is required to confirm the identities of the compounds of interest and their potential mechanistic contributions toward vasorelaxation.

5.2 Introduction

In Chapter 4, it was reported that consuming darker-coloured beans, specifically red kidney beans and black beans, induced acute relaxation of blood vessels in healthy adults. In this unique study, we compared the postprandial vascular responses of dried bean varieties with contrasting seed coat colours [navy (white), pinto (tan), red kidney, black], and white rice, in healthy adults. It was assumed that each of the food articles had a distinct phytochemical profile, which would contribute to their respective biological effects in relation to vasorelaxation. Since the greatest effect was obtained after consuming the darker-coloured beans, but not the lighter-coloured beans, bioactive compounds specific to the darker-coloured beans may be responsible for inducing relaxation of the smooth muscle cells within the blood vessel wall.

The pigmented seed coat of beans contains a variety of phytochemical compounds, including polyphenols such as flavonols, anthocyanins, and tannins (Pitura & Arntfield 2019). However, it is difficult to detect these compounds in serum samples due to many factors, including their degradation during the extraction process, low oral bioavailability, and extensive metabolic modifications in the host, which may produce unknown metabolites (D'Archivio *et al* 2010; Manach *et al* 2004). Furthermore, profiling the parent bioactive compounds in foods and their metabolites in the host does not give indication of the physiological actions of the bioactive compounds. Therefore, an alternative approach is to analyze the serum levels of the host's endogenous metabolites as they change in response to the intake of bioactive compounds when foods, such as beans, are consumed. These changes in the endogenous metabolome, reflective of the postprandial actions of bioactive compounds, can potentially elucidate biochemical pathways that are modified by the consumption of foods and their bioactive compounds.

Studies have shown that plant compounds can produce vascular relaxation through many endogenous mechanisms, such as inhibition or activation of certain pathway activators (e.g., prostaglandin, nitric oxide, cyclic adenosine monophosphate), blocking or releasing ions (K^+, Ca^{2+}), blocking or activating ion channels, and others (Luna-Vázquez *et al* 2013). The endogenous metabolites related to these pathways may be detected using metabolomics. Furthermore, through using an untargetted metabolomics approach, changes in endogenous metabolites, potentially including, but not limited to, the above pathways, could provide insight into the mechanism(s) of action for vascular relaxation induced by the consumption of darker-coloured beans.

Therefore, using serum samples obtained from our previous study, we employed an untargetted metabolomics approach to analyze metabolites within the human endogenous metabolome that change in response to the consumption of darker-coloured beans in relation to lighter-coloured beans. The endogenous serum metabolites were analyzed for changes among samples obtained at baseline, 2 hours, and 6 hours post-consumption to correspond with the postprandial vasorelaxation previously observed after the consumption of darker-coloured beans (Chapter 4). We hypothesized that consuming darker-coloured beans would modulate endogenous compounds related to acute vascular function, either directly or indirectly, as compared to lighter-coloured beans.

5.3 Materials and Methods

Chemical, Materials, and Reagents

HPLC-grade acetonitrile (LC-MS Chromasolv®), methanol, spectroscopic-grade formic acid, and D-norvaline were purchased from Sigma-Aldrich (Oakville, ON, Canada). HPLC-MS-grade water (Pierce®) was purchased from Thermo Scientific (Mississauga, ON, Canada).

Serum Sample Preparation

Serum was collected during the randomized crossover study described in Chapter 4. Briefly, the study compared the postprandial effects of dried bean varieties with contrasting seed coat colours [navy (white), pinto (tan), red kidney, black] or white rice, on vascular and metabolic responses in healthy individuals (n =8). Serum was prepared from blood samples collected at baseline, 2, and 6 hours after consumption of the food articles.

The method for performing sample extractions followed the protocol outlined by Wang (2016). Serum aliquots (350 µL) were thawed at room temperature, vortexed (10 seconds) and 100 µL transferred to new microfuge tubes. Acetonitrile (200 µL) was added to each aliquot. The mixture was vortexed (30 seconds) and centrifuged at 10,000*g* at 4°C for 10 min. Supernatants were transferred to new microfuge tubes and dried under a steady stream of N_2 gas in a N-EVAP 111 nitrogen evaporator (Organomation Associates Inc, MA, USA) for 30-60 min and kept at -20°C prior to reconstitution in 200 µL of 16:3:1 acetonitrile:LC-MS Grade H_2O:D-norvaline [0.03 mg/mL]. The samples were then transferred to 250 µL glass inserts for brown LC vials for LC-QTOF-MS analysis. Blank samples were prepared with a 4:1 acetonitrile:LC-MS Grade H_2O mixture. All extractions were performed in triplicate.

Six quality control (QC) mixtures (each consisting of 5 samples) were prepared by pooling serum (23 µL) randomly chosen from 120 samples (3 time points × 8 participants × 5 diet groups) and they were analyzed in a random manner among all the other samples. Pooled QC samples were used to assess the variance observed in the data throughout sample preparation (extraction process), sample running (HPLC-MS method), and data acquisition.

HPLC-QTOF-MS

Samples were analyzed using a 1260 Infinity HPLC coupled to a 6538 Ultra High Definition Accurate QTOF-MS system (Agilent Technologies, CA, USA) operated with a dual electrospray ionization source in positive mode. Serum metabolites were separated on a 2.1 mm × 50 mm, 1.8 μm ZORBAX Extend-C18 column (Agilent Technologies) with the column temperature maintained at 60°C. Mobile phases A and B were water and acetonitrile with 0.1% formic acid, respectively. The gradient duration was 0, 0.5, and 16 min with 30%, 30%, and 100% of solvent B, respectively. A post-run time of 2 min was used prior to new injections. The autosampler injected 2 μL of sample per run. The column flow rate was kept at 0.7 mL/min with temperature maintained at 5°C.

The instrument acquired data using the following parameters: drying gas (N_2) temperature, 300°C; drying gas flow, 11 L/min; nebulizer, 45 psig; capillary voltage, 4,000 V; fragmentor, 175 V; skimmer, 50 V; and octopole 1 radio frequency peak-to-peak (OCT1 RF pp), 750 V. Spectra were acquired over the 50-1,700 *m/z* range, with mass detection operated by using electrospray with reference ions of *m/z* 121.050873 and 922.009798 (ESI+).

Data Processing

Compounds were detected and reported from accurate-mass scan data using Agilent MassHunter Qualitative Analysis (MHQ version B.07) software. *Homo sapiens* was selected as the organism from which the data were obtained; thus, the search objective for MHQ was refined to endogenous compounds for *Homo sapiens*. The workflow applied for data processing is described in **Table 5.1**. The raw data files were first acquired by Agilent MassHunter Acquisition software (version B.07) and stored as "*.d" files. Molecular Feature Extraction (MFE) was the first algorithm applied to the "*.d" files, and features with absolute abundances ≥

5,000 counts were extracted based on set parameters, providing further information regarding $[M+H]^+$, isomers, and all corresponding Na^+ adducts. Potential formulas, retention time, exact mass, and ion abundance summaries were generated for the extracted ion features. The resultant data were converted into compound exchange format ("*.cef") files and exported to MPP for further comparative and statistical analyses. All data were transformed to Log2 scale. The individual "*.cef" files were exported into Mass Profiler Professional (MPP version 12.6) for statistical analysis. A frequency filtration was used to accept features that were detected in at least one condition. This filtration step was employed to ensure elimination of the potential feature extraction artifacts; entities were accepted if detected in at least 70% of one of the three time points. The retention time compound alignment parameters were set to 0.1 min with a mass tolerance of 15 ppm.

Table 5.1 Metabolomics workflow summary.

Step 1: Metabolomics of human serum (LC-QTOF-MS and MHQ B.07)
Non-targetted analysis of all serum samples by LC-QTOF-MS in ESI positive mode.
Homo sapiens was selected as the organism for the origin source of the data set.
Molecular Feature Extraction (MFE) algorithm was used to extract all detectable endogenous compounds.
Generate Formulas algorithm was used to obtain potential formulas for extracted compounds.
Export to CEF algorithm was used to convert "*.d" files to "*.cef" files for analyses.
Step 2: Statistical Analysis (MPP 12.6.1)
Filter by Frequency algorithm was used to accept entities detected in at least 70% of one of the three conditions (baseline, 2 hours, and 6 hours). This generated 407, 355, 406, 388, and 401 entities for white rice, navy beans, pinto beans, red kidney beans, and black beans, respectively.
Statistical analyses were conducted individually for each food sample and time-point comparison. This generated 5 statistical data sets.
One-way ANOVA ($P < 0.05$) for unequal variance was performed for each data set followed by a post-hoc test (Tukey HSD) for comparing between time-point pairs (baseline *vs* 2 hours, baseline *vs* 6 hours, and 2 hours *vs* 6 hours). Bonferroni FWER was performed to correct the asymptotic *P*-value (P=0.05 divided by the number of compounds after filtration) to prevent false positive significant results. This generated 96, 131, 129, 102, and 132 significant (corrected *P*-value < 0.05) compounds for white rice, navy beans, pinto beans, red kidney beans, and black beans, respectively (**Figure 5.1**).
Step 3: Metabolite Identification & Pathway Analysis
Identification of patterns (4) showing changes in compounds between baseline, 2 hours, and 6 hours (**Figure 5.2**). Patterns were selected if they presented changes at time points relevant to the previously observed vascular outcomes. This pattern organization refined the compound list down to 88 compounds. Of these 88 compounds, 13, 21, 16, 12, and 35 were unique to white rice, navy beans, pinto beans, red kidney beans, and black beans, respectively (**Table 5.2**).
Comparison of compound identities between MHQ, Human Metabolome Database, and PubChem using *m/z* ratios. Of the 88 compounds, only 5 were matched between HMDB and MHQ, and were related to black bean consumption (**Table 5.3**).
Literature search and interpretation of biochemical pathways for the suggested identities of metabolites present in serum of participants who consumed black beans (**Table 5.4)**. Of the 5 compounds of interest, acylcarnitines may have links to improved vascular function.

Metabolite Identification

Using an untargetted metabolomics approach, a list of endogenous metabolites significantly changing in response to the consumption of each food article was generated. MHQ generated identities for the endogenous compounds by searching METLIN as a Personal Compound Database and Library. The list was refined according to the direction of change patterns occurring between the three time points, i.e. increased or decreased between: (i) baseline *vs* 2 hours; (ii) baseline *vs* 6 hours; (iii) 2 hours *vs* 6 hours. These patterns, and their respective metabolites, were further investigated if the pattern represented changes in metabolites at time points relevant to the vascular outcomes previously observed in Chapter 4. The list of identities generated by MHQ was also searched against publicly available, widely used databases such as Human Metabolome Database (HMDB) and PubChem to corroborate identities of compounds using *m/z* ratios provided in the output by MHQ.

Biological Interpretation and Pathway Analysis

Select compounds were investigated for their biological relevance to the current research project using HMDB and by performing a search of the literature using PubMed (**Figure S5.1**). Biochemical pathways of selected metabolites were searched and analyzed (if available) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

Statistical Analysis

Each food sample data set was analyzed individually for the time point comparisons (Table 5.1). The final processed list contained 407, 355, 406, 388, and 401 total compounds in ESI+ mode, for white rice, navy beans, pinto beans, red kidney beans, and black beans, respectively. One-way ANOVA ($P<0.05$) for unequal variance (Welch), followed by a post-hoc test (Tukey Honest Significant Difference (HSD)), an asymptotic *P*-value computation, and a

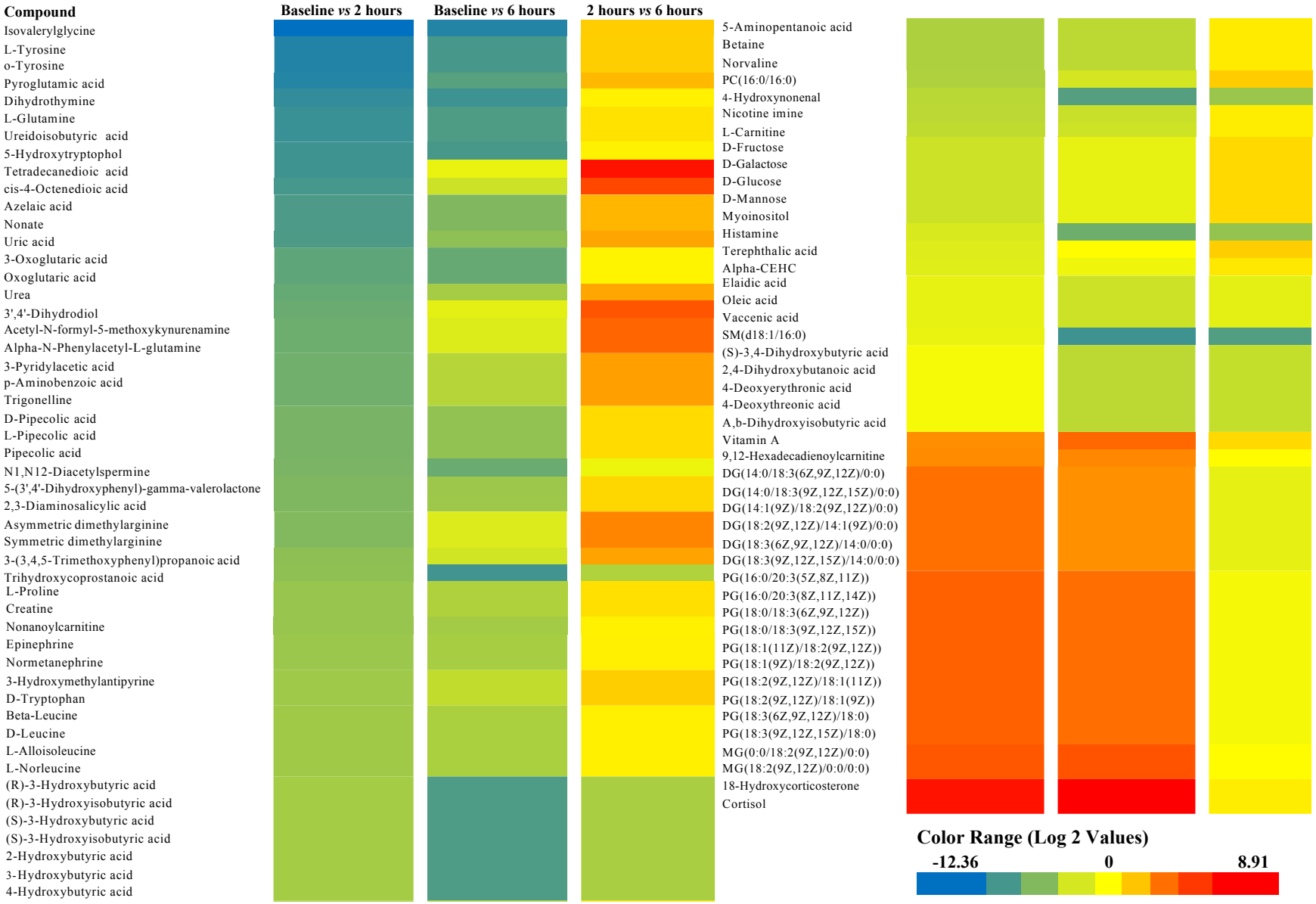
multiple correction test (Bonferroni Family-Wise Error Rate (FWER)) were used to determine the serum metabolites changing between the time points for each food article (white rice *n*=96, navy beans *n*=131, pinto beans *n*=129, red kidney beans *n*=102, and black beans *n*=132).

5.4 Results

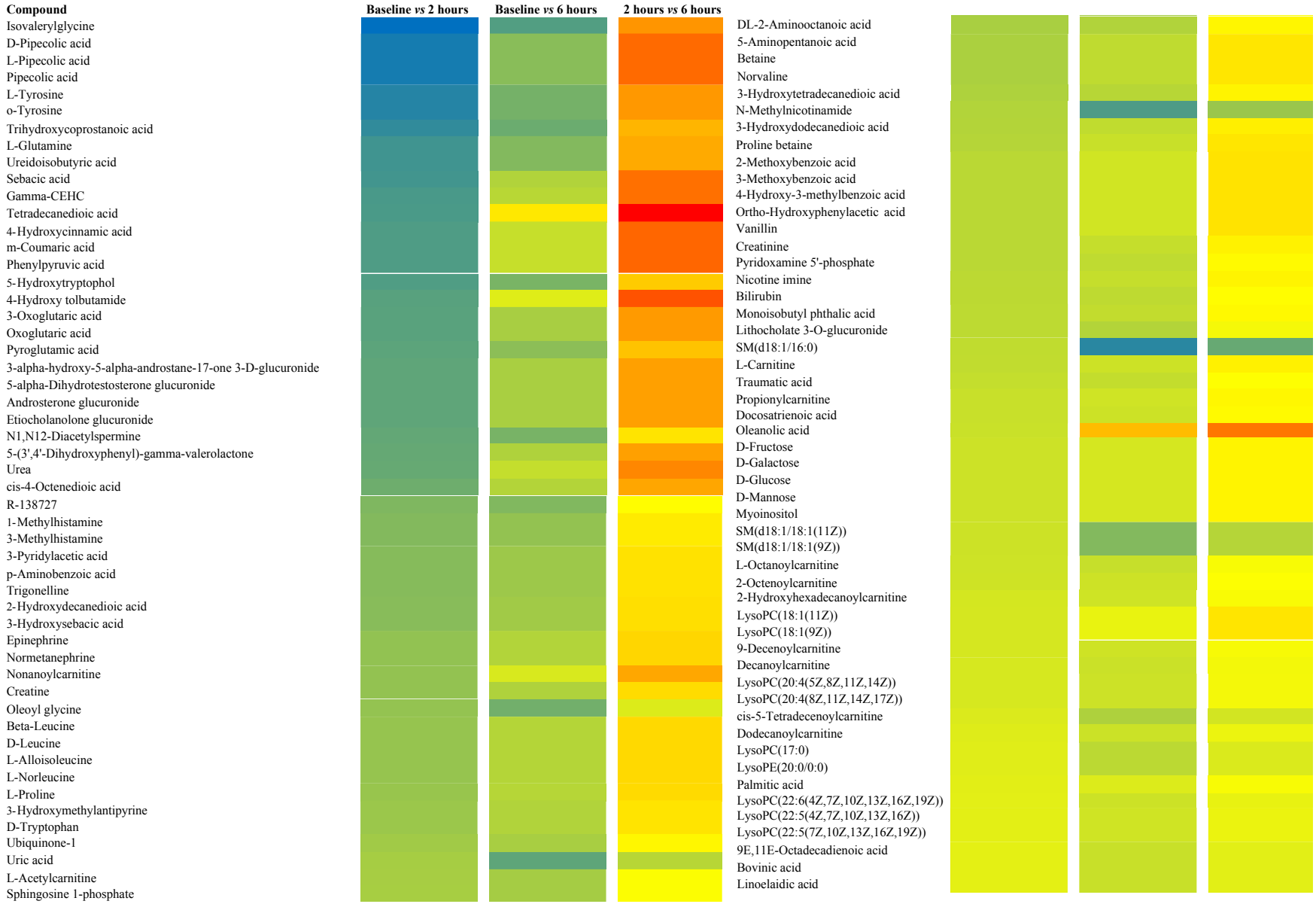
Detection of Endogenous Metabolites and Their Postprandial Modulation by Beans and White Rice

The untargetted metabolomics approach employed in our study detected 96, 131, 129, 102, and 132 endogenous compounds considered significantly modified (their serum levels increased or decreased at 2 and 6 hours relative to baseline) as a result of consuming white rice, navy beans, pinto beans, red kidney beans, or black beans, respectively. These data are plotted as heatmaps for each food article, presented in descending order of direction (up/positive or down/negative) and magnitude of change (reported as Log 2 values) for each metabolite (**Figure 5.1a-e**).

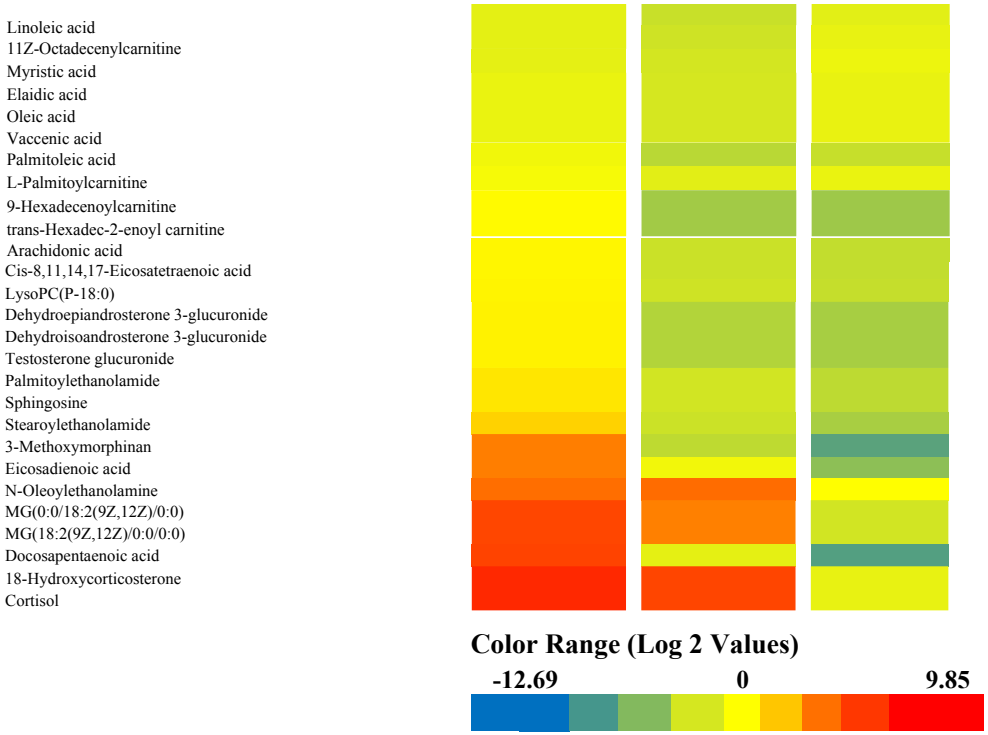
A White Rice


Compound
Baseline vs 2 hours
Baseline vs 6 hours
2 hours vs 6 hours
Isovalerylglycine
L-Tyrosine
o-Tyrosine
Pyroglutamic acid
Dihydrothymine
L-Glutamine
Ureidoisobutyric acid
5-Hydroxytryptophol
Tetradecanedioic acid
cis-4-Octenedioic acid
Azelaic acid
Nonate
Uric acid
3-Oxoglutaric acid
Oxoglutaric acid
Urea
3',4'-Dihydrodiol
Acetyl-N-formyl-5-methoxykynurenamine
Alpha-N-Phenylacetyl-L-glutamine
3-Pyridylacetic acid
p-Aminobenzoic acid
Trigonelline
D-Pipecolic acid
L-Pipecolic acid
Pipecolic acid
N1,N12-Diacetylspermine
5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone
2,3-Diaminosalicylic acid
Asymmetric dimethylarginine
Symmetric dimethylarginine
3-(3,4,5-Trimethoxyphenyl)propanoic acid
Trihydroxycoprostanoic acid
L-Proline
Creatine
Nonanoylcarnitine
Epinephrine
Normetanephrine
3-Hydroxymethylantipyrine
D-Tryptophan
Beta-Leucine
D-Leucine
L-Alloisoleucine
L-Norleucine
(R)-3-Hydroxybutyric acid
(R)-3-Hydroxyisobutyric acid
(S)-3-Hydroxybutyric acid
(S)-3-Hydroxyisobutyric acid
2-Hydroxybutyric acid
3-Hydroxybutyric acid
4-Hydroxybutyric acid
5-Aminopentanoic acid
Betaine
Norvaline
PC(16:0/16:0)
4-Hydroxynonenal
Nicotine imine
L-Carnitine
D-Fructose
D-Galactose
D-Glucose
D-Mannose
Myoinositol
Histamine
Terephthalic acid
Alpha-CEHC
Elaidic acid
Oleic acid
Vaccenic acid
SM(d18:1/16:0)
(S)-3,4-Dihydroxybutyric acid
2,4-Dihydroxybutanoic acid
4-Deoxyerythronic acid
4-Deoxythreonic acid
A,b-Dihydroxyisobutyric acid
Vitamin A
9,12-Hexadecadienoylcarnitine
DG(14:0/18:3(6Z,9Z,12Z)/0:0)
DG(14:0/18:3(9Z,12Z,15Z)/0:0)
DG(14:1(9Z)/18:2(9Z,12Z)/0:0)
DG(18:2(9Z,12Z)/14:1(9Z)/0:0)
DG(18:3(6Z,9Z,12Z)/14:0/0:0)
DG(18:3(9Z,12Z,15Z)/14:0/0:0)
PG(16:0/20:3(5Z,8Z,11Z))
PG(16:0/20:3(8Z,11Z,14Z))
PG(18:0/18:3(6Z,9Z,12Z))
PG(18:0/18:3(9Z,12Z,15Z))
PG(18:1(11Z)/18:2(9Z,12Z))
PG(18:1(9Z)/18:2(9Z,12Z))
PG(18:2(9Z,12Z)/18:1(11Z))
PG(18:2(9Z,12Z)/18:1(9Z))
PG(18:3(6Z,9Z,12Z)/18:0)
PG(18:3(9Z,12Z,15Z)/18:0)
MG(0:0/18:2(9Z,12Z)/0:0)
MG(18:2(9Z,12Z)/0:0/0:0)
18-Hydroxycorticosterone
Cortisol
Color Range (Log 2 Values)
-12.36
0
8.91

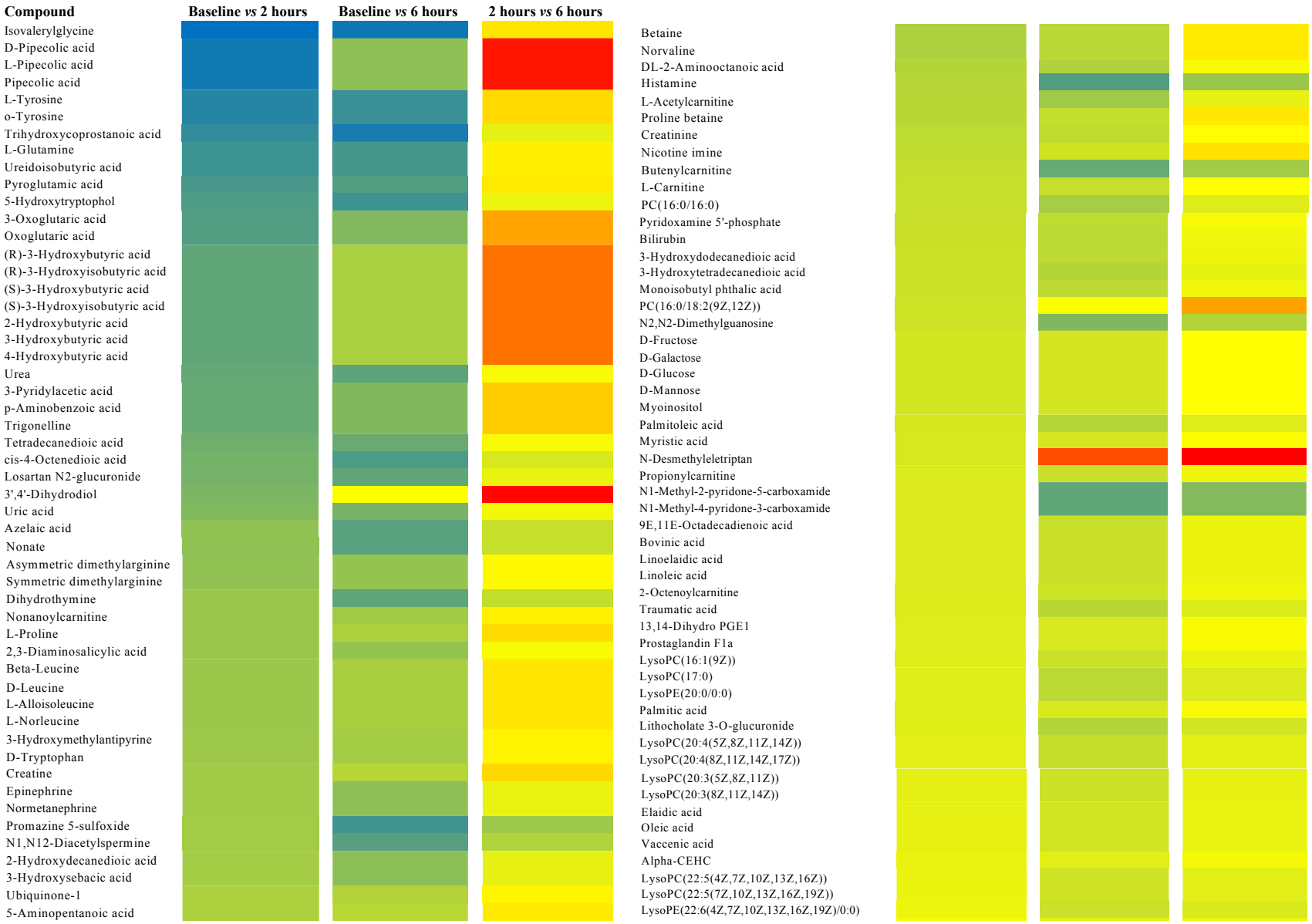
B Navy Bean


Compound
Baseline vs 2 hours
Baseline vs 6 hours
2 hours vs 6 hours
Isovalerylglycine
D-Pipecolic acid
L-Pipecolic acid
Pipecolic acid
L-Tyrosine
o-Tyrosine
Trihydroxycoprostanoic acid
L-Glutamine
Ureidoisobutyric acid
Sebacic acid
Gamma-CEHC
Tetradecanedioic acid
4-Hydroxycinnamic acid
m-Coumaric acid
Phenylpyruvic acid
5-Hydroxytryptophol
4-Hydroxy tolbutamide
3-Oxoglutaric acid
Oxoglutaric acid
Pyroglutamic acid
3-alpha-hydroxy-5-alpha-androstane-17-one 3-D-glucuronide
5-alpha-Dihydrotestosterone glucuronide
Androsterone glucuronide
Etiocholanolone glucuronide
N1,N12-Diacetylspermine
5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone
Urea
cis-4-Octenedioic acid
R-138727
1-Methylhistamine
3-Methylhistamine
3-Pyridylacetic acid
p-Aminobenzoic acid
Trigonelline
2-Hydroxydecanedioic acid
3-Hydroxysebacic acid
Epinephrine
Normetanephrine
Nonanoylcarnitine
Creatine
Oleoyl glycine
Beta-Leucine
D-Leucine
L-Alloisoleucine
L-Norleucine
L-Proline
3-Hydroxymethylantipyrine
D-Tryptophan
Ubiquinone-1
Uric acid
L-Acetylcarnitine
Sphingosine 1-phosphate
DL-2-Aminooctanoic acid
5-Aminopentanoic acid
Betaine
Norvaline
3-Hydroxytetradecanedioic acid
N-Methylnicotinamide
3-Hydroxydodecanedioic acid
Proline betaine
2-Methoxybenzoic acid
3-Methoxybenzoic acid
4-Hydroxy-3-methylbenzoic acid
Ortho-Hydroxyphenylacetic acid
Vanillin
Creatinine
Pyridoxamine 5'-phosphate
Nicotine imine
Bilirubin
Monoisobutyl phthalic acid
Lithocholate 3-O-glucuronide
SM(d18:1/16:0)
L-Carnitine
Traumatic acid
Propionylcarnitine
Docosatrienoic acid
Oleanolic acid
D-Fructose
D-Galactose
D-Glucose
D-Mannose
Myoinositol
SM(d18:1/18:1(11Z))
SM(d18:1/18:1(9Z))
L-Octanoylcarnitine
2-Octenoylcarnitine
2-Hydroxyhexadecanoylcarnitine
LysoPC(18:1(11Z))
LysoPC(18:1(9Z))
9-Decenoylcarnitine
Decanoylcarnitine
LysoPC(20:4(5Z,8Z,11Z,14Z))
LysoPC(20:4(8Z,11Z,14Z,17Z))
cis-5-Tetradecenoylcarnitine
Dodecanoylcarnitine
LysoPC(17:0)
LysoPE(20:0/0:0)
Palmitic acid
LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))
LysoPC(22:5(4Z,7Z,10Z,13Z,16Z))
LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))
9E,11E-Octadecadienoic acid
Bovinic acid
Linoelaidic acid

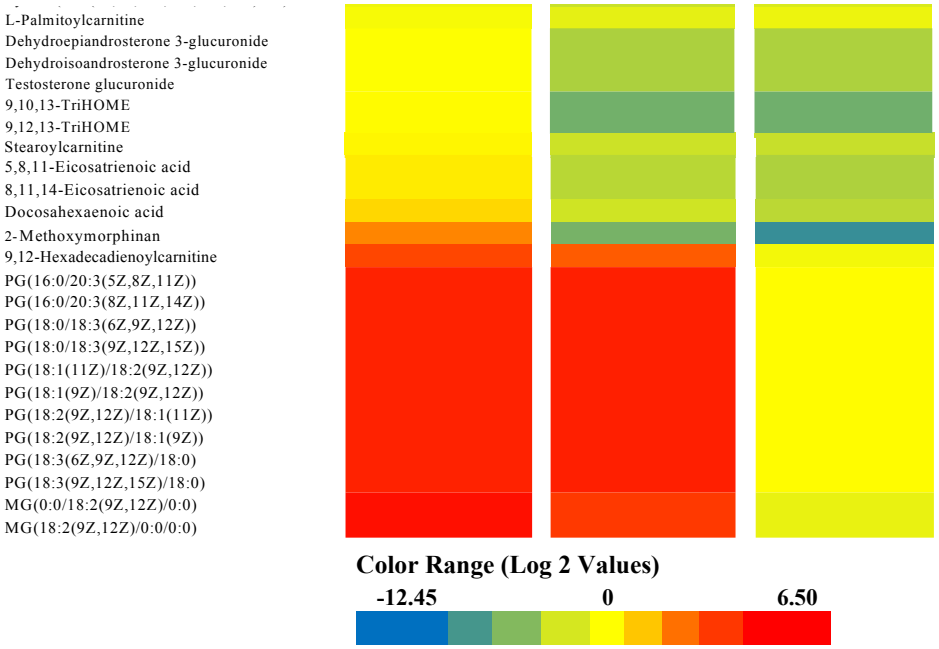
B Navy Bean (continued)



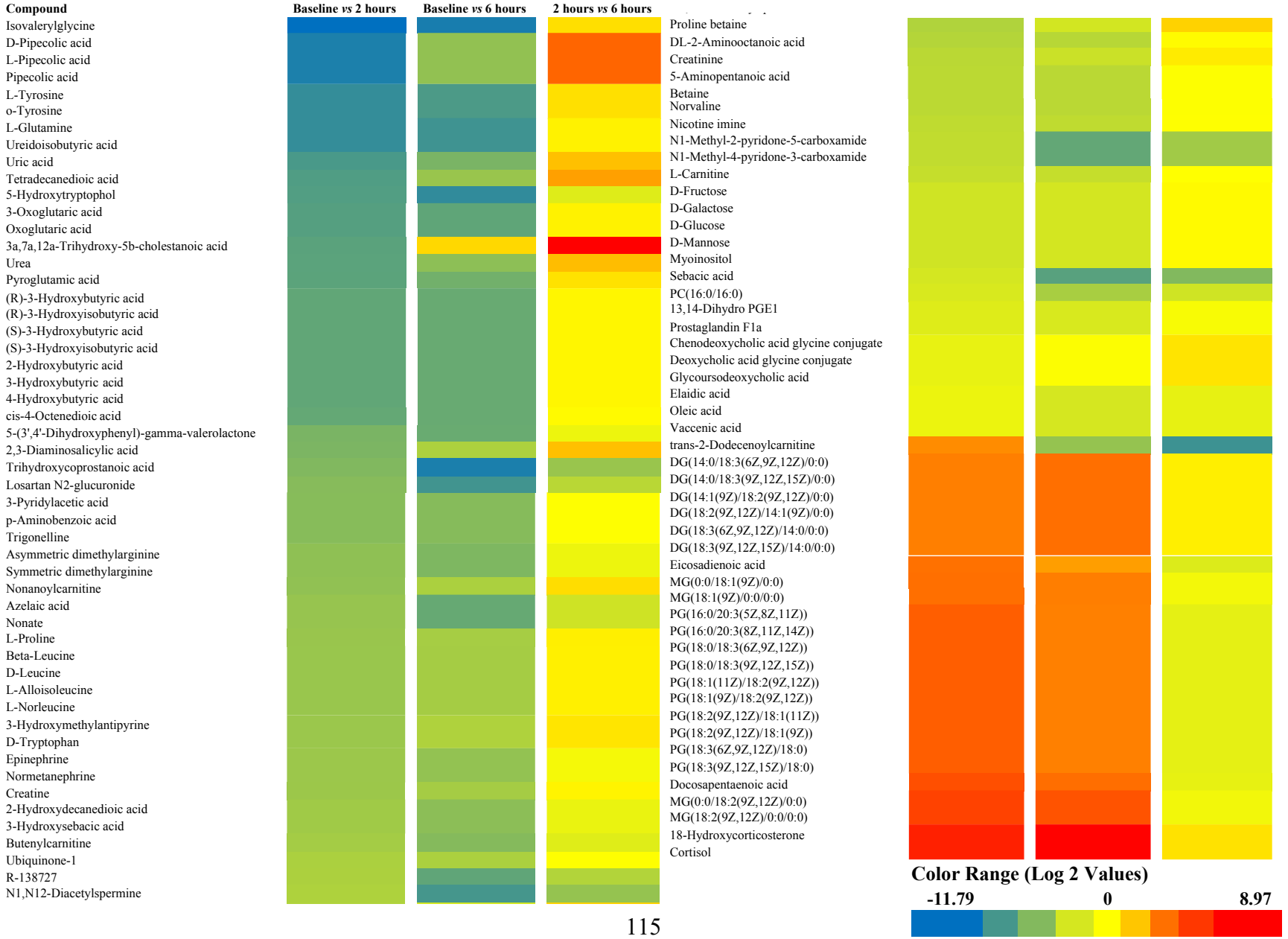
C Pinto Bean


Compound
Baseline vs 2 hours
Baseline vs 6 hours
2 hours vs 6 hours
Isovalerylglycine
D-Pipecolic acid
L-Pipecolic acid
Pipecolic acid
L-Tyrosine
o-Tyrosine
Trihydroxycoprostanoic acid
L-Glutamine
Ureidoisobutyric acid
Pyroglutamic acid
5-Hydroxytryptophol
3-Oxoglutaric acid
Oxoglutaric acid
(R)-3-Hydroxybutyric acid
(R)-3-Hydroxyisobutyric acid
(S)-3-Hydroxybutyric acid
(S)-3-Hydroxyisobutyric acid
2-Hydroxybutyric acid
3-Hydroxybutyric acid
4-Hydroxybutyric acid
Urea
3-Pyridylacetic acid
p-Aminobenzoic acid
Trigonelline
Tetradecanedioic acid
cis-4-Octenedioic acid
Losartan N2-glucuronide
3',4'-Dihydrodiol
Uric acid
Azelaic acid
Nonate
Asymmetric dimethylarginine
Symmetric dimethylarginine
Dihydrothymine
Nonanoylcarnitine
L-Proline
2,3-Diaminosalicylic acid
Beta-Leucine
D-Leucine
L-Alloisoleucine
L-Norleucine
3-Hydroxymethylantipyrine
D-Tryptophan
Creatine
Epinephrine
Normetanephrine
Promazine 5-sulfoxide
N1,N12-Diacetylspermine
2-Hydroxydecanedioic acid
3-Hydroxysebacic acid
Ubiquinone-1
5-Aminopentanoic acid
Betaine
Norvaline
DL-2-Aminooctanoic acid
Histamine
L-Acetylcarnitine
Proline betaine
Creatinine
Nicotine imine
Butenylcarnitine
L-Carnitine
PC(16:0/16:0)
Pyridoxamine 5'-phosphate
Bilirubin
3-Hydroxydodecanedioic acid
3-Hydroxytetradecanedioic acid
Monoisobutyl phthalic acid
PC(16:0/18:2(9Z,12Z))
N2,N2-Dimethylguanosine
D-Fructose
D-Galactose
D-Glucose
D-Mannose
Myoinositol
Palmitoleic acid
Myristic acid
N-Desmethyleletriptan
Propionylcarnitine
N1-Methyl-2-pyridone-5-carboxamide
N1-Methyl-4-pyridone-3-carboxamide
9E,11E-Octadecadienoic acid
Bovinic acid
Linoelaidic acid
Linoleic acid
2-Octenoylcarnitine
Traumatic acid
13,14-Dihydro PGE1
Prostaglandin F1a
LysoPC(16:1(9Z))
LysoPC(17:0)
LysoPE(20:0/0:0)
Palmitic acid
Lithocholate 3-O-glucuronide
LysoPC(20:4(5Z,8Z,11Z,14Z))
LysoPC(20:4(8Z,11Z,14Z,17Z))
LysoPC(20:3(5Z,8Z,11Z))
LysoPC(20:3(8Z,11Z,14Z))
Elaidic acid
Oleic acid
Vaccenic acid
Alpha-CEHC
LysoPC(22:5(4Z,7Z,10Z,13Z,16Z))
LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))
LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)

C Pinto Bean (continued)



D Red Kidney Bean



E Black Bean


Compound
Baseline vs 2 hours
Baseline vs 6 hours
2 hours vs 6 hours
Isovalerylglycine
L-Tyrosine
o-Tyrosine
D-Pipecolic acid
L-Pipecolic acid
Pipecolic acid
L-Glutamine
Ureidoisobutyric acid
5-Hydroxytryptophol
Gamma-CEHC
Tetradecanedioic acid
Uric acid
3a,7a,12a-Trihydroxy-5b-cholestanoic acid
Pyroglutamic acid
Sebacic acid
3-Oxoglutaric acid
Oxoglutaric acid
3-Pyridylacetic acid
p-Aminobenzoic acid
Trigonelline
5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone
4-Hydroxycinnamic acid
m-Coumaric acid
Phenylpyruvic acid
Urea
Nonanoylcarnitine
L-Proline
Beta-Leucine
D-Leucine
L-Alloisoleucine
L-Norleucine
Asymmetric dimethylarginine
Symmetric dimethylarginine
3-Hydroxymethylantipyrine
D-Tryptophan
2-Hydroxydecanedioic acid
3-Hydroxysebacic acid
Creatine
Epinephrine
Normetanephrine
Stearic acid
Trihydroxycoprostanoic acid
5-Aminopentanoic acid
Betaine
Norvaline
Proline betaine
2,3-Diaminosalicylic acid
Nicotine imine
L-Acetylcarnitine
DL-2-Aminooctanoic acid
1-Methylhistamine
3-Methylhistamine
Creatinine
Pyridoxamine 5'-phosphate
L-Carnitine
3-Hydroxytetradecanedioic acid
3-Hydroxydodecanedioic acid
D-Fructose
D-Galactose
D-Glucose
D-Mannose
Myoinositol
5,8,11-Eicosatrienoic acid
8,11,14-Eicosatrienoic acid
Butyrylcarnitine
Isobutyryl-L-carnitine
Myristic acid
Resolvin D1
LysoPC(17:0)
LysoPE(20:0/0:0)
Lithocholate 3-O-glucuronide
Traumatic acid
Monoisobutyl phthalic acid
9E,11E-Octadecadienoic acid
Bovinic acid
Linoelaidic acid
Linoleic acid
Propionylcarnitine
LysoPC(20:4(5Z,8Z,11Z,14Z))
LysoPC(20:4(8Z,11Z,14Z,17Z))
Terephthalic acid
Palmitoleic acid
Alpha-CEHC
LysoPC(20:3(5Z,8Z,11Z))
LysoPC(20:3(8Z,11Z,14Z))
Elaidic acid
Oleic acid
Vaccenic acid
Chenodeoxycholic acid glycine conjugate
Deoxycholic acid glycine conjugate
Glycoursodeoxycholic acid
13,14-Dihydro PGE1
Prostaglandin F1a
Atropine
Donepezil metabolite M4
N1-Methyl-2-pyridone-5-carboxamide
N1-Methyl-4-pyridone-3-carboxamide
LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)
Isovalerylcarnitine
Valerylcarnitine
cis-5-Tetradecenoylcarnitine
3-Hydroxy-9-hexadecenoylcarnitine
(S)-3,4-Dihydroxybutyric acid
2,4-Dihydroxybutanoic acid

E Black Bean (continued)

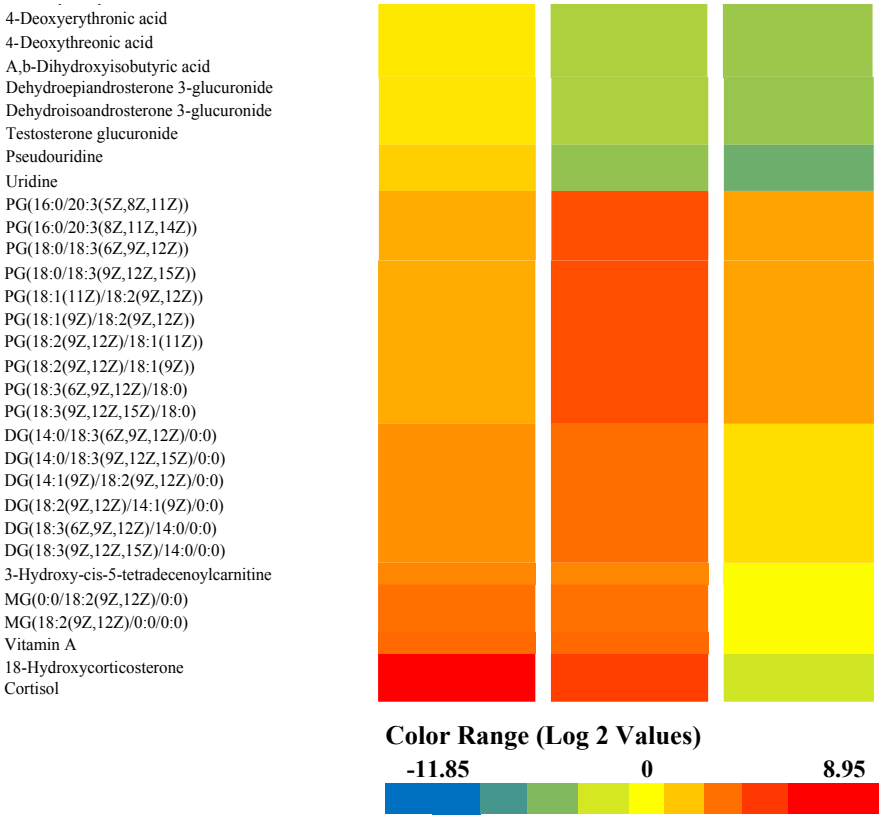


Figure 5.1 Heatmaps for metabolites significantly changed in serum of healthy adults fed white rice or beans.

(A) White rice; **(B)** Navy beans; **(C)** Pinto beans; **(D)** Red kidney beans; **(E)** Black beans. The three columns display baseline versus 2 hours, baseline versus 6 hours, and 2 hours versus 6 hours. Each row represents a metabolite. All values are Log-2 normalized values of the detected abundance for each metabolite. The *color range* scale represents relative abundance from low (blue) to high (red). Compound identities are tentative (determined by MassHunter Qualitative software version B.07) until verified against authentic reference standards.

Changes in Endogenous Compound Levels in Response to White Rice or Beans

To determine which metabolites could be relevant to the vascular outcomes previously observed in Chapter 4, the data were organized into discrete patterns as determined by the levels found at each of the three time points (baseline, 2 hours, and 6 hours). Decreases or increases in endogenous compounds occurred between i) baseline *vs* 2 hours; ii) baseline *vs* 6 hours; or iii) 2 hours *vs* 6 hours. Of these time point comparisons, four patterns were considered relevant (**Figure 5.2**) to the vascular outcomes previously observed in Chapter 4: Pattern 1, down at 2 hours compared to baseline, and down at 6 hours compared to 2 hours (Down-Down); Pattern 2, down at 2 hours compared to baseline and up at 6 hours compared to 2 hours (Down-Up); Pattern 3, up at 2 hours compared to baseline and down at 6 hours compared to 2 hours (Up-Down); and Pattern 4, up at 2 hours compared to baseline and up at 6 hours compared to 2 hours (Up-Up).

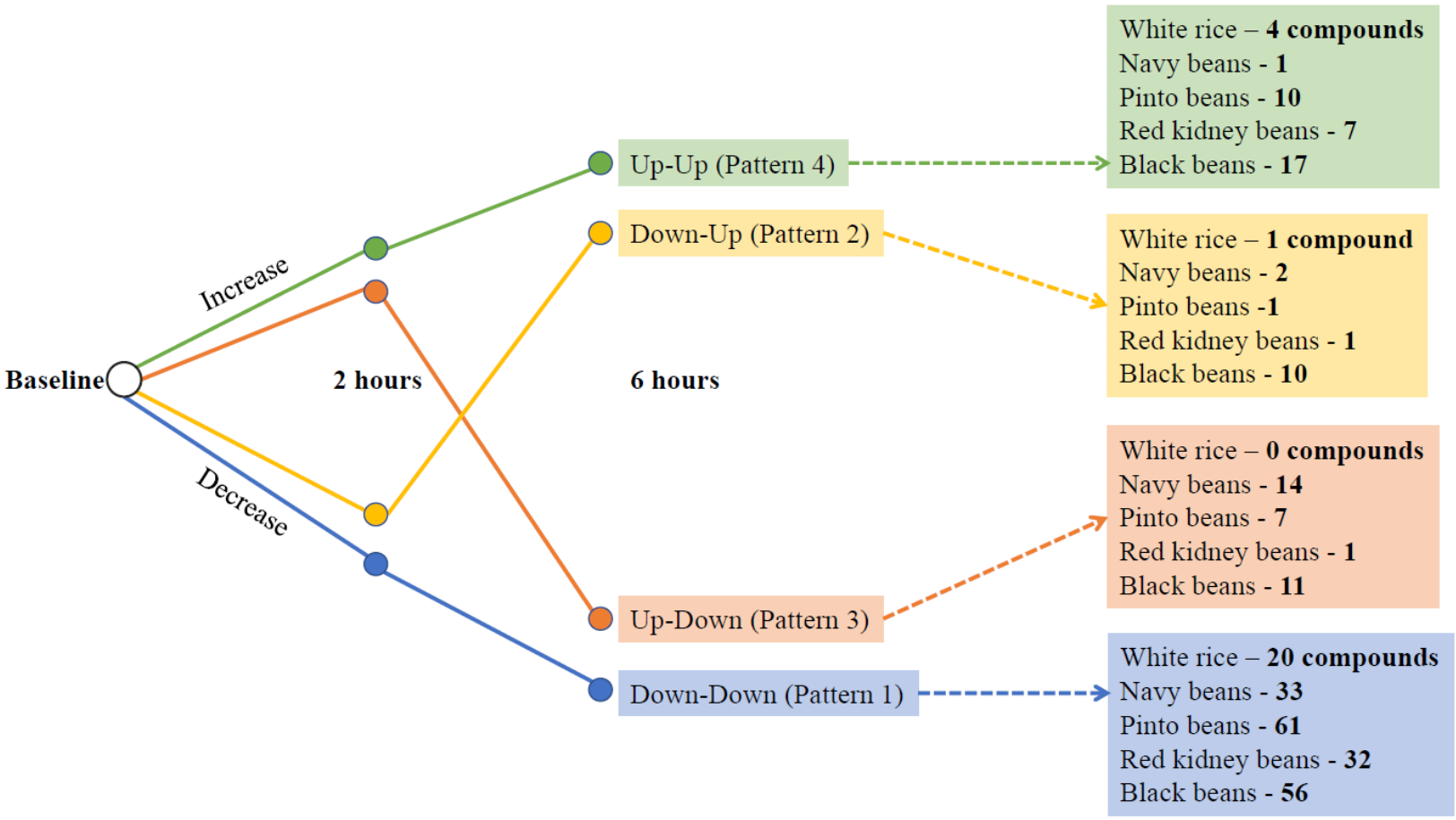

Baseline
Increase
Decrease
2 hours
6 hours
Up-Up (Pattern 4)
Down-Up (Pattern 2)
Up-Down (Pattern 3)
Down-Down (Pattern 1)
White rice – 4 compounds
Navy beans - 1
Pinto beans - 10
Red kidney beans - 7
Black beans - 17
White rice – 1 compound
Navy beans - 2
Pinto beans -1
Red kidney beans - 1
Black beans - 10
White rice – 0 compounds
Navy beans - 14
Pinto beans - 7
Red kidney beans - 1
Black beans - 11
White rice – 20 compounds
Navy beans - 33
Pinto beans - 61
Red kidney beans - 32
Black beans - 56

Figure 5.2 Direction of change patterns for endogenous serum metabolites.

Serum endogenous compounds were increased (up) or decreased (down) after consumption of white rice, navy beans, pinto beans, red kidney beans, or black beans. These increases or decreases occurred between baseline *vs* 2 hours, baseline *vs* 6 hours, or 2 hours *vs* 6 hours. Four patterns were considered physiologically relevant to the previously observed vasorelaxation at 6 hours post-consumption of darker-coloured beans. The number of compounds changing in response to each food article is displayed beside each respective pattern. One-way ANOVA (MHQ B.07) was used to determine significant differences ($p<0.05$) between time points.

The following describes the changes in endogenous metabolites in response to consuming white rice (WR), navy beans (NB), pinto beans (PB), red kidney beans (RKB), or black beans (BB), respectively, by healthy adults (Figure 5.2). There were 20 (WR), 33 (NB), 61 (PB), 32 (RKB), and 56 (BB) endogenous compounds consistently reduced over 6 hours (Pattern 1: Down-Down); 1 (WR), 2 (NB), 1 (PB), 1 (RKB), and 10 (BB) compounds transiently reduced at 2 hours then increased to baseline levels or higher at 6 hours (Pattern 2: Down-Up); 0 (WR), 14 (NB), 7 (PB), 1 (RKB), and 11 (BB) compounds transiently increased at 2 hours then decreased to baseline levels or lower at 6 hours (Pattern 3: Up-Down); and 4 (WR), 1 (NB), 10 (PB), 7 (RKB), and 17 (BB) compounds consistently increased over 6 hours (Pattern 4: Up-Up).

The following describes the endogenous metabolites changing uniquely in response to each food article. There were 13 (WR), 8 (NB), 8 (PB), 9 (RKB), and 16 (BB) unique compounds decreased at 6 hours compared to baseline (Pattern 1: Down-Down) (**Table 5.2**); 2 (NB), 1 (PB), 1 (RKB), and 10 (BB) unique compounds following Pattern 2 (Down-Up); 10 (NB), 6 (PB), 1 (RKB), and 8 (BB) unique compounds following Pattern 3 (Up-Down); and 1(NB), 1 (RKB), and 1 (BB) unique compounds that were increased at 6 hours compared to baseline (Pattern 4: Up-Up). No unique compounds were changed in response to white rice consumption for Patterns 2, 3, and 4.

Table 5.2 Serum metabolites of endogenous compounds significantly modulated by consumption of beans or rice

Food type	Metabolites	P (Corr)*	Baseline *vs* 2 hours			Baseline *vs* 6 hours			2 hours *vs* 6 hours			*m/z*
			Log 2* FC	FC	Direction of change (D)	Log 2* FC	FC	D	Log 2* FC	FC	D	
White rice	(R)-3-Hydroxybutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	(R)-3-Hydroxyisobutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	(S)-3-Hydroxybutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	(S)-3-Hydroxyisobutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	(S)-3,4-Dihydroxybutyric acid	<0.05	-0.42	1	↓	-3.24	9	↓	-2.82	7	↓	121.0508
	2-Hydroxybutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	2,4-Dihydroxybutanoic acid	< 0.05	-0.42	1	↓	-3.24	9	↓	-2.82	7	↓	121.0508
	3-Hydroxybutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	4-Deoxyerythronic acid	< 0.05	-0.42	1	↓	-3.24	9	↓	-2.82	7	↓	121.0508
	4-Deoxythreonic acid	< 0.05	-0.42	1	↓	-3.24	9	↓	-2.82	7	↓	121.0508
	4-Hydroxybutyric acid	< 0.01	-4.40	21	↓	-8.55	376	↓	-4.15	18	↓	231.0796
	4-Hydroxynonenal	< 0.01	-3.38	10	↓	-8.24	302	↓	-4.85	29	↓	330.2593

	A,b-Dihydroxyisobutyric acid	< 0.05	-0.42	1	↓	-3.24	9	↓	-2.82	7	↓	121.0508
Navy beans	Decanoylcarnitine	< 0.01	-2.04	4	↓	-2.62	6	↓	-0.58	1	↓	1538.0164
	Dodecanoylcarnitine	< 0.01	-1.62	3	↓	-2.56	6	↓	-0.94	2	↓	249.1518
	L-Octanoylcarnitine	< 0.01	-2.47	6	↓	-2.80	7	↓	-0.33	1	↓	669.5365
	LysoPC(22:5(7Z,10Z,13Z,16Z,19Z))	< 0.05	-1.37	3	↓	-2.36	5	↓	-0.99	2	↓	651.5985
	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))	< 0.01	-1.39	3	↓	-2.50	6	↓	-1.11	2	↓	623.578
	N-Methylnicotinamide	< 0.01	-3.79	14	↓	-8.81	448	↓	-5.02	32	↓	695.5625
	Oleoyl glycine	< 0.05	-5.31	40	↓	-7.01	129	↓	-1.70	3	↓	n/a
	Oleanolic acid	< 0.01	-2.66	6	↓	2.62	6	↑	5.28	39	↑	617.5072
	Tetradecanedioic acid	< 0.01	-8.96	498	↓	0.89	2	↑	9.85	923	↑	169.0492
	cis-5-Tetradecenoylcarnitine	< 0.01	-1.79	3	↓	-4.01	16	↓	-2.22	5	↓	n/a
	9-Hexadecenoylcarnitine	< 0.01	0.20	1	↑	-4.62	25	↓	-4.82	28	↓	734.4983
	Arachidonic acid	< 0.05	0.37	1	↑	-2.62	6	↓	-2.99	8	↓	887.5628

	Docosapentaenoic acid	< 0.01	7.26	154	↑	-1.26	2	↓	-8.52	367	↓	812.6938
	Eicosadienoic acid	< 0.01	5.02	32	↑	-0.63	2	↓	-5.65	50	↓	786.1494
	cis-8,11,14,17-Eicosatetraenoic acid	< 0.05	0.37	1	↑	-2.62	6	↓	-2.99	8	↓	n/a
	LysoPC(P-18:0)	< 0.05	0.46	1	↑	-2.41	5	↓	-2.87	7	↓	695.5625
	Palmitoylethanolamide	< 0.05	0.99	2	↑	-2.25	5	↓	-3.24	9	↓	615.5020
	Sphingosine	< 0.05	0.99	2	↑	-2.25	5	↓	-3.24	9	↓	275.1856
	Stearoylethanolamide	< 0.01	1.76	3	↑	-2.55	6	↓	-4.31	20	↓	409.1834
	trans-Hexadec-2-enoyl carnitine	< 0.01	0.20	1	↑	-4.62	25	↓	-4.82	28	↓	673.4691
	N-Oleoylethanolamine	< 0.01	5.63	50	↑	5.70	52	↑	0.07	1	↑	679.4722
Pinto beans	Alpha-CEHC	<0.05	-1.02	2	↓	-1.46	3	↓	-0.44	1	↓	n/a
	Bilirubin	< 0.01	-2.62	6	↓	-3.27	10	↓	-0.66	2	↓	n/a
	Dehydroepiandrosterone 3-glucuronide	< 0.01	-0.02	1	↓	-4.02	16	↓	-4.00	16	↓	771.5189
	Dehydroisoandrosterone 3-glucuronide	< 0.01	-0.02	1	↓	-4.02	16	↓	-4.00	16	↓	773.5291
	Dihydrothymine	< 0.01	-4.92	30	↓	-7.82	226	↓	-2.89	7	↓	626.3578

	N2,N2-Dimethylguanosine	< 0.05	-2.38	5	↓	-6.14	70	↓	-3.75	13	↓	615.5020
	cis-4-Octenedioic acid	< 0.01	-6.77	109	↓	-8.71	418	↓	-1.94	4	↓	897.4833
	Promazine 5-sulfoxide	< 0.01	-4.51	23	↓	-9.28	622	↓	-4.77	27	↓	121.0509
	N-Desmethyleletriptan	<0.05	-1.96	4	↓	4.54	23	↑	6.50	90	↑	563.4653
	5,8,11-Eicosatrienoic acid	< 0.01	0.51	1	↑	-3.42	11	↓	-3.94	15	↓	836.5439
	8,11,14-Eicosatrienoic acid	< 0.01	0.51	1	↑	-3.42	11	↓	-3.94	15	↓	734.4983
	9,10,13-TriHOME	< 0.01	0.11	1	↑	-6.84	115	↓	-6.96	124	↓	709.4889
	9,12,13-TriHOME	< 0.01	0.11	1	↑	-6.84	115	↓	-6.96	124	↓	351.2154
	Docosahexaenoic acid	< 0.05	1.02	2	↑	-2.30	5	↓	-3.32	10	↓	644.4977
	Stearoylcarnitine	< 0.05	0.24	1	↑	-2.49	6	↓	-2.73	7	↓	582.2433
Red kidney beans	3-Pyridylacetic acid	<0.05	-5.51	46	↓	-5.55	47	↓	-0.04	1	↓	785.6561
	5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone	< 0.01	-6.11	69	↓	-6.90	119	↓	-0.79	2	↓	n/a
	5-Aminopentanoic acid	< 0.01	-3.11	9	↓	-3.17	9	↓	-0.06	1	↓	1641.6960
	Betaine	< 0.01	-3.11	9	↓	-3.17	9	↓	-0.06	1	↓	n/a
	p-Aminobenzoic acid	< 0.05	-5.51	46	↓	-5.55	47	↓	-0.04	1	↓	n/a
	R-138727	< 0.01	-3.80	14	↓	-7.34	162	↓	-3.54	12	↓	452.2325

	Sebacic acid	< 0.01	-1.97	4	↓	-7.72	211	↓	-5.75	54	↓	121.0508
	Trigonelline	< 0.05	-5.51	46	↓	-5.55	47	↓	-0.04	1	↓	709.4889
	Ubiquinone-1	< 0.01	-3.86	14	↓	-3.86	15	↓	0.00	1	↓	673.4691
	3a,7a,12a-Trihydroxy-5b-cholestanoic acid	< 0.01	-7.60	194	↓	1.38	3	↑	8.98	505	↑	641.4519
	trans-2-Dodecenoylcarnitine	< 0.01	4.06	17	↑	-4.89	30	↓	-8.96	497	↓	733.6143
	18-Hydroxycorticosterone	< 0.01	7.89	236	↑	8.94	490	↑	1.05	2	↑	857.7596
Black beans	3-Methylhistamine	< 0.05	-2.86	7	↓	-7.37	165	↓	-4.50	23	↓	126.1007
	3-Oxoglutaric acid	< 0.01	-7.04	132	↓	-9.22	596	↓	-2.17	5	↓	184.9851
	5,8,11-Eicosatrienoic acid	< 0.01	-2.31	5	↓	-3.54	12	↓	-1.23	2	↓	651.4649
	8,11,14-Eicosatrienoic acid	< 0.01	-2.31	5	↓	-3.54	12	↓	-1.23	2	↓	651.4649
	Butyrylcarnitine	<0.01	-2.27	5	↓	-3.12	9	↓	-0.85	2	↓	463.2867
	Creatinine	<0.05	-2.77	7	↓	-2.82	7	↓	-0.05	1	↓	227.1252
	Isobutyryl-L-carnitine	< 0.01	-2.27	5	↓	-3.12	9	↓	-0.85	2	↓	463.2867
	Isovalerylcarnitine	< 0.01	-0.96	2	↓	-3.38	10	↓	-2.42	5	↓	508.3576
	Isovalerylglycine	< 0.01	-11.23	2406	↓	-11.85	3688	↓	-0.62	2	↓	319.1795

	L-Carnitine	< 0.01	-2.71	7	↓	-2.88	7	↓	-0.17	1	↓	361.1856
	L-Glutamine	< 0.01	-9.17	578	↓	-9.45	701	↓	-0.28	1	↓	293.1362
	Oxoglutaric acid	< 0.01	-7.04	132	↓	-9.22	596	↓	-2.17	5	↓	184.9851
	Pyroglutamic acid	< 0.01	-7.40	169	↓	-11.34	2586	↓	-3.93	15	↓	259.0937
	Ureidoisobutyric acid	< 0.01	-9.17	578	↓	-9.45	701	↓	-0.28	1	↓	293.1362
	Valerylcarnitine	< 0.01	-0.96	2	↓	-3.38	10	↓	-2.42	5	↓	508.3576
	4-Hydroxycinnamic acid	< 0.05	-6.46	88	↓	1.59	3	↑	8.05	265	↑	329.1026
	Atropine	< 0.01	-1.46	3	↓	4.92	30	↑	6.38	83	↑	579.3378
	Chenodeoxycholic acid glycine conjugate	< 0.01	-1.48	3	↓	0.01	1	↑	1.49	3	↑	916.6448
	Deoxycholic acid glycine conjugate	< 0.01	-1.48	3	↓	0.01	1	↑	1.49	3	↑	916.6448
	Donepezil metabolite M4	< 0.01	-1.46	3	↓	4.92	30	↑	6.38	83	↑	579.3378
	Glycoursodeoxycholic acid	< 0.01	-1.48	3	↓	0.01	1	↑	1.49	3	↑	916.6448
	m-Coumaric acid	< 0.05	-6.46	88	↓	1.59	3	↑	8.05	265	↑	329.1026
	Phenylpyruvic acid	< 0.05	-6.46	88	↓	1.59	3	↑	8.05	265	↑	329.1026
	Stearic acid	< 0.01	-3.95	15	↓	3.55	12	↑	7.50	181	↑	591.5406

	(S)-3,4-Dihydroxybutyric acid	< 0.01	0.81	2	↑	-3.78	14	↓	-4.60	24	↓	121.0508
	2,4-Dihydroxybutanoic acid	< 0.01	0.81	2	↑	-3.78	14	↓	-4.60	24	↓	121.0508
	4-Deoxyerythronic acid	< 0.01	0.81	2	↑	-3.78	14	↓	-4.60	24	↓	121.0508
	4-Deoxythreonic acid	< 0.01	0.81	2	↑	-3.78	14	↓	-4.60	24	↓	121.0508
	A,b-Dihydroxyisobutyric acid	< 0.01	0.81	2	↑	-3.78	14	↓	-4.60	24	↓	121.0508
	cis-5-Tetradecenoylcarnitine	< 0.01	0.09	1	↑	-3.74	13	↓	-3.83	14	↓	739.6061
	Pseudouridine	< 0.01	1.71	3	↑	-4.99	32	↓	-6.70	104	↓	245.0783
	Uridine	< 0.01	1.71	3	↑	-4.99	32	↓	-6.70	104	↓	245.0783
	3-Hydroxy-9-hexadecenoylcarnitine	< 0.01	0.49	1	↑	6.51	91	↑	6.02	65	↑	865.5935

Metabolites in serum of healthy adults showing significant change (One-way ANOVA; $p < 0.05$) at baseline versus 2 hours or 6 hours, or 2 hours versus 6 hours after consuming each food article as detected using electrospray ionization (ESI) in positive mode.
Abbreviations: ↓, decreased; ↑, increased; D, direction of change; FC, fold change.

Identifying Compounds of Interest

MHQ/METLIN provides a putative identity for each unique entity based on its *m/z* ratio. However, this identity cannot be confirmed without comparison against a defined standard. Since this is not always possible, an alternative approach was employed. In this case, confirmation of the identity was accepted if the *m/z* ratio resulted in selection of the same compound in the Human Metabolome Database (HMDB) or PubChem. Out of the 88 total compounds changing in response to the consumption of specific food articles, only five compounds had identities matched by the HMDB based on *m/z* ratios (**Table 5.3**). No compounds had similar identities and *m/z* ratios when compared among all three databases (MHQ/METLIN, HMDB, and PubChem).

The levels of 47 compounds were changed in response to the consumption of the darker-coloured beans (12 and 35 for red kidney beans and black beans, respectively) that may be of interest for further investigation based on their possible roles in vasorelaxation. Of these, only five that changed in response to black beans had *m/z* ratios corroborated by the HMDB: 3-oxoglutaric acid, oxoglutaric acid, isovalerylcarnitine, valerylcarnitine, and 3-hydroxy-9-hexadecenoylcarnitine.

Table 5.3 Comparison of compound identities and *m/z* ratios across three databases.

MassHunter Qualitative Software[1]		**Human Metabolome Database (HMDB)**[3]		**PubChem**[5]
Detected *m/z*[2]	Suggested identities	Best-matched *m/z*	Suggested identities[4] (HMDB ID)	Queried compound (suggested *m/z*)
121.0508	• (S)-3,4-Dihydroxybutyric acid • 2,4-Dihydroxybutanoic acid • 4-Deoxyerythronic acid • 4-Deoxythreonic acid • A,b-Dihydroxyisobutyric acid • Sebacic acid	121.0509	• Purine (HMDB0001366)	• Sebacic acid (203.128)
126.1007	• 3-Methylhistamine	126.1002	• Diethylenetriamine (HMDB0031413) • Methylamine (HMDB0000164)	• 3-Methylhistamine (126.1)
147.02885	• **3-Oxoglutaric acid** • **Oxoglutaric acid**	147.0288	• 3-Oxoglutaric acid (HMDB0013701) • Oxoglutaric acid (HMDB0000208)	• Oxoglutaric acid (145.0142)*
227.1252	• Creatinine	227.1251	• Creatinine (HMDB0000562) • N,N'-dinitrosopiperazine (HMDB0041941)	• Creatinine (114.0664)

235.1684	• Betaine	235.1681	• 3-Dehydroteasterone (HMDB0041527) • Secasterone (HMDB0040999) • (3beta,5alpha,6beta,7alpha,22E,24R)-Ergosta-8,22-diene-3,5,6,7-tetrol (HMDB0032107) • (3beta,5alpha,6alpha,9alpha,22E,24R)-Ergosta-7,22-diene-3,5,6,9-tetrol (HMDB0032123) • 3,5,9-Trihydroxyergost-7-en-6-one (HMDB0032875)	• Betaine (118.0864)
231.0796	• (R)-3-Hydroxybutyric acid • (R)-3-Hydroxyisobutyric acid • (S)-3-Hydroxybutyric acid • (S)-3-Hydroxyisobutyric acid • 2-Hydroxybutyric acid • 3-Hydroxybutyric acid • 4-Hydroxybutyric acid	231.0798	• 2-Oxo-4-methylthiobutanoic acid (HMDB0001553) • N1-Methyl-4-pyridone-3-carboxamide (HMDB0004194) • N1-Methyl-2-pyridone-5-carboxamide (HMDB0004193)	• (R)-3-Hydroxybutyric acid (105.0546) • 3-Hydroxybutyric acid (103.0388)* • 4-Hydroxybutyric acid (103)*
245.0783	• Pseudouridine • Uridine	245.0783	• Cystathionine sulfoxide (HMDB0002399)	• Pseudouridine (243.1)* • Uridine (245.0767)
257.1234	• Dihydrothymine	257.1236	• 1,4’-Bipiperdine-1’carboxylic acid (HMDB0060336) • Galactonic acid (HMDB0000565) • Gluconic acid (HMDB0000625) • Gulonic acid (HMDB0003290)	• Dihydrothymine (129.0665)
259.0937	• Pyroglutamic acid	259.0936	• Distichonic acid A (HMDB0038752) • Distichonic acid B (HMDB0038753)	• Pyroglutamic acid (130.05)

273.12988	• N-Methylnicotinamide	273.1301	• Glycylglycylglycine (HMDB0029419) • Asparaginyl-Glycine (HMDB0028731) • Glycyl-Asparagine (HMDB0028836)	• *m/z* not available
292.1216	• p-Aminobenzoic acid • Trigonelline • 3-Pyridylacetic acid	292.1213	• 2-(2,6-dihydroxy-3,4-dimethoxycyclohexylidene)acetonitrile (HMDB0125517) • {[1-(4-methoxyphenyl)pentan-3-yl]oxy}sulfonic acid (HMDB0133010)	• p-Aminobenzoic acid (138.055) • Trigonelline (138.06) • 3-Pyridylacetic acid (138.055)
293.1362	• L-Glutamine • Ureidoisobutyric acid	293.1359	• Phytolaccoside D2 (HMDB0040879) • Elatoside H (HMDB0041356) • Cynarasaponin E (HMDB0039562) • 15-Oxo-21-hydroxymabiogenin 3-[rhamnosyl-(1->6)-glucoside] (HMDB0040840) • Lucyoside J (HMDB0031774)	• L-Glutamine (145.0607)*
301.14075	• Promazine 5-sulfoxide	121.0509	• 2-(4-hydroxy-3-methylbut-2-en-1-yl)-4-(3-methylbut-2-en-1-yl)benzene-1,3,5-triol (HMDB0133045) • bis(3-methylbut-2-en-1-yl)benzene-1,2,3,5-tetrol (HMDB0133047) • 2-[(3,3-dimethyloxiran-2-yl)methyl]-4-(3-methylbut-2-en-1-yl)benzene-1,3,5-triol (HMDB0133048) • 4-(3,7-dimethylocta-2,6-dien-1-yl)benzene-1,2,3,5-tetrol (HMDB0133084) • 2-{[3-methyl-3-(4-methylpent-3-en-1-yl)oxiran-2-yl]methyl}benzene-1,3,5-triol (HMDB0133085)	• *m/z* not available

309.2781	• Eicosadienoic acid	309.2780	• TG (22:0/15:0/18:1(11Z)) (HMDB0046285) • TG (24:0/15:0/16:1(9Z)) (HMDB0047031) • TG (22:0/15:0/18:1(9Z)) (HMDB0046286) • TG (20:0/15:0/20:1(11Z)) (HMDB0045510) • TG (18:0/15:0/22:1(13Z)) (HMDB0044705)	• Eicosadienoic acid (307.3)*
312.132	• N2,N2-Dimethylguanosine	312.1319	• Hydroxylated N-acetyl desmethyl frovatriptan (HMDB0061151) • 6-Hydroxymelatonin (HMDB0004081) • cyclic 3-Hydroxymelatonin (HMDB0060069) • 2-Oxomelatonin (HMDB0060721)	• N2,N2-Dimethylguanosine (312.1298)
319.1795	• Isovalerylglycine	319.1794	• Notoginsenoside R1 (HMDB0035363)	• N-Isovaleryl-glycine (158.081)*

329.1026	• 4-Hydroxycinnamic acid • m-Coumaric acid • Phenylpyruvic acid	329.1026	• 5,7-dihydroxy-2-(3,4,5-trimethoxyphenyl)-3,4-dihydro-2H-1-benzopyran-4-one (HMDB0124860) • 1-(2,4,6-trihydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (HMDB0124863) • 2-hydroxy-2-(4-hydroxyphenyl)ethyl 3-(2,4-dihydroxy-3-methoxyphenyl)prop-2-enoate (HMDB0135789) • 2-(3,4-dihydroxyphenyl)-2-hydroxyethyl 3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate (HMDB0135790) • 2-hydroxy-2-(4-hydroxyphenyl)ethyl (2Z)-2-hydroxy-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate (HMDB0135787)	• 4-Hydroxycinnamic acid (163.0418)* • m-Coumaric acid (163.0422)* • Phenylpyruvic acid (163.0391)*
330.2593	• 4-Hydroxynonenal	330.2598	• CerP(d18:1/16:0) (HMDB0001366)	• 4-Hydroxynonenal (157.1223)
361.1856	• L-Carnitine	361.1857	• 3-Hydroxy-4-isopropylbenzyl alcohol 3-glucoside (HMDB0039385) • Cymorcin monoglucoside (HMDB0029777) • Perilloside B (HMDB0040465) • Cistocardin (HMDB0038992) • Isoeruboside B (HMDB0029769)	• L-Carnitine (163)*
398.32697	• trans-Hexadec-2-enoyl carnitine • 9-Hexadecenoylcarnitine	398.3271	• 3-Hydroxyhexadecanoylcarnitine (HMDB0013336)	• *m/z* not available

417.163	• 5-(3',4'-Dihydroxyphenyl)-gamma-valerolactone	417.1634	• N-Methylcalystegine B2 (HMDB0036604) • N-Lactoylvaline (HMDB0036604) • 1-Hydroxyhexanoylglycine (HMDB0094718) • 2-Hydroxyhexanoylglycine (HMDB0094719) • 3-Hydroxyhexanoylglycine (HMDB0094720)	• *m/z* not available
428.37424	• Stearoylcarnitine	428.3742	• Glycerol 1,3-dihexadecanoate 2-(9Z-octadecenoate) (HMDB0030969) • Glycerol 1,2-dihexadecanoate 3-(9Z-octadecenoate) (HMDB0031119) • TG (14:0/22:1(13Z)/14:0) (HMDB0042451) • TG (15:0/20:1(11Z)/15:0) (HMDB0043279) • TG (16:0/18:1(11Z)/16:0) (HMDB0044081)	• *m/z* not available
457.3636	• Oleanolic acid	457.3636	• 3-Hydroxyhexadecanoylcarnitine (HMDB0013336)	• *m/z* not available
463.2867	• Butyrylcarnitine • Isobutyryl-L-carnitine	463.2877	• Persicaxanthin (HMDB0034952) • Persicachrome (HMDB0036425)	• *m/z* not available
465.24557	• Dehydroepiandrosterone 3-glucuronide • Dehydroisoandrosterone 3-glucuronide	465.2459	• Prostaglandin G2 2-glyceryl Ester (HMDB0062591)	• *m/z* not available

508.37524	• LysoPC(P-18:0)	508.3761	• 3-beta-Hydroxy-4-beta-methyl-5-alpha-cholest-7-ene-4-alpha-carboxylate (HMDB0011662) • 4alpha-Carboxy-4beta-methyl-5alpha-cholesta-8-en-3beta-ol (HMDB0012165) • (3beta,5alpha,6beta,22E,24R)-23-Methylergosta-7,22-diene-3,5,6-triol (HMDB0033633) • Schleicherastatin 5 (HMDB0035804) • 4α-carboxy-4β-methyl-5α-cholesta-8-en-3β-ol (HMDB0062384)	• LysoPC(P-18:0) (508.3762)
508.3576	• **Isovalerylcarnitine** • **Valerylcarnitine**	508.3592	• 2-Methylbutyroylcarnitine (HMDB0000378) • Isovalerylcarnitine (HMDB0000688) • Pivaloylcarnitine (HMDB0041993) • Valerylcarnitine (HMDB0013128)	• Isovalerylcarnitine (246.17) • Valerylcarnitine (246.17)
555.3402	• Tetradecanedioic acid	555.3378	• Ganglioside GA2 (d18:1/16:0) (HMDB0004890)	• Tetradecanedioic acid (259.1904)
579.3378	• Atropine • Donepezil metabolite M4	579.3381	• Blumenol C O-[rhamnosyl-(1->6)-glucoside] (HMDB0031935)	• Atropine (290.1751)

591.5406	• Stearic acid	591.5412	• TG (a-25:0/a-25:0/i-22:0)[rac] (HMDB0070069) • TG (24:0/24:0/24:0) (HMDB0047149) • TG (22:0/a-25:0/a-25:0)[rac] (HMDB0070851) • TG (a-25:0/a-25:0/22:0)[rac] (HMDB0070696) • TG (i-22:0/a-25:0/a-25:0)[rac] (HMDB0070290)	• Stearic acid (283.2654)*
595.2727	• Alpha-CEHC	595.2731	• Sandoricin (HMDB0037555) • Udenafil (HMDB0015628)	• Alpha-CEHC (277.19)*
597.404	• L-Octanoylcarnitine	597.4043	• Alpha-Cryptoxanthin (HMDB0002268) • 1,2-Epoxy-1,2-dihydrolycopene (HMDB0035138) • (S)-1’,2’-Epoxy-1’,2’-dihydro-b,y-carotone (HMDB0030607) • Flavochrome (HMDB0036917) • Epsilon,gamma-Caroten-3-ol (HMDB0030576)	• *m/z* not available
616.5862	• Palmitoylethanolamide • Sphingosine	616.5880	• Cer(t18:0/16:0) (HMDB0010697) • Armillaramide (HMDB0037105)	• Palmitoylethanolamide (348.29)* • Sphingosine (*m/z* not available)
647.442	• cis-8,11,14,17-Eicosatetraenoic acid	647.4421	• Ganglioside GM3 (d18:1/26:0) (HMDB0004850) • Ganglioside GM3 (d18:1/26:1(17Z)) (HMDB0011927)	• cis-8,11,14,17-Eicosatetraenoic acid (327.26)
651.4649	• 5,8,11-Eicosatrienoic acid • 8,11,14-Eicosatrienoic acid	651.4619	• 3,6-Epoxy-5,5',6,6'-tetrahydro-b,b-carotene-3',5,5',6'-tetrol (HMDB0029654)	• 5,8,11-Eicosatrienoic acid (305.24806)* • 8,11,14-Eicosatrienoic acid (307.2632)

651.5851	• N-Oleoylethanolamine	651.5823	• CE(12:0) (HMDB0002262)	• N-Oleoylethanolamine (326.311)
655.6395	• Stearoylethanolamide	655.6347	• N,N-Dimethylsphingosine (HMDB0012645)	• m/z not available
661.4852	• 9,10,13-TriHOME • 9,12,13-TriHOME	661.4832	• Neoxanthin (HMDB0003020) • Violaxanthin (HMDB0003101) • Cucurbitaxanthin B (HMDB0035320) • Cucurbitachrome 1 (HMDB0041581) • Beta-Carotene (HMDB0039023)	• *m/z* not available
695.45984	• Docosahexaenoic acid	695.4606	• PS(14:0/14:1(9Z)) (HMDB0012331) • PS(14:1(9Z)/14:0) (HMDB0012341)	• Docosahexaenoic acid (327.2327)
699.225	• R-138727	699.2261	• (+/-)-threo-1-(p-Hydroxyphenyl)propylene glycol 4'-glucoside (HMDB0033068) • (+/-)-3-(4-Hydroxyphenyl)-1,2-propanediol 4'-O-glucoside • 3-(3,4-Dihydroxyphenyl)-1-propanol 3'-glucoside	• *m/z* not available
699.48474	• Docosapentaenoic acid	699.4864	• Glucosylceramide Mosinone A (d18:1/24:0) (HMDB003122804978) • Glucosylceramide (HMDB0000140)	• *m/z* not available
717.5028	• Oleoyl glycine	717.5047	• PA (15:0/22:4(7Z,10Z,13Z,16Z)) (HMDB0114827) • PA (22:5(7Z,10Z,13Z,16Z)/15:0) (HMDB0115325)	• N-Oleoyl glycine (340.2846)

721.4973	• trans-2-Dodecenoylcarnitine	721.4965	• CL(a-13:0/i-16:0/18:2(9Z,11Z)/18:2(9Z,11Z))[rac] (HMDB0079068) • CL(i-13:0/i-16:0/18:2(9Z,11Z)/18:2(9Z,11Z)) (HMDB0079073) • CL(i-12:0/i-17:0/18:2(9Z,11Z)/18:2(9Z,11Z)) (HMDB0080092) • CL(i-12:0/a-17:0/18:2(9Z,11Z)/18:2(9Z,11Z))[rac] (HMDB0080093) • CL(i-12:0/18:2(9Z,11Z)/a-17:0/18:2(9Z,11Z))[rac] (HMDB0080663)	• *m/z* not available
725.5035	• Dodecanoylcarnitine	725.5036	• CE (18:2/(9Z,12Z) (HMDB0000610)	• Dodecanoylcarnitine (342.264)*
737.31104	• N-Desmethyleletriptan	737.3110	• Capsicoside E (HMDB0031443)	• *m/z* not available
739.6061	• cis-5-Tetradecenoylcarnitine	739.6058	• MG (18:0/0:0/0:0) (HMDB0011131) • MG (0:0/18:0/0:0) (HMDB0011535) • MG (i-18:0/0:0/0:0) (HMDB0072842) • MG (0:0/i-18:0/0:0) (HMDB0072877) • Campesteryl alpha-linolenate (HMDB0036286)	• *m/z* not available
742.4546	• 18-Hydroxycorticosterone	742.4554	• Propoxyphene (HMDB0014785) • Noracymethadol (HMDB0061036) • nor-Levomethadyl acetate (HMDB0061169)	• *m/z* not available

865.5935	• **3-Hydroxy-9-hexadecenoylcarnitine**	865.5914	• 3-Hydroxy-9-hexadecenoylcarnitine (HMDB0013333)	• *m/z* not available
887.5628	• Arachidonic acid	887.5644	• Latanoprost (HMDB0014792) • PI (16:0/22:4(10Z,13Z,16Z,19Z)) (HMDB0009793) • PI (16:0/22:4(7Z,10Z,13Z,16Z)) (HMDB0009794) • PI (18:0/20:4(5Z,8Z,11Z,14Z)) (HMDB0009815) • PI (18:0/20:4(8Z,11Z,14Z,17Z)) (HMDB0009816)	• Arachidonic acid (305.2475)
897.4833	• cis-4-Octenedioic acid	897.4842	• Momordin le (HMDB0041020)	• *m/z* not available
916.6448	• Chenodeoxycholic acid glycine conjugate • Deoxycholic acid glycine conjugate • Glycoursodeoxycholic acid	916.6460	• PC (18:0/22:4(7Z,10Z,13Z,16Z)) (HMDB0008054) • PC (18:2(9Z,12Z)/22:2(13Z,16Z)) (HMDB0008152) • PC (18:3(6Z,9Z,12Z)/22:1(13Z)) (HMDB0008184) • PC (18:3(9Z,12Z,15Z)/22:1(13Z)) (HMDB0008217) • PC (18:4(6Z,9Z,12Z,15Z)/22:0) (HMDB0008249)	• Chenodeoxycholic acid glycine conjugate (450.322) • Deoxycholic acid glycine conjugate (450.1)

923.6462	• 3a,7a,12a-Trihydroxy-5b-cholestanoic acid	923.6525	• TG(15:0/20:5(5Z,8Z,11Z,14Z,17Z)/20:5(5Z,8Z,11Z,14Z,17Z)) (HMDB0043739) • TG(18:4(6Z,9Z,12Z,15Z)/15:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z) (HMDB0055367) • TG(15:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:4(6Z,9Z,12Z,15Z)) (HMDB0043795)	• *m/z* not available
1135.661	• LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))	1135.6628	• Melilotin (HMDB0034527)	• *m/z* not available
1169.5239	• Bilirubin	1169.5271	• Notoginsenoside I (HMDB0031371)	• Bilirubin (583.2)*
1538.0164	• Decanoylcarnitine	1538.0045	• PS (15:0/18:0) (HMDB0112322) • PS (18:0/15:0) (HMDB0112372)	• *m/z* not available
1641.6960	• 5-Aminopentanoic acid	1641.7167	• Quillaic acid 3-[galactosyl-(1->2)-[rhamnosyl-(1->3)]-glucuronide] 28-[6-acetyl-glucosyl-(1->3)-[xylosyl-(1->4)-rhamnosyl-(1->2)]-4-acetyl-fucosyl] ester (HMDB0036949)	• 5-Aminopentanoic acid (118.0865)

[1] Software searched Metlin database for compound identification.

[2] *m/z* ratio detected by MassHunter Qualitative Analysis software (version B.07).

[3] HMDB LC-MS Spectra Search using positive ion mode and unknown adduct; retrieved from http://www.hmdb.ca

[4] Displaying the first five suggested metabolites for the nearest *m/z* ratio.

[5] Data retrieved from https://pubchem.ncbi.nlm.nih.gov

**m/z* ratio not confirmed by HMDB.

Boldface delineates compounds whose *m/z* ratios and suggested identities matched between MassHunter Qualitative Analysis software and HMDB.

Biological Interpretation

Five select compounds (3-oxoglutaric acid, oxoglutaric acid, isovalerylcarnitine, valerylcarnitine, and 3-hydroxy-9-hexadecenoylcarnitine) were investigated for their biological relevance to the vascular outcomes observed in Chapter 4. Using the methodological approach described in Figure S5.1, a literature search was performed using HMDB, PubMed, and KEGG (if applicable) to examine the biochemical pathways and relevance of these five metabolites. A summary of the literature results is provided in **Table 5.4**.

Overall, there were four primary findings from the literature search: (1) elevated plasma levels of acylcarnitine metabolites (3-hydroxy-9-hexadecenoylcarnitine, isovalerylcarnitine, and valerylcarnitine) showed links to metabolic diseases such as type 2 diabetes (Mihalik *et al* 2010), insulin resistance, metabolic syndrome (Rousseau *et al* 2019), or alcohol hepatitis (Ascha *et al* 2016); (2) oxoglutaric acid (alpha-ketoglutarate) is an endogenous intermediary metabolite of the Krebs cycle (Wu *et al* 2016) and is involved in branched-chain amino acid (BCAA) metabolism (Adeva-Andany *et al* 2017); (3) BCAA metabolism is involved with the production of certain short-chain acylcarnitines (Crown *et al* 2015); and (4) 3-oxoglutaric acid is often measured in urine for indication of yeast growth (The Great Plains Laboratory 2019). The biological relevance of plasma levels of 3-oxoglutaric acid was not disclosed in the literature. For this reason, 3-oxoglutaric acid was not considered as a further compound of interest. The reduction in the circulating levels of the three acylcarnitine metabolites and oxoglutaric acid following black bean consumption suggests a potentially favourable effect of black beans on the production and metabolic actions of short-chain acylcarnitines.

Table 5.4 Literature summary and biological relevance of metabolites of interest.

Metabolite	Synonym(s)[1]	Biological roles and/or pathways[1]	Related literature
3-hydroxy-9-hexadecenoylcarnitine	3-Hydroxy-9(Z)-octadecenoylcarnitine; methylmalonylcarnitine[2]	• Beta-hydroxy acid derivative • Methylmalonylcarnitine contributes toward plasma C4-dicarboxylcarnitine levels[3]	• Increased plasma C4-dicarboxylcarnitine levels in type 2 diabetes[3]
3-oxoglutaric acid	Acetonedicarboxylic acid; acetone 1,3-dicarboxylic acid[4]; beta-ketoglutaric acid[4]	• Short-chain keto-acid derivative • Breakdown produces acetoacetic acid + carbon dioxide[5] • Acetonedicarboxylic acid used in synthesis of heterocyclic rings[6]	• Increased urinary 3-oxoglutaric acid indicative of yeast overgrowth in gastrointestinal tract[7]
Oxoglutaric acid	2-oxoglutaric acid; alpha-ketoglutarate	• Rate-determining intermediate in Krebs cycle[8] • Source of glutamate and glutamine for protein synthesis in skeletal muscle[8] • Involved in BCAA transamination reactions[9]	• Leucine and isoleucine catabolism involved in production of acetyl-CoA of Krebs cycle and β-oxidation[10]
Isovalerylcarnitine	3-Methylbutyrylcarnitine	• Odd- and short-chain acylcarnitine (SCAC; C5 acylcarnitine) • Leucine catabolite[10]	• Increased circulating SCACs associated with metabolic disturbances (e.g. insulin resistance, metabolic syndrome)[11] • Consumption of pulses decreased circulating SCACs[12] • Increased plasma pentanoylcarnitine a biomarker for alcohol hepatitis[14]
Valerylcarnitine	C5-carnitine; pentanoylcarnitine	• Odd- and SCAC • Pentanoylcarnitine is a by-product of valproate mitochondrial metabolism[13]	

[1]Information obtained from HMDB unless otherwise stated; [2]The Milk Composition Database; [3]Mihalik *et al* 2010; [4]Sigma-Aldrich 2019; [5]Larson & Lister 1968; [6]Stanovnik & Grošelj 2010; [7]The Great Plains Laboratory 2019; [8]Wu *et al* 2016; [9]Adeva-Andany *et al* 2017; [10]Crown *et al* 2015; [11]Rousseau *et al* 2019; [12]Zahradka *et al* 2013; [13]Silva *et al* 2002; [14]Ascha *et al* 2016. *Abbreviations:* BCAA, branched-chain amino acid; SCAC, short-chain acylcarnitine.

5.5 Discussion

In this study, our purpose was to determine if endogenous metabolites were modified in response to the consumption of darker-coloured beans and if these metabolites could be related to the postprandial vasorelaxation previously reported in Chapter 4. The outcome of the current metabolomics analysis was the detection of 12 and 35 compounds uniquely present in the serum when the participants consumed red kidney beans or black beans, respectively. These compounds were increased or decreased at time points corresponding with the postprandial vasorelaxation reported in Chapter 4. Additionally, black beans appeared to elicit the greatest changes in the endogenous metabolome, as indicated by a greater number of compounds increasing or decreasing compared to the other beans and white rice. However, the identities of these compounds are speculative, as they have not been verified. This study also presents the discrepancies observed between MHQ software and well-known, publicly available metabolite databases, such as HMDB and PubChem. Overall, the results from this study indicate that consuming darker-coloured beans substantially modifies endogenous compounds at time points when vasorelaxation occurred (as previously reported). Thus, our observations support the need for further investigation into these compounds of interest and their potential involvement in the mechanism(s) that lead to vasorelaxation.

The untargetted approach employed in this study required several choices to be made as the analysis progressed. One-way ANOVA determined 96, 131, 129, 102, and 132 compounds were significantly increased or decreased among the three time points for white rice, navy beans, pinto beans, red kidney beans, and black beans, respectively. The list of potential compounds of interest was narrowed down by prioritizing the changes occurring between pairs of the three time points. This prioritization was determined based on the previously reported vasorelaxant effects

observed 6 hours after the consumption of darker-coloured beans (Chapter 4). Therefore, four regulation patterns were considered relevant: 1) consistently decreased over 6 hours; 2) transiently decreased between baseline and 2 hours, then increased to baseline levels or higher at 6 hours; 3) transiently increased between baseline and 2 hours, then decreased to baseline levels or lower at 6 hours; or 4) consistently increased over 6 hours. Within these four prioritized patterns, the compounds were compared between the five food articles, and only 12 and 35 metabolites were unique to red kidney beans and black beans, respectively. From this narrowed list, attempts were made to verify the identities of these compounds by searching the *m/z* ratios generated by MHQ in external databases. Eleven databases were reviewed (**Table S5.1**); HMDB and METLIN were the most user-friendly and publicly available databases for *m/z* queries and, thus, were preferred for these searches. HMDB and METLIN produced similar results; therefore, HMDB, being the database not utilized by MHQ, was used as the main comparator in Table 5.3. Additionally, compound names suggested by MHQ were searched in PubChem (a commonly used chemical database) to verify *m/z* ratios. Of these various searches, five compounds had matching *m/z* ratios in MHQ and HMDB, and these compounds were detected in the serum of participants fed black beans. No compounds were matched by PubChem using the *m/z* ratios. It was not possible to verify the remaining 83 compounds using these databases. None of the compounds were compared against authentic reference standards to verify absolute identities.

The objective of the current study was to determine the endogenous compounds of the human metabolome that changed in response to the consumption of darker-coloured beans. HMDB presented five compounds for which their suggested identities, based on the *m/z* query, matched the identities proposed by MHQ. Of these compounds, suggested identities included

two circulating short-chain acylcarnitines (isovalerylcarnitine and valerylcarnitine), which were reduced in response to black beans. There were other acylcarnitines detected in the serum and the majority of these were lowered in response to consuming each of the four bean types (data not shown); however, their *m/z* ratios could not be matched by the metabolite databases. Interestingly, this is not the first to study to indicate consumption of pulses, including beans, reduces serum short-chain acylcarnitine levels. Zahradka *et al* (2013) reported pulse consumption significantly reduced circulating short-chain acylcarnitines in individuals with peripheral artery disease. The circulating acylcarnitine levels were detected and verified using direct flow injection MS and isotope-labelled internal standards, respectively (Zahradka *et al* 2013). While isovalerylcarnitine and valerylcarnitine were not among those acylcarnitines detected and verified by Zahradka *et al* (2013), these results do suggest a precedent for pulses, including black beans, to reduce circulating acylcarnitine levels in otherwise healthy individuals. The reduction in circulating acylcarnitines indicates improved fatty acid metabolism by the skeletal muscles (Strand *et al* 2017; Zahradka *et al* 2013). Furthermore, this improvement in fatty acid metabolism was attributed to better arterial blood flow induced by pulse consumption (Zahradka *et al* 2013). While the study in Chapter 4 did not measure arterial blood flow directly, we reported postprandial vasorelaxation, an indication of improved vascular tone, was induced by the consumption of darker-coloured beans. Vascular tone modulates blood flow (Jackson 2000), and improved vascular tone might result in increased blood flow. Therefore, our results, in combination with those previously reported by Zahradka *et al* (2013), suggest further investigation into the potential mechanism(s) involved with acylcarnitines and vascular function.

Consumption of black beans also reduced 3-hydroxy-9-hexadecenoylcarnitine, a metabolite suggested to contribute to the plasma levels of C4-dicarboxylcarnitine which is

positively associated with type 2 diabetes (Mihalik *et al* 2010). Vascular disturbances, such as endothelial dysfunction and vasoconstriction, are likely to occur with diabetes, thus increasing the risk for vascular diseases (e.g., atherosclerosis, retinopathy, nephropathy) which are the primary cause of morbidity and mortality in people with diabetes (Creager *et al* 2003). The reduction of 3-hydroxy-9-hexadecenoylcarnitine, as well as isovalerylcarnitine and valerylcarnitine, indicate a favourable effect of black beans on short-chain acylcarnitines. It is unknown how or why black beans affect these short-chain acylcarnitines; however, certain short-chain acylcarnitines are byproducts of BCAA catabolism (e.g. isovalerylcarnitine from leucine breakdown) (Crown *et al* 2015) where oxoglutaric acid is involved upstream of acylcarnitine production (Adeva-Andany *et al* 2017). Therefore, it is possible that oxoglutaric acid is a rate-limiting step for BCAA metabolism; as a result, decreased levels of oxoglutaric acid (due to black bean consumption) may reduce or inhibit catabolism of certain BCAAs, resulting in a lower production of short-chain acylcarnitines catabolites (**Figure S5.2**). The mechanism by which black beans decrease levels of oxoglutaric acid (alpha-ketoglutarate) is unknown, but it is possible that black beans inhibit or reduce activity of enzymes involved in the synthesis of oxoglutaric acid during Krebs cycle. However, it must be reiterated that the identities of these compounds, regardless of the matching database responses, are tentative as they have not been verified against pure reference standards.

Possible reasons for the discrepancy between the databases include the need for better search information to assist with locating potential compounds. For example, when searching HMDB and METLIN using the same *m/z* ratio (361.1856), METLIN yields five compounds with identities matching the search response for HMDB. However, while these five compounds have the same *m/z* ratio and mass, they have different adduct species, which is one parameter

accounting for their multiple identities. Additionally, the same adduct species presented have more than one identity option. Therefore, to retrieve the most accurate identity using these database repositories, as much mass spectra information as possible should be inputted, including, but not limited to, retention time, retention index, mass fragment pattern, parent ion mass, and elemental composition (Scalbert *et al* 2009). There is also the issue that the presence of endogenous and exogenous compounds in databases such as HMDB and METLIN may confound the search results. For example, when searching the *m/z* ratio of L-glutamine, HMDB suggests Phytolaccoside D2, a metabolite found in fruit, as a potential match. Given the human origin of the metabolites in this data set, Phytolaccoside D2 is not a realistic match. Therefore, a useful addition to database search engines would be the option to restrict matching to certain kinds of compounds (e.g. endogenous, exogenous) within the mass spectra search (Scalbert *et al* 2009). While HMDB provides the option to search metabolites with a given origin (e.g. endogenous, drug, food, etc.), this search condition is not optional when searching by *m/z* ratio. Overall, search results within and among databases may be improved by not only providing as much mass spectra information as possible but also by being able to search this information within a given origin.

The current study refined and prioritized a list of potential compounds for further verification of their metabolite identities, with the goal of subsequently investigating their possible mechanistic contributions toward vasorelaxation induced by darker-coloured beans. This framework follows that which is suggested by Scalbert *et al* (2009), in which an untargetted approach is employed first for hypothesis testing, followed secondly by a targetted approach to validate the mechanism(s) of action. Additionally, authentic standards should be utilized as reference compounds to determine the absolute identity of metabolites (Scalbert *et al* 2009; Xiao

et al 2012). While authentic standards would verify the identity of the compounds, it is important to note that changes occurring in serum are not simply translated to changes occurring within an organ or tissue (Scalbert *et al* 2009). Therefore, it is also recommended that human intervention studies analyzing metabolite profiles be followed by an animal model study to associate the changes in serum metabolite levels to the physiology of the target tissue(s) (Scalbert *et al* 2009).

Based on the information observed during the current study and the information obtained from the literature (Scalbert *et al* 2009; Xiao *et al* 2012), it is of this author's opinion that metabolite databases be used as platforms for obtaining information related to metabolites and for assisting, but not confirming, metabolite identification. Xiao *et al* (2012) stated that computational approaches assist with metabolite identification, but they cannot replace experimental verification. To this end, these databases supply suggested identities of the queried compound, not absolute identities; researchers performing such studies should therefore be cognizant of this limitation, and it should be meticulously stated that identities are relative, or suggested, unless verified by authentic standards.

In conclusion, using an untargetted metabolomics approach, we detected unique changes in the endogenous metabolome induced by the consumption of darker-coloured beans. Furthermore, black beans reduced compounds putatively identified as acylcarnitines, which may be associated with improved vascular function. However, absolute identities were not verified at this time. In addition, the numerous identities produced for single metabolites in this study reinforces the need for identifications to be verified against authentic standards. Therefore, further investigation is required to verify these compounds of interest regulated by darker-coloured beans and their potential mechanistic contributions within the vasculature.

Acknowledgements

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5.6 Supplementary Material

Table S5.1 Summary of components found in databases searched for endogenous compounds.

Database[1,2,3]	Data coverage	Search (S) functions and result (R) output					Additional information	
		Compound name (S) (R)	***m/z* ratio** (S) (R)	**Molecular weight** (S) (R)	**Chemical formula** (S) (R)	**Other**	**Links to other databases**	**Comments**
ChemSpider	> 67 million structures	✓ ✓	**X X**	**X** ✓	✓ ✓	• n/a	**X**	• Provides compound IDs for other databases
Golm Metabolome Database	1,000 metabolites	✓ ✓	**X X**	✓ ✓	✓ ✓	• CAS • KEGG	**X**	• n/a
Human Metabolome Database	114,100 metabolites	✓ ✓	✓ ✓	✓ ✓	✓ ✓	• Sequences • CAS • Pathways	✓	• n/a
KEGG	618,519 pathways 18,505 compounds	✓ **X**	**X X**	**X X**	**X** ✓	• Pathways • Pathway maps • Genes • Diseases • Other	✓	• n/a

Madison Metabolomics Consortium Database	20,306 compounds	✓✓	✓ **X**	✓✓	✓✓	• Other database IDs • CAS	✓	• Require adduct species for *m/z* search • Mass search requires inputting batch files
MassBank	n/a	✓✓	✓✓	✓✓	✓✓	• CAS	✓	• n/a
MetaCyc	2,698 pathways 20,351 compounds	✓✓	**X X**	✓ ✓	✓ ✓	• Pathways • Genes • Proteins	✓	• Molecular weight search yields compounds ≥ searched molecular weight
METLIN	>1 million metabolites	✓✓	✓✓	✓✓	✓✓	• Other database IDs • CAS	✓	• Requires registration • Requires adduct species information for accurate *m/z*

mzCloud	16,236 compounds	✓X	X X	X X	X ✓	•CAS	✓	• Does not provide best-match *m/z* ratios • Requires software download • Mass spectra data only available after software installation; unable to consistently access
PubChem	97,126,025 compounds	✓✓	✓	✓✓	✓✓	•BioAssays •Substances •Pathways	✓	• n/a
Small Molecule Pathway Database	> 30,000 small molecule pathways	✓X	X X	✓X	✓✓	•Pathways •Other database IDs •Sequence •Concentrations	✓	• Can search compounds for their pathways

[1] Databases were accessible free-of-charge

[2] Databases were discovered based on internet search and Metabolomics Workbench (UC San Diego).

[3] Websites: ChemSpider, http://www.chemspider.com/Default.aspx; Golm Metabolome Database, http://gmd.mpimp-golm.mpg.de; Human Metabolome Database, www.hmdb.ca; KEGG, https://www.genome.jp/kegg/; Madison Metabolomics Consortium Database, http://mmcd.nmrfam.wisc.edu; MassBank, http://mona.fiehnlab.ucdavis.edu/; MetaCyc, http://metacyc.ai.sri.com; METLIN, https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage; mzCloud, https://www.mzcloud.org; PubChem, https://pubchem.ncbi.nlm.nih.gov; Small Molecule Pathway Database, www.smpdb.ca.

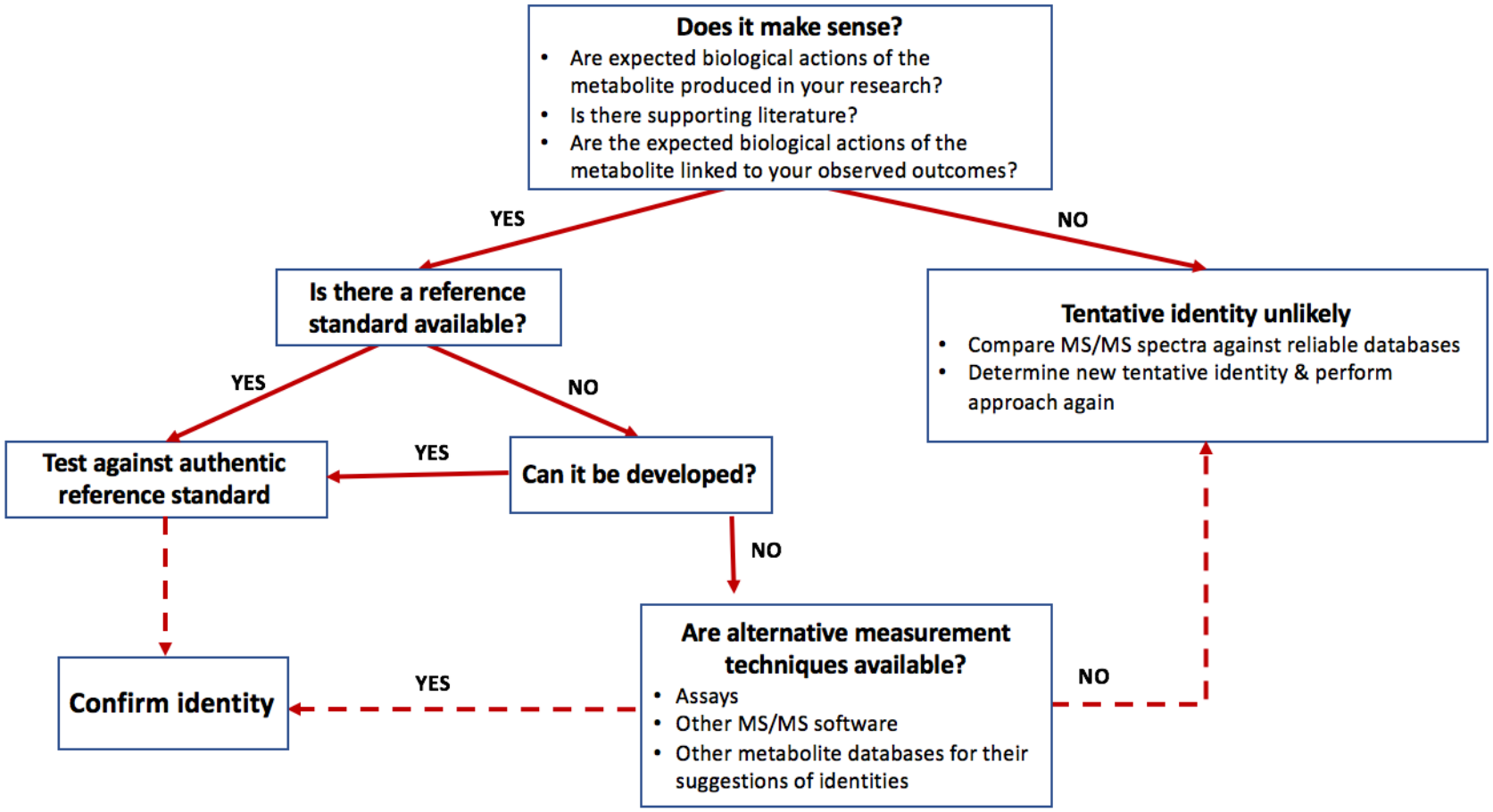

Figure S5.1 Methodological approach for determining metabolite identities.

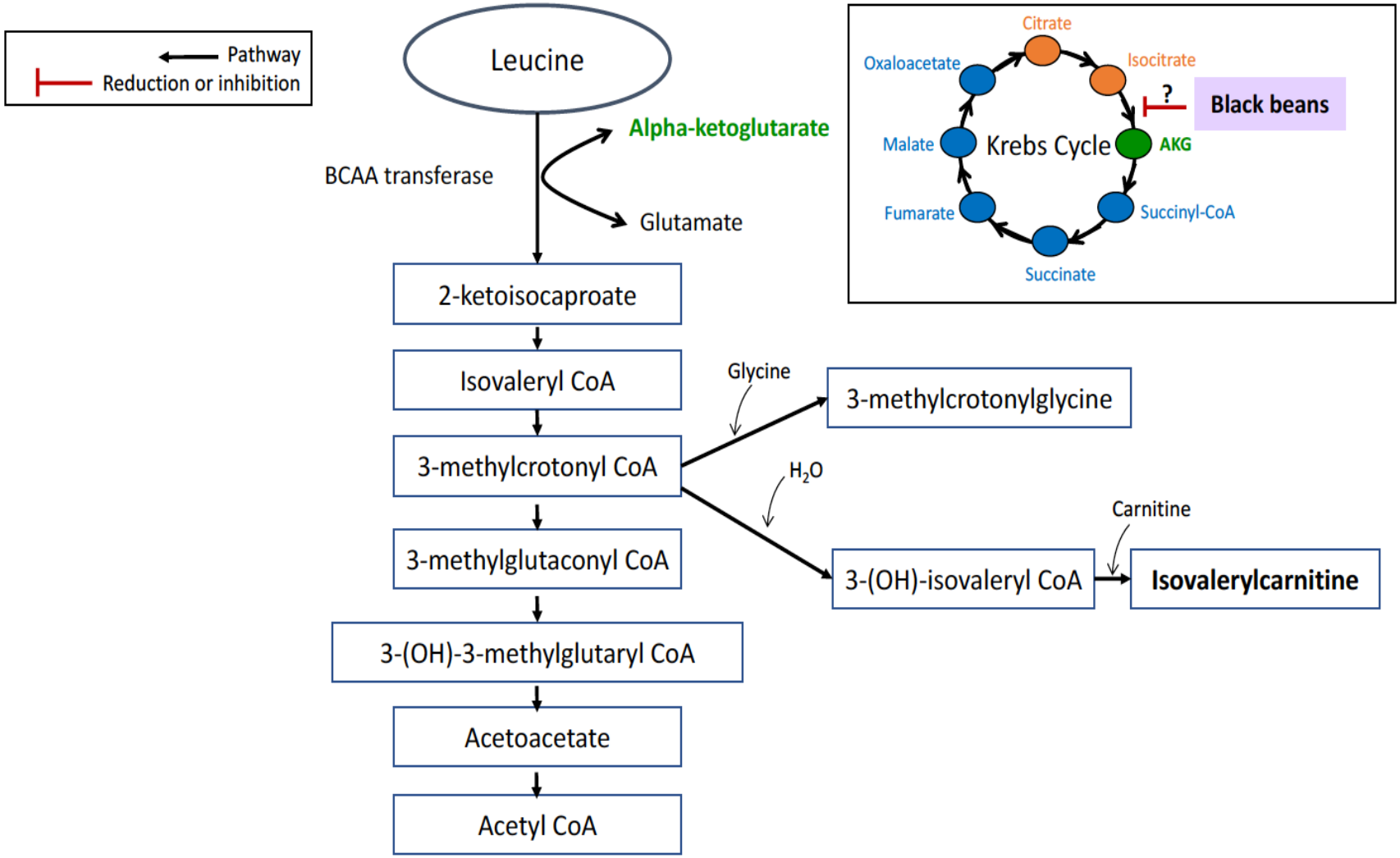
Pathway
Reduction or inhibition
Leucine
Alpha-ketoglutarate
BCAA transferase
Glutamate
2-ketoisocaproate
Isovaleryl CoA
3-methylcrotonyl CoA
3-methylglutaconyl CoA
3-(OH)-3-methylglutaryl CoA
Acetoacetate
Acetyl CoA
Glycine
3-methylcrotonylglycine
H2O
Carnitine
3-(OH)-isovaleryl CoA
Isovalerylcarnitine
Citrate
Isocitrate
Oxaloacetate
Malate
Krebs Cycle
AKG
Fumarate
Succinyl-CoA
Succinate
?
Black beans

Figure S5.2 Potential mechanism of action for black beans modulating BCAA acylcarnitine catabolites.

Abbreviations: AKG, alpha-ketoglutarate; BCAA, branched-chain amino acid. Adapted from Berg *et al* 2002; Brosnan & Brosnan 2006; Crown *et al* 2015.

Boldface indicates key compounds.

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Transition Statement Two

Chapter 5 addressed the second hypothesis of this dissertation, which proposed that consumption of darker-coloured beans would differentially modulate endogenous compounds related to acute vascular function, either directly or indirectly, compared to the consumption of lighter-coloured beans. Using an untargetted metabolomics approach, we detected unique changes in the endogenous metabolome induced by the consumption of darker-coloured beans. Furthermore, black beans reduced compounds putatively identified as acylcarnitines, which may be associated with improved vascular function. However, confirmation of compound identities by testing against pure standards was not performed at this time.

As previously discussed in earlier chapters, key studies have shown that long-term consumption of pulses (4-8 weeks) can improve vascular function in subjects with hypertension and/or arterial stiffness (Hanson *et al* 2014; Hanson *et al* 2016; Zahradka *et al* 2013). However, it is unknown if beans can improve hypertension-induced structural and functional changes in the vasculature of a rodent model. Additionally, it is unknown if beans need to be habitually consumed to maintain their beneficial effects within the vasculature, or if their biological effects are retained after long-term consumption. Thus, the next chapter explores (1) whether chronic consumption (8 weeks) of darker-coloured beans *vs* lighter-coloured beans (black beans *vs* navy beans) can modulate vascular structure and function in SHR, a rodent model of hypertension; and (2) if the vascular improvements from chronic consumption of beans are sustained after bean consumption is discontinued during a washout phase (2 weeks and 4 weeks).

Chapter 6: Regular Black Bean Consumption is Necessary to Sustain Improvements in Small-Artery Vascular Compliance in the Spontaneously Hypertensive Rat

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6.1 Abstract

Vascular remodelling and arterial stiffness are structural and functional changes, respectively, underlying the clinical symptoms of hypertension. Pulses, such as dried beans, may be useful in treating hypertension by attenuating vascular remodelling and arterial stiffness through mechanisms related to their bioactive phytochemical compounds. It was hypothesized that darker-coloured beans, with extensive phytochemical composition (greater amount and/or variety) as indicated by their dark seed coat colour, have a greater potential for vascular improvements. Thus, the objective of this study was to compare the effects of black bean versus navy (white) bean consumption on hypertension, vascular remodelling, and vascular compliance in SHR, with a follow-up phase to determine how long the alterations in vascular properties are maintained after bean consumption had stopped. Assessments of BP, PWV, vessel compliance (small-artery) and morphology (large-artery) were conducted to ascertain the vascular effects of the beans. Results showed that neither black beans nor navy beans influenced BP, PWV, or vessel geometry in SHR. Black bean consumption for 8 weeks improved vascular compliance by 11% compared to the bean-free control diet, and this improvement was reversed within 2 weeks after cessation of bean consumption ($p<0.05$). Black beans, but not navy beans, improved vascular compliance, indicating that the phytochemical content of beans, reflected by seed coat colour, is likely responsible for their efficacy on vascular function. Thus, regular consumption of black beans, due to their phytochemical content, could potentially be a therapeutic dietary strategy for combating the remodelling and diminished compliance of blood vessels that underlies hypertension.

6.2 Introduction

Hypertension occurs in more than 1 in 5 Canadian adults with 95% of cases having an unknown etiology (known as essential hypertension), making treatment difficult (Carretero *et al* 2000; Padwal *et al* 2016). Current prescription drug therapy for hypertension focuses on controlling high BP, which unfortunately is unachievable for 30% of individuals (Padwal *et al* 2016). This gap in treatment efficacy suggests that it may be beneficial to pursue therapeutic strategies that target the vascular structural changes underlying hypertension (i.e. vascular remodelling (Feihl *et al* 2008)), rather than the clinical symptoms such as elevated SBP and/or DBP.

Although essential hypertension has no known etiology, diet is identified as a modifiable risk factor (Hypertension Canada 2018). A meta-analysis has shown that various pulses (beans, peas, chickpeas, lentils) exert a BP-lowering effect in hypertensive individuals (Jayalath *et al* 2014). Lentils, consumed whole (Hanson *et al* 2014 and 2016) or as a polyphenol-rich extract (Yao *et al* 2012), have been reported to mitigate structural and functional changes to hypertensive blood vessels. While various pulses have similar macronutrient and micronutrient content, it is possible that differences in their phytochemical composition, including polyphenols, could affect their therapeutic potential with respect to vascular function. As indicated by their dark seed coat colour, black beans typically have a greater total phenolic content and antioxidant activity than other bean types (Xu *et al* 2012), but they have yet to be examined for their vascular remodelling effects.

The first aim of this study was to compare beans with contrasting seed coat colours (black beans *vs* white navy beans) for their effects on BP, vascular function, and vascular remodelling after 8 weeks of intervention in a rodent model of essential hypertension. The

second aim was to determine if regular consumption of the effective bean (black or navy) is required for maintenance of the vascular effects by performing assessments at 2 and 4 weeks of a washout phase for this objective. We hypothesized that black beans, with greater phytochemical content as suggested by their dark seed coat colour, would attenuate the progression of hypertension and improve blood vessel function and structure in SHR. Additionally, we hypothesized that the effective bean would need to be consumed regularly to maintain their vascular benefits.

6.3 Materials and Methods

Animals

Fifteen-week old male SHR and WKY rats (Charles River Laboratories, Saint-Constant, QC, Canada) were housed individually in standard plastic-bottomed cages in a room maintained at 25ºC with 40-60% relative humidity and a 12 h light – 12 h dark cycle. The rats were acclimated for one week, and thus were 16 weeks old for baseline assessments. In Phase 1, the rats were randomized into five groups (n=10/group) for 8 weeks: i) normotensive control WKY rats fed bean-free control diet (WKY-CTRL); ii) SHR fed bean-free control diet (SHR-CTRL); iii) SHR fed a black bean diet (SHR-BB); and iv) SHR fed a navy bean diet (SHR-NB). In Phase 2, the SHR were fed a black bean diet for 8 weeks (n=10) and subsequently were randomized into two washout groups: i) fed bean-free control diet for 2 weeks (n=5) or ii) fed bean-free control diet for 4 weeks (n=5). Black beans were used in Phase 2 since they were considered the more effective bean based on preliminary vascular compliance results obtained from Phase 1. These experiments were carried out in accordance to proper animal care and experimentation as outlined by the Canadian Council on Animal Care and a protocol approved by the University of Manitoba Animal Care Committee.

Diet

Diets were formulated and prepared as previously described (Hanson *et al* 2014) (**Table 6.1**). Black beans (~90% Eclipse, ~10% unknown variety) and navy beans (~75% T9905, ~20% Envoy, ~5% unknown variety) were sourced from Legumex Walker (Plum Coulee, MB). Whole beans were soaked overnight, boiled, freeze-dried, milled to a fine powder and then added to the diet at a 30% w/w inclusion which represents approximately 35% of total energy from beans. Diets were provided *ad libitum*. The previous day's unconsumed feed was weighed to determine daily feed intake.

Table 6.1 Experimental diet formulations.

	Control diet[1]	Navy bean diet	Black bean diet
Ingredients[2,3] (g/kg)			
Casein	200	118	121.5
Maize starch	397	229.5	226
Maltodextrin	132	132	132
Sucrose	100	100	100
Cellulose	50	0	50
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
AIN-93G-MX mineral mix	35	35	35
AIN-93-VX vitamin mix	10	10	10
Soybean oil[4]	70	70	70
Pulse powders[5] (g/kg)			
Navy bean	-	300	-
Black bean	-	-	300

[1]American Institute of Nutrition-93G formulation (Reeves *et al* 1993).

[2]Ingredients (except pulse powders) from Dyets, Inc (Bethlehem, PA, USA).

[3]Adjustments to casein, maize starch, and cellulose for pulse diets were based on proximate analysis of the pulse powders (see **Table S6.1**).

[4]With 0.02% tert-butylhydroquinone.

[5]Pulse powders were prepared as described in the Methods section.

Body Weight and Composition

Body weights were measured weekly during both Phases. Whole body composition (fat mass and lean body mass) was assessed *in vivo* using the EchoMRI-700 whole body quantitative magnetic resonance instrument (EchoMRI, Houston, TX, USA) at baseline and 8 weeks in Phase 1 and at baseline, 8, 10, and 12 weeks in Phase 2.

Serum Biochemistry

Prior to sample collection, rats were fasted for 6 hours in metabolic cages with free access to water. Fasting blood samples were collected from the saphenous vein at baseline and 8 weeks for Phases 1 and 2, with additional collections at 10 and 12 weeks for Phase 2. Serum HDL-C, LDL-C, TC, and TG concentrations were determined using a Cobas c111 auto analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Blood Pressure and Pulse Wave Velocity

BP was measured by tail-cuff plethysmography (CODA system; Kent Scientific, Torrington, CT, USA) at 0, 4, and 8 weeks for Phases 1 and 2, and at 10 and 12 weeks in Phase 2. A minimum of five values were obtained per animal per time point for SBP, DBP and MAP. PWV of the femoral artery of anesthetized rats was measured at baseline and 8 weeks for Phases 1 and 2, and at 10 and 12 weeks in Phase 2, with a 10-MHz electrocardiogram-triggered Doppler probe (Indus Instruments, Houston, TX, USA). Three trace measurements were obtained per animal per time point. Analysis of the PWV data was done in a blinded manner using the Doppler Signal Processing Workstation program (DSPW Version 1.624; Indus Instruments, Houston, TX, USA) to determine peak velocity, mean flow velocity and minimum flow velocity.

Tissue Collection

At endpoints for each Phase (8, or 10 and 12 weeks), rats were euthanized by intraperitoneal injection of sodium pentobarbital, followed by decapitation (with rats held horizontally during blood collection to minimize contamination of the blood sample by gastric fluid (Stricker *et al* 2007)). The aorta and mesenteric bed were excised for further analyses. The aortae were placed in Optimal Cutting Temperature Compound embedding media (Sakura Finetek USA Inc., Torrence, CA, USA), frozen in a dry ice/ethanol bath, and stored at -80^{o}C until analyzed.

Pressure Myography of Resistance Arteries

Immediately following dissection, a third order vessel was isolated from the first 10 cm of mesenteric fat and mounted on a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA), pressurized to 45 mmHg, allowed to equilibrate for 1 h in Krebs–Henseleit (KH) buffer (25 mM $NaHCO_3$, 5.5 mM glucose, 2.7 μM NaEDTA, 120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH 7.4) at 37°C, then challenged with 125 mM KCl solution (in KH buffer) to constrict and confirm arterial viability. After 30 min in Ca^{2+}-free KH buffer containing 10 mM EGTA, triplicate measurements of lumen diameter and left and right wall thickness were recorded at 3, 10, 20, 30, 40, 60, 80, 100, 120 and 140 mmHg.

Aortic Morphology

Using a Thermo Shandon Cryotome (Thermo Fisher Scientific, Waltham, MA, USA), 5 μm sections of aortae were prepared. The slides were kept frozen at -20^{o}C until stained using an Elastin Stain Kit (Sigma-Aldrich; St. Louis, MO, USA). The slides were fixed in 1% paraformaldehyde for 10 min, washed in 1× phosphate buffer solution (137 mM NaCl, 3 mM

KCl, 10 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) for 10 min, hydrated in double-distilled water for 15 min, and then stained according to the manufacturer's protocol. Differentiation in the working ferric chloride solution was done for 3 min, and staining in the Van Gieson solution was for 90 seconds. Slides were dehydrated in xylene, air-dried for 1 hour, and then mounted using VectaMount Permanent Mounting Medium. Slides were imaged with an EVOS® microscope (AMG Solutions) under 4× and 20× objectives. ImageJ software was used to determine vessel morphology (4× objective), and elastin and collagen content (stained black and red, respectively; 20× objective) (Schneider *et al* 2012).

Statistical Analyses

Data were analyzed using repeated-measures ANOVA for time-course data (body weight, body composition, serum analysis, BP, PWV) and one-way ANOVA for endpoint data using SAS statistical software (version 9.3). Duncan's multiple range post hoc test was used to determine significance among the means. Data that were not normal after log transformation (serum LDL-C, serum TG, body weight, aortic lumen diameter and CSA, aortic collagen content (total and relative), and aortic collagen:elastin ratio), as determined by the Shapiro-Wilk normality test, were analyzed using the Wilcoxon Rank-Sum test for non-parametric data followed by Tukey-Kramer multiple comparison test to determine differences between least-squares means. A Box-Cox analysis was used to determine the most appropriate power transformation for nonlinear data (media stress and strain) and this resulted in the square-root transformation being used for media stress. Results were considered statistically significant at $P<0.05$.

6.4 Results

Phase 1: Comparing the Effects of Navy and Black Beans

Feed Intake & Body Composition

WKY rats consumed less feed throughout the trial but had a higher body weight compared to SHR, regardless of the dietary intervention (**Table 6.2**). There was a distinct separation in whole body fat mass between WKY and SHR animals at baseline and week 8 (**Figure 6.1a**). The rightward direction of the linear relationship (positive slope) between fat mass and lean mass indicates an increase in lean mass over time, whereas the upward direction (steepest slope) indicates an increase in fat mass over time. The WKY rats had the steepest slope, which was significantly different from the slope of the SHR consuming the navy bean diet (**Figure 6.1b**). Interestingly, the relationship between fat mass and lean mass shifted rightward for SHR-NB (presenting as a negative slope), indicating an increase in lean mass, but not fat mass, over time. There were no differences in the slope of the fat-lean mass relationship among WKY, SHR-CTRL, and SHR-BB groups.

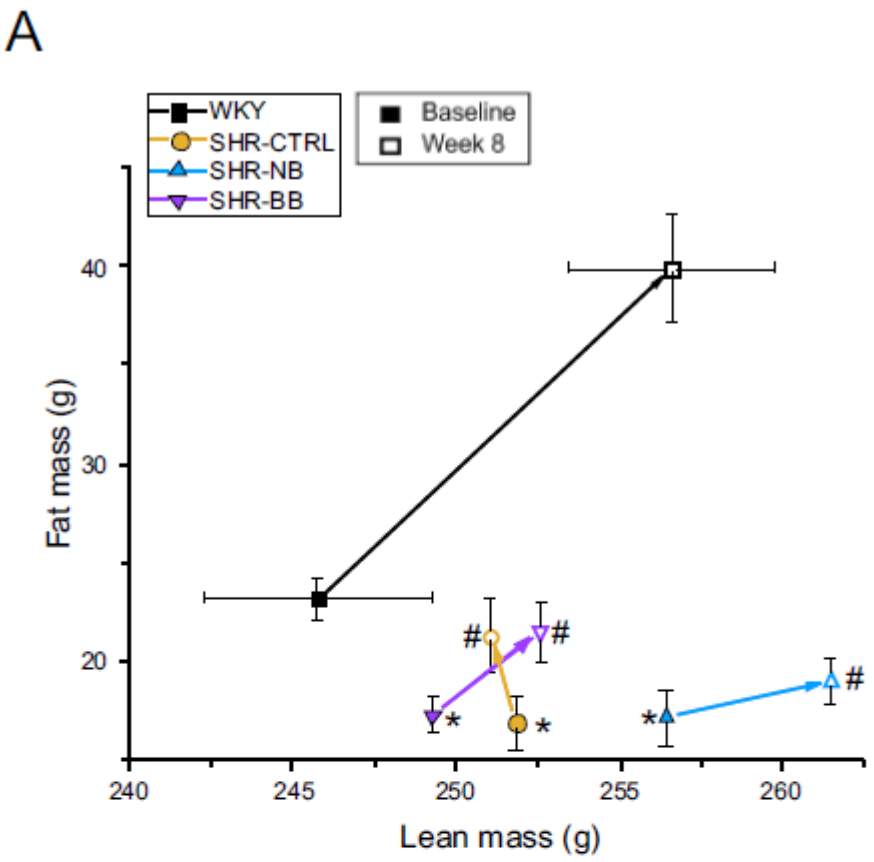
Serum Lipid Analysis

At baseline and week 8, WKY rats had higher serum TG, TC, HDL-C, and LDL-C than SHR (Table 6.2). There were no differences in serum lipids among SHR groups after 8 weeks of bean consumption.

Table 6.2 Phase 1: Metabolic parameters.

	WKY	SHR		
	Control	Control	Navy bean	Black bean
Feed intake (g/day)				
Week 1	17.6 ± 0.6	20.9 ± 1.5	18.4 ± 1.8	21.3 ± 1.7
Week 8	24.8 ± 2.4^{b}	43.5 ± 2.7^{a}	34.9 ± 4.1^{a}	39.2 ± 4.5^{a}
Body weight (g)				
Baseline	336 ± 6	324± 6	338 ± 3	335 ± 4
Week 8	407 ± 8^{a}	370 ± 5^{b}	379 ± 5^{b}	373 ± 6^{b}
Triglycerides (mmol/L)				
Baseline	1.54 ± 0.17^{a}	0.58 ± 0.10^{b}	0.64 ± 0.05^{b}	0.60 ± 0.08^{b}
Week 8	1.41 ± 0.10^{a}	0.59 ± 0.06^{b}	0.69 ± 0.09^{b}	0.62 ± 0.07^{b}
Total cholesterol (mmol/L)				
Baseline	2.64 ± 0.09^{a}	1.63 ± 0.05^{b}	1.69 ± 0.05^{b}	1.63 ± 0.03^{b}
Week 8	3.51 ± 0.09^{a}	1.68 ± 0.08^{b}	1.58 ± 0.06^{b}	1.50 ± 0.04^{b}
HDL-cholesterol (mmol/L)				
Baseline	2.07 ± 0.08^{a}	1.34 ± 0.03^{b}	1.33 ± 0.04^{b}	1.31 ± 0.03^{b}
Week 8	2.57 ± 0.06^{a}	1.24 ± 0.06^{b}	1.40 ± 0.24^{b}	1.15 ± 0.04^{b}
LDL-cholesterol (mmol/L)				
Baseline	0.40 ±0.03^{a}	0.35 ± 0.03^{ab}	0.30 ± 0.02^{b}	0.30 ± 0.03^{b}
Week 8	0.77 ± 0.05^{a}	0.40 ± 0.04^{b}	0.32 ± 0.04^{b}	0.32 ± 0.01^{b}

Data are expressed as mean ± SEM (n=8-10/group). For means within the same row, different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* HDL, high-density lipoprotein; LDL, low-density lipoprotein.


A
WKY
SHR-CTRL
SHR-NB
SHR-BB
Baseline
Week 8
Fat mass (g)
Lean mass (g)
40
30
20
240
245
250
255
260
#
*

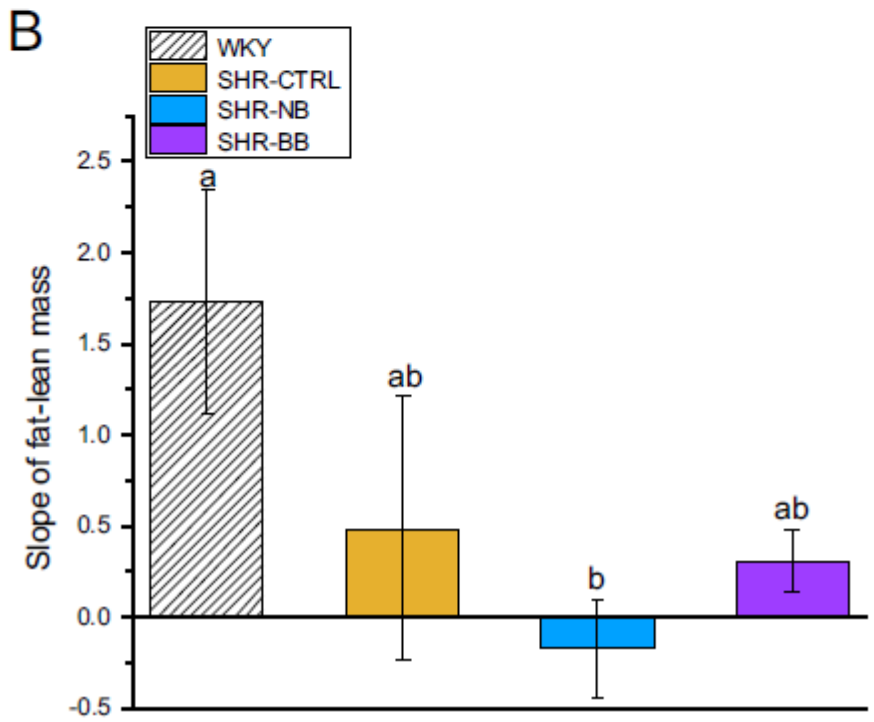

B
WKY
SHR-CTRL
SHR-NB
SHR-BB
Slope of fat-lean mass
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
a
ab
b
ab

Figure 6.1 Phase 1: Whole body fat mass and lean mass.

(A) Whole body fat-lean mass relationship at baseline compared to week 8; **(B)** Slope of the whole body fat-lean mass relationship. Measurements of fat mass and lean mass were obtained *in vivo* using an EchoMRI whole body quantitative magnetic resonance instrument and plotted with fat mass on the Y axis and lean mass on the X axis with closed symbols for baseline and open symbols for week 8. In Panel A, arrows between time points indicate direction of change from baseline to week 8. Data are expressed as mean ± SEM (n=9-10/group); SEM bars for lean mass are too small to be visible in the figure. *****, fat mass significantly different (p<0.05) compared to WKY at baseline; **#**, fat mass significantly different (p<0.05) compared to WKY at week 8. In Panel B, columns with different letters are significantly different (p<0.05). *Abbreviations:* BB, black bean diet; CTRL, bean-free control diet; NB, navy bean diet; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Blood Pressure and Arterial Stiffness

At baseline, week 4 and week 8, all SHR had higher SBP, DBP, and MAP compared to WKY rats (**Table 6.3**). There were no changes over time among the WKY and SHR groups. There were no diet-related differences in BP among the SHR groups. There were no differences for heart rate and heart rate variability among groups. PWV measurements (peak flow velocity, minimum flow velocity, mean flow velocity, pulsatility index, and resistivity index) did not differ between any group, including SHR *vs* WKY, at week 8.

Table 6.3 Phase 1: Haemodynamic properties and arterial stiffness.

	WKY	SHR		
	Control	Control	Navy bean	Black bean
Haemodynamics				
SBP (mmHg)[1]				
Baseline	153 ± 7[b]	197 ± 6[a]	181 ± 8[a]	187 ± 4[a]
Week 4	153 ± 4[b]	188 ± 5[a]	194 ± 7[a]	194 ± 5[a]
Week 8	142 ± 6[b]	184 ± 5[a]	199 ± 6[a]	199 ± 6[a]
DBP (mmHg)[1]				
Baseline	99 ± 4[b]	149 ± 7[a]	134 ± 8[a]	140 ± 5[a]
Week 4	103 ± 5[b]	140 ± 4[a]	145 ± 7[a]	142 ± 7[a]
Week 8	94 ± 4[b]	138 ± 4[a]	146 ± 9[a]	144 ± 7[a]
MAP (mmHg)[1]				
Baseline	116 ± 5[b]	165 ± 6[a]	149 ± 8[a]	155 ± 5[a]
Week 4	119 ± 4[b]	156 ± 4[a]	161 ± 7[a]	159 ± 6[a]
Week 8	110 ± 5[b]	153 ± 4[a]	163 ± 8[a]	162 ± 6[a]
HR (bpm)[2]				
Baseline	397 ± 7	436 ± 20	435 ± 20	375 ± 7
Week 8	387 ± 14	442 ± 22	423 ± 22	423 ± 16
HRV (bpm)[2]				
Baseline	153 ± 2	145 ± 5	147 ± 4	162 ± 3
Week 8	158 ± 4	145 ± 6	148 ± 5	147 ± 3
Femoral artery stiffness				
PFV (cm/s)				
Baseline	26.4 ± 1.8	28.3 ± 0.6	27.6 ± 1.1	27.6 ± 0.9
Week 8	24.6 ± 1.3	27.9 ± 1.1	27.3 ± 1.9	27.7 ± 1.4
MinFV (cm/s)				
Baseline	1.60 ± 0.78	1.90 ± 1.10	3.32 ± 0.35	2.98 ± 0.54
Week 8	2.74 ± 0.32	3.25 ± 0.73	2.00 ± 0.76	3.85 ± 0.59
MFV (cm/s)				
Baseline	6.37 ± 0.59[b]	7.44 ± 0.88[ab]	8.55 ± 0.44[a]	8.67 ± 0.48[a]
Week 8	6.85 ± 0.45	8.20 ± 0.79	7.30 ± 0.56	8.69 ± 0.72
Pulsatility index				
Baseline	4.2 ± 0.4	3.7 ± 0.6	2.9 ± 0.1	3.1 ± 0.3
Week 8	2.7 ± 0.4	3.0 ± 0.3	3.3 ± 0.2	3.2 ± 0.3
Resistivity index				
Baseline	0.94 ± 0.03	0.92 ± 0.03	0.88 ± 0.01	0.90 ± 0.02
Week 8	0.92 ± 0.02	0.88 ±0.03	0.92 ± 0.03	0.87 ± 0.02

Measurements obtained from [1] tail and [2] femoral arteries. Data are expressed as mean ± SEM (n=5-10/group). For means within the same row, different letter superscripts represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* DBP, diastolic blood pressure; HR, heart rate; HRV, heart rate variability; MAP, mean arterial pressure; MinFV, minimum flow velocity; MFV, mean flow velocity; PFV, peak flow velocity.

Vascular Remodelling & Geometry

Morphometric analyses of aortic sections revealed no differences among any of the groups with respect to aortic lumen diameter, lumen cross-sectional area (CSA), external diameter, adventitia thickness, or adventitia CSA (**Table 6.4**). The WKY group had a smaller aortic media wall thickness, media CSA, and media:lumen ratio than all the SHR groups. Representative sections of aorta stained for elastin and collagen are shown in **Figure 1a.** There were no differences in total aortic elastin content or elastin content relative to media area between the groups (**Figure 6.2b,c**). SHR-NB had greater aortic collagen content (total and relative to media area) compared to WKY and SHR-CTRL. SHR-NB had greater total aortic collagen compared to SHR-BB, but there was no difference for aortic collagen relative to media area (**Figure 6.2d,e**). SHR-NB also had a greater collagen:elastin ratio compared to WKY, SHR-CTRL, and SHR-BB (**Figure 6.2f**).

Wall thickness and lumen measurements (vascular geometry) were obtained via myography at a constant intraluminal pressure of 45 mmHg in mesenteric resistance arteries perfused with a Ca^{2+}-free KH solution to deactivate myogenic tone (Schmid *et al* 2014). The WKY group had a larger lumen diameter, smaller media thickness and media:lumen ratio, larger external diameter, and similar media CSA compared to SHR (Table 6.4). There were no diet-related differences in vascular geometry measurements of mesenteric arteries among the SHR groups.

Table 6.4 Phase 1: Vascular geometry.

	WKY	SHR		
	Control	Control	Navy bean	Black bean
Aorta				
Lumen diameter (μm)	1680 ± 33	1710 ± 20	1680 ± 37	1670 ± 21
Lumen CSA (μm^2)	1970 ± 90	2040 ± 72	1980 ± 86	1990 ± 54
Media thickness (μm)	63.1 ± 1.9^b	93.4 ± 3.3^a	85.9 ± 5.0^a	84.7 ± 2.1^a
Media CSA (μm^2)	326 ± 12^b	498 ± 24^a	478 ± 27^a	444 ± 19^a
Media:lumen ratio (× 100)	3.76 ± 0.14^b	5.48 ± 0.21^a	5.12 ± 0.32^a	5.08 ± 0.12^a
External diameter (μm)	1810 ± 34	1900 ± 19	1860 ± 38	1840 ± 23
Adventitia thickness (μm)	81.4 ± 9.7	64.8 ± 9.3	62.5 ± 10.9	61.0 ± 4.9
Adventitia CSA (μm^2)	358 ± 20	302 ± 49	337 ± 51	384 ± 28
Mesenteric resistance arteries				
Lumen diameter (μm)	340 ± 8^a	250 ± 7^b	260 ± 13^b	270 ± 11^b
Media thickness (μm)	25.5 ± 1.2^b	34.5 ± 1.4^a	35.5 ± 1.4^a	35.9 ± 1.5^a
Media CSA (μm^2)	29380 ± 1892	30510 ± 2058	33440 ± 2603	35080 ± 2602
Media:lumen ratio	7.54 ± 0.28^b	13.54 ± 0.41^a	13.71 ± 0.63^a	13.26 ± 0.37^a
External diameter (μm)	390 ± 9^a	320 ± 9^b	330 ± 14^b	340 ± 14^b

Data are expressed as mean ± SEM (n=5-10/group). For means within the same row, different letter superscripts represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* CSA, cross-sectional area.

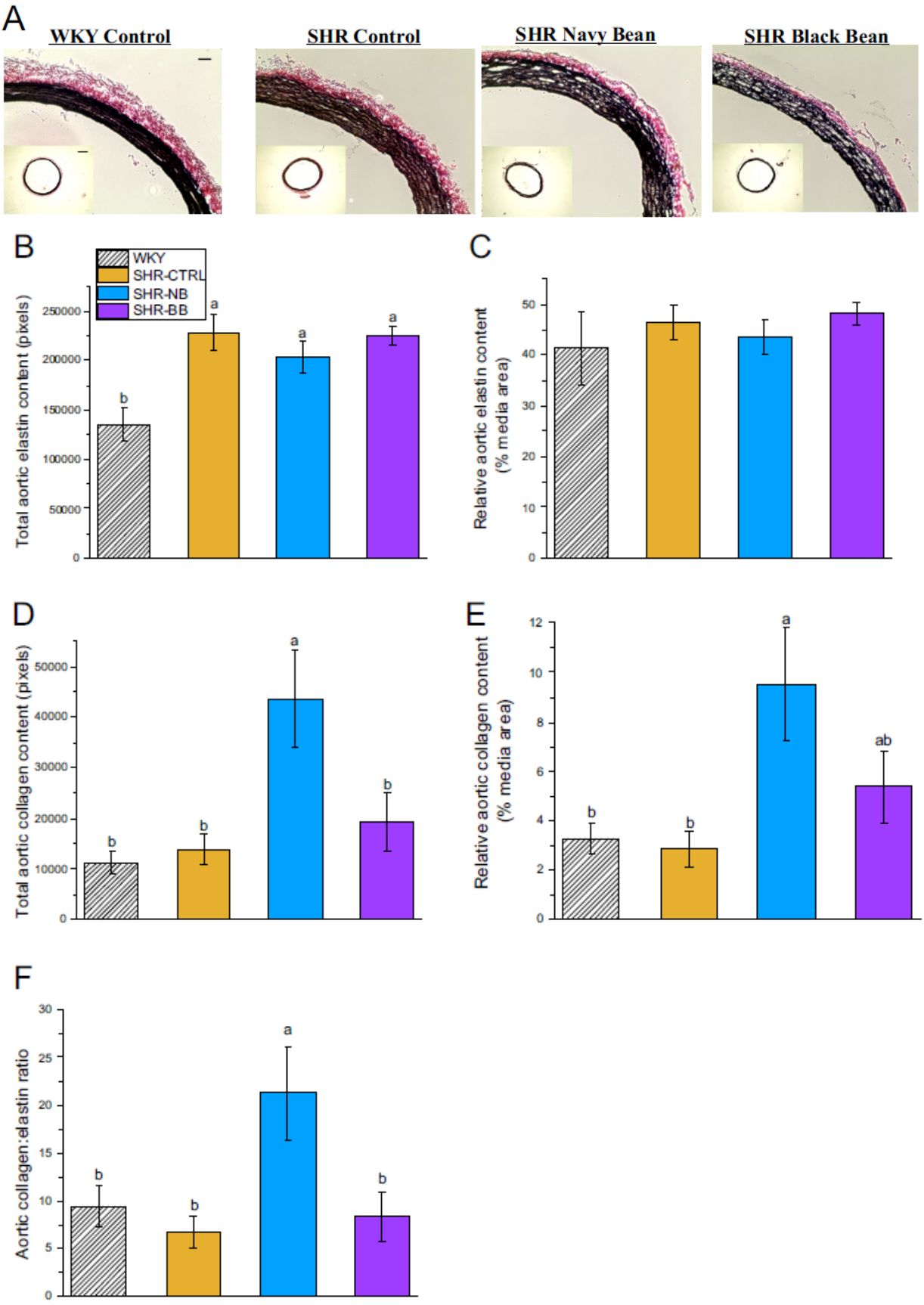

A
WKY Control
SHR Control
SHR Navy Bean
SHR Black Bean
B
WKY
SHR-CTRL
SHR-NB
SHR-BB
Total aortic elastin content (pixels)
0
50000
100000
150000
200000
250000
b
a
a
a
C
Relative aortic elastin content (% media area)
0
10
20
30
40
50
D
Total aortic collagen content (pixels)
0
10000
20000
30000
40000
50000
b
b
a
b
E
Relative aortic collagen content (% media area)
0
2
4
6
8
10
12
b
b
a
ab
F
Aortic collagen:elastin ratio
0
5
10
15
20
25
30
b
b
a
b

Figure 6.2 Phase 1: Large-artery elastin and collagen.

(A) Representative cross-sections of aorta stained for elastin (black) and collagen (red); **(B)** Elastin content in aorta as total area; **(C)** Elastin content in aorta as % media area; **(D)** Collagen content in aorta as total area; **(E)** Collagen content in aorta as % media area; **(F)** Aortic collagen:elastin ratio. Aorta cross-sections were stained with an Elastin Stain Kit (Sigma); elastin and collagen contents were determined using ImageJ to quantify the area of blackness (elastin) and redness (collagen). Data are expressed as mean ± SEM (n=6-9/group). Different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* BB, black bean diet; CTRL, bean-free control diet; NB, navy bean diet; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Vascular Compliance

Vascular compliance, the ability of a vessel to buffer changes in pressure (Intengan *et al* 1999), was measured by plotting the relationship between media stress and media strain (Lee *et al* 2017) for the mesenteric resistance arteries. There was a distinct separation of the stress-strain curves among the four animal groups. SHR-CTRL and SHR-NB show a leftward shift, indicating reduced vascular compliance, while SHR-BB and WKY rats are shifted towards the right, indicating improved vascular compliance (**Figure 6.3a**). The SHR-NB had a greater slope for the stress-strain curve compared to SHR-BB and WKY rats, indicating reduced elasticity (less medial strain) of the arterial wall at a lower intraluminal pressure for SHR-NB compared to that of SHR-BB and WKY rats (**Figure 6.3b**). Furthermore, the slope of the stress-strain curve for the SHR-BB group was not significantly different from the WKY group.

Unlike vascular compliance, incremental elastic modulus depends upon the stiffness of the vessel wall components independent of vessel geometry (Intengan *et al* 1999). When incremental elastic modulus is plotted against media stress, the slope of elastic modulus *vs* stress indicates the stiffness of wall components such as elastin, collagen, and SMC (Lee *et al* 2017). There were no differences in the slope of the elastic modulus *vs* media stress among the four groups (**Figure 6.3c,d**), despite there being differences in arterial compliance.

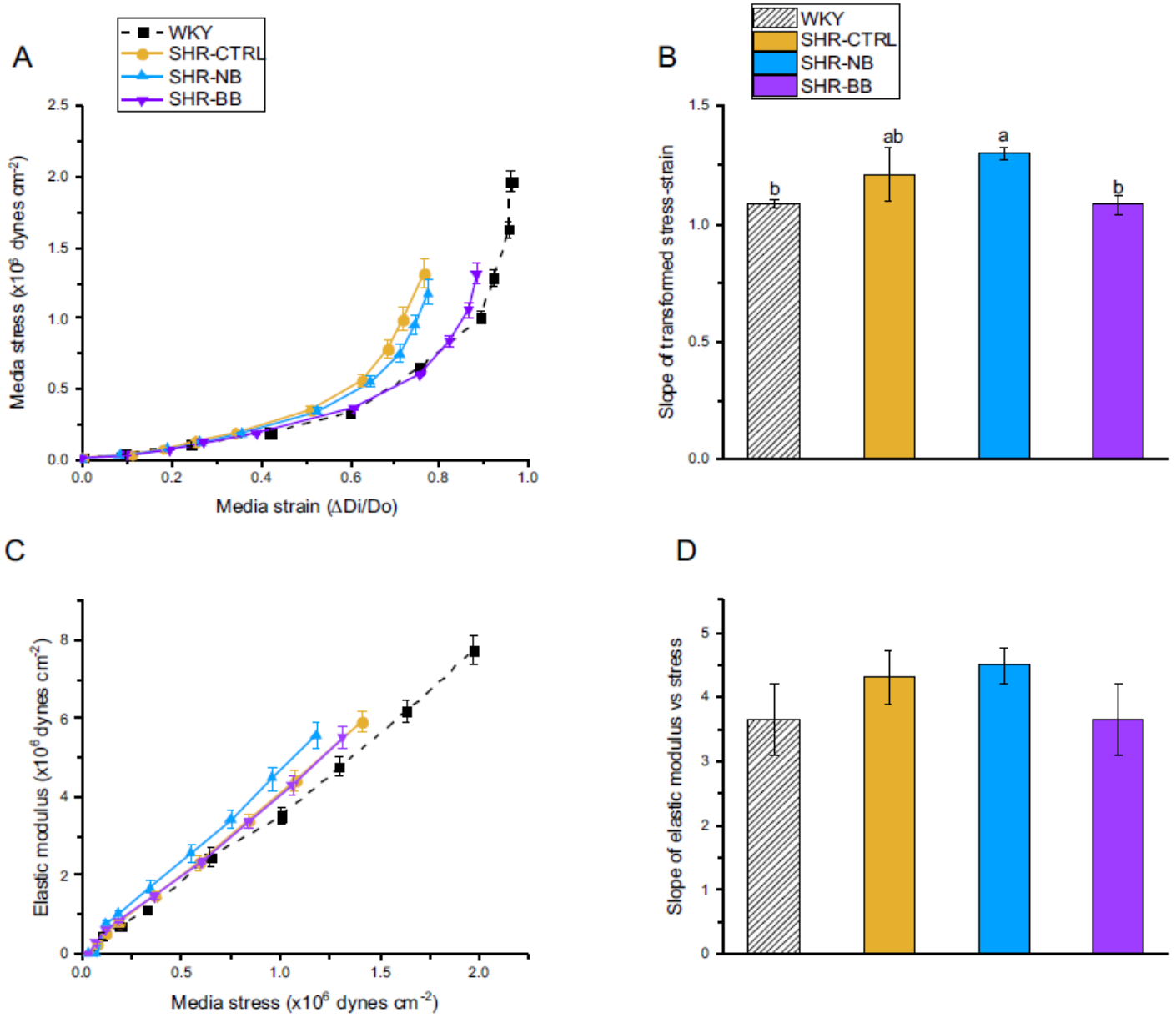

A
WKY
SHR-CTRL
SHR-NB
SHR-BB
Media stress (x10⁶ dynes cm⁻²)
Media strain (ΔDi/Do)
B
WKY
SHR-CTRL
SHR-NB
SHR-BB
Slope of transformed stress-strain
b
ab
a
b
C
Elastic modulus (x10⁶ dynes cm⁻²)
Media stress (x10⁶ dynes cm⁻²)
D
Slope of elastic modulus vs stress

Figure 6.3 Phase 1: Small-artery vascular compliance.

(A) Media stress-strain relationship; **(B)** Slope of the transformed media stress-strain relationship; **(C)** Elastic modulus *vs* media stress; **(D)** Slope of elastic modulus *vs* media stress. Vascular compliance measurements of mesenteric resistance arteries were calculated from data obtained at 3, 10, 20, 30, 40, 60, 80, 100, 120, and 140 mmHg on the pressure myograph. The non-linear stress-strain data were transformed using a power transformation calculation (square-root of media stress) determined by Box-Cox analysis (SAS version 9.2). Data are expressed as mean ± SEM (n=7-10/group). Different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* BB, black bean diet; BW, body weight; CTRL, bean-free control diet; NB, navy bean diet; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

Phase 2: Determining the Retention of Beneficial Effects from Black Beans During a Washout Period

Feed Intake & Body Composition

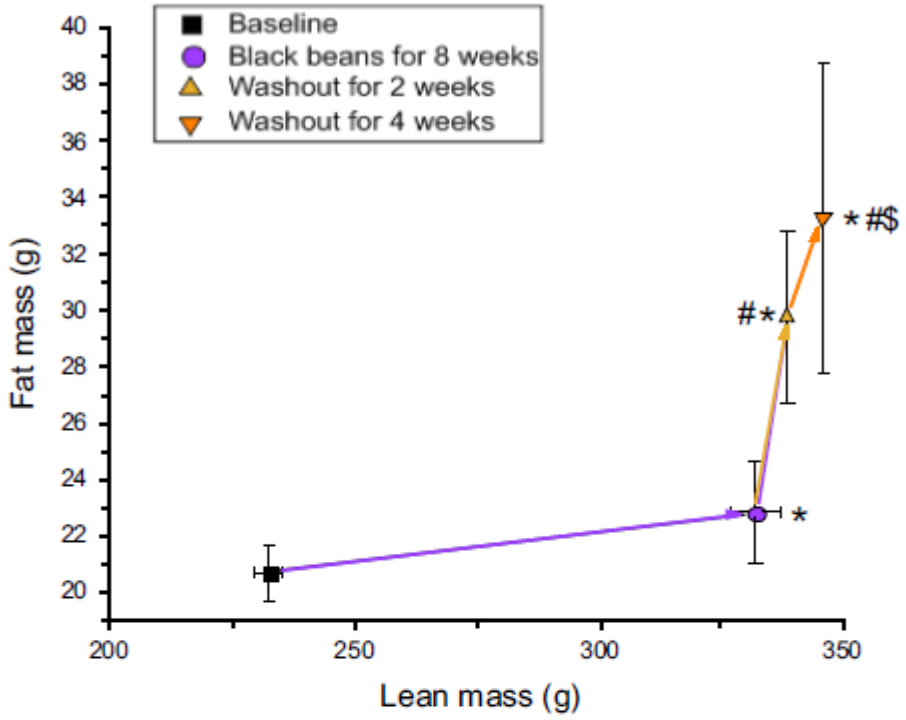
SHR fed the black bean diet had an increased daily feed intake at 8 weeks compared to baseline, but there were no differences in daily feed intake between 8 to 12 weeks when they consumed the bean-free diet (**Table 6.5**). Similar to Phase 1, SHR gained weight during the 8 weeks of black bean feeding. There was a further increase in body weight gain during the 4 weeks when SHR were switched from the black bean diet to the bean-free control diet. Whole body fat mass was plotted relative to whole body lean mass (**Figure 6.4a).** The rightward direction of the linear relationship (positive slope) between fat mass and lean mass indicates an increase in lean mass over time, as was observed between baseline and 8 weeks of bean consumption. The upward direction (steepest slope) indicates an increase in fat mass over time. Whole body fat mass did not change during the 8 weeks of black bean feeding; however, after 2 weeks of a bean-free diet, fat mass was increased compared to baseline. Additionally, after 4 weeks of a bean-free diet, fat mass was increased compared to baseline and 8 weeks of black bean feeding. There was no difference in fat mass between the two washout groups. The slopes of the fat-lean mass relationship were greater for the two washout phases compared to week 8 due to the increase in fat mass, but not lean mass, after bean consumption was discontinued (**Figure 6.4b**).

Table 6.5 Phase 2: Metabolic parameters.

	SHR			
	Baseline	Black beans for 8 weeks	Washout for 2 weeks	Washout for 4 weeks
Feed intake (g/day)	26.8 ± 2.4^{b}	38.7 ± 2.1^{a}	44.0 ± 2.3^{a}	46.0 ± 1.9^{a}
Body weight (g)	301 ± 5^{c}	370 ± 6^{b}	389 ± 6^{ab}	403 ± 8^{a}
Triglycerides (mmol/L)	0.77 ± 0.09^{a}	0.51 ± 0.03^{b}	0.54 ± 0.03^{b}	0.53 ± 0.04^{b}
Total cholesterol (mmol/L)	1.91 ± 0.10^{a}	1.60 ± 0.06^{b}	1.84 ± 0.08^{ab}	1.71 ± 0.08^{ab}
HDL-cholesterol (mmol/L)	1.29 ± 0.09	1.17 ± 0.04	1.36 ± 0.03	1.20 ± 0.05
LDL-cholesterol (mmol/L)	0.20 ± 0.07^{b}	0.37 ± 0.03^{ab}	0.44 ± 0.02^{a}	0.39 ± 0.04^{a}

Data are expressed as mean ± SEM (n=10/group at baseline and 8 weeks; n=5/group for washout for 2 or 4 weeks). For means within the same row, different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* HDL, high-density lipoprotein; LDL, low-density lipoprotein; SHR, spontaneously hypertensive rat.

A


Baseline
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
Fat mass (g)
Lean mass (g)
40
38
36
34
32
30
28
26
24
22
20
200
250
300
350
*#$
#*
*

B

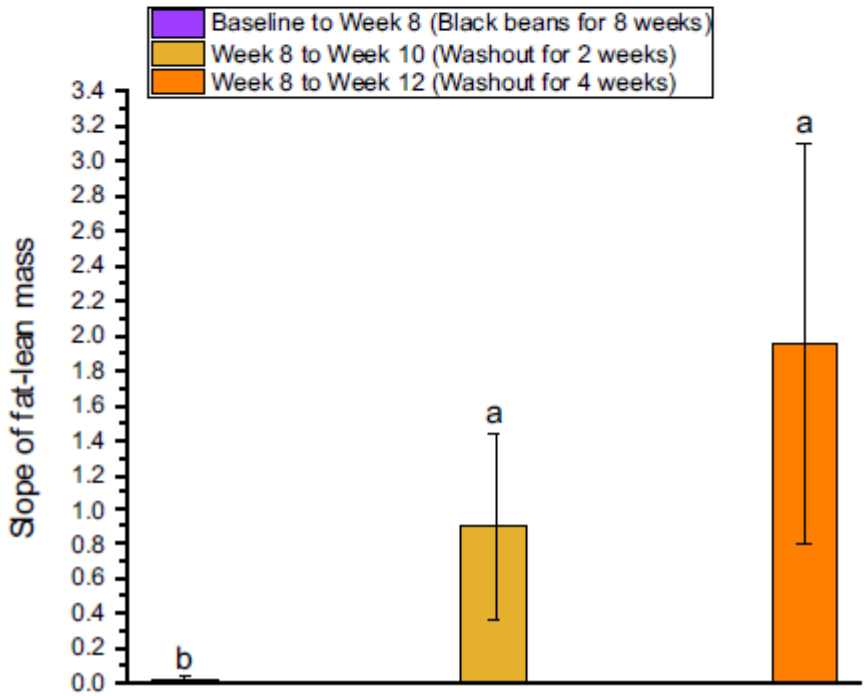

Baseline to Week 8 (Black beans for 8 weeks)
Week 8 to Week 10 (Washout for 2 weeks)
Week 8 to Week 12 (Washout for 4 weeks)
Slope of fat-lean mass
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b

Figure 6.4. Phase 2: Whole body fat mass and lean mass.

(A) Whole body fat-lean mass relationships from baseline to week 8, week 8 to week 10, and week 10 to week 12; **(B)** Slope of whole body fat-lean mass relationship. Measurements were obtained *in vivo* using an EchoMRI whole body quantitative magnetic resonance instrument. In Panel A, arrows between time points indicate direction of change. Data are expressed as mean ± SEM (n=4-9/group); some SEM bars for lean mass are too small to be visible in the figure. *****, lean mass significantly different (p<0.05) from baseline; **#**, fat mass significantly different (p<0.05) from baseline; **$**, fat mass significantly different (p<0.05) from week 8. In Panel B, different letters represent significant differences (p<0.05). *Abbreviations:* BB, black bean diet; BW, body weight; CTRL, bean-free control diet; NB, navy bean diet; SHR, spontaneously hypertensive rat.

Serum Lipid Analysis

Consumption of the black bean diet for 8 weeks decreased serum TG by 34% and TC by 16% compared to baseline (Table 6.5). TG and TC were unchanged during the washout phase. HDL-C was unchanged during this experiment whereas LDL-C increased by 120% during the washout period compared to baseline.

Blood Pressure and Arterial Stiffness

SBP, DBP, and MAP did not change after 8 weeks of black bean feeding, nor did they change during the 4 weeks of washout period (**Table 6.6**). Heart rate was increased compared to baseline with 8 weeks of black bean feeding, and this increase was maintained during the washout periods. In contrast, heart rate variability was reduced with black bean feeding and this reduction was maintained during the washouts, with no differences between the 2 and 4 week washout periods. Measurements of arterial stiffness (PWV), such as minimum and mean flow velocity, were reduced after black bean consumption for 8 weeks and remained reduced during the washout periods. Peak flow velocity decreased over time, but was not different between the black bean phase and washout phase. Pulsatility index increased following black bean consumption, but was decreased during the washout periods, with no difference between the washout time-points. Resistivity index also increased following 8 weeks of black bean consumption, but it did not significantly decline during the washout periods.

Table 6.6 Phase 2: Haemodynamic properties and arterial stiffness.

	SHR			
	Baseline	Black beans for 8 weeks	Washout for 2 weeks	Washout for 4 weeks
Haemodynamics				
DBP (mmHg)[1]	123 ± 4	136 ± 6	141 ± 8	135 ± 12
SBP (mmHg)[1]	165 ± 5	187 ± 6	188 ± 10	187 ± 11
MAP (mmHg)[1]	137 ± 4	153 ± 6	156 ± 9	152 ± 12
HR (bpm)[2]	377 ± 9[b]	466 ± 14[a]	427 ± 14[a]	455 ± 34[a]
HRV (bpm)[2]	161 ± 3[a]	139 ± 3[b]	150 ± 3[b]	143 ± 7[b]
Femoral artery stiffness				
PFV (cm/sec)	31.8 ± 0.6[a]	28.9 ± 1.6[ab]	25.2 ± 0.9[b]	27.3 ± 1.7[b]
MinFV (cm/sec)	3.5 ± 0.4[a]	0.2 ± 0.7[b]	1.6 ± 0.5[b]	1.5 ± 0.7[b]
MFV (cm/sec)	9.7 ± 0.5[a]	5.9 ± 0.5[b]	7.5 ± 0.6[b]	7.4 ± 1.2[b]
Pulsatility index	3.0 ± 0.1[b]	6.0 ± 1.3[a]	3.4 ± 0.3[b]	3.8 ± 0.4[b]
Resistivity index	0.89 ± 0.01[b]	0.99 ± 0.02[a]	0.94 ± 0.02[ab]	0.95 ± 0.02[ab]

Measurements obtained from [1]tail artery, [2]femoral artery. Data are expressed as mean ± SEM (n=10/group at baseline and 8 weeks; n=5/group for washout for 2 or 4 weeks). For means within the same row, different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* DBP, diastolic blood pressure; HR, heart rate; HRV, heart rate variability; MAP, mean arterial pressure; MinFV, minimum flow velocity; MFV, mean flow velocity; PFV, peak flow velocity; SHR, spontaneously hypertensive rats.

Vascular Geometry

Morphometric analyses of aortic sections revealed no differences among SHR fed black beans for 8 weeks and the two washout groups with respect to aortic lumen diameter, lumen CSA, external diameter, adventitia thickness, and adventitia CSA (**Table 6.7**). Media thickness and CSA were increased in the two washout groups compared to the black bean group. Media:lumen ratio was increased after four weeks of washout diet but was not different between 8 weeks of black bean feeding and 2 weeks of washout diet. There were no differences in total aortic elastin content or elastin content relative to media area among SHR fed black beans for 8 weeks and the two washout groups (**Figure 6.5b,c**). Total aortic collagen content, collagen content relative to media area (**Figure 6.5d,e**), and collagen:elastin ratio (**Figure 6.5f**) were higher in the two washout groups compared to the black bean group.

There was a significant effect of the bean washout period on media thickness and media CSA of the mesenteric resistance arteries. Media thickness and CSA began to decrease within two weeks on the bean-free control diet, and after four weeks on the washout diet, these parameters were different from the rats which had consumed black beans for 8 weeks (**Figure 6.6**). Lumen diameter, external diameter, and media:lumen ratio were unchanged.

Table 6.7 Phase 2: Large-artery geometry[1].

	SHR		
	Black beans for 8 weeks	Washout for 2 weeks	Washout for 4 weeks
Lumen diameter (µm)	1670 ± 21	1847 ± 107	1732 ± 31
Lumen CSA (µm^2)	1990 ± 54	2487 ± 286	2252 ± 119
Media thickness (µm)	84.7 ± 2.1^b	108 ± 8^a	122 ± 15^a
Media CSA (µm)	444 ± 19^b	670 ± 84^a	712 ± 90^a
Media:lumen ratio (× 100)	5.08 ± 0.12^b	5.9 ± 0.3^b	7.1 ± 0.9^a
External diameter (µm)	1840 ± 23	2187 ± 137	2104 ± 54
Adventitia thickness (µm)	61.0 ± 4.9	62 ± 10	64 ± 11
Adventitia CSA (µm^2)	384 ± 28	396 ± 74	353 ± 55

[1]Measurements obtained from aorta cross-sections. Data are expressed as mean ± SEM (n=4-7/group). For means within the same row, different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* CSA, cross-sectional area; group at baseline and 8 weeks; n=5/group for washout for 2 or 4 weeks.

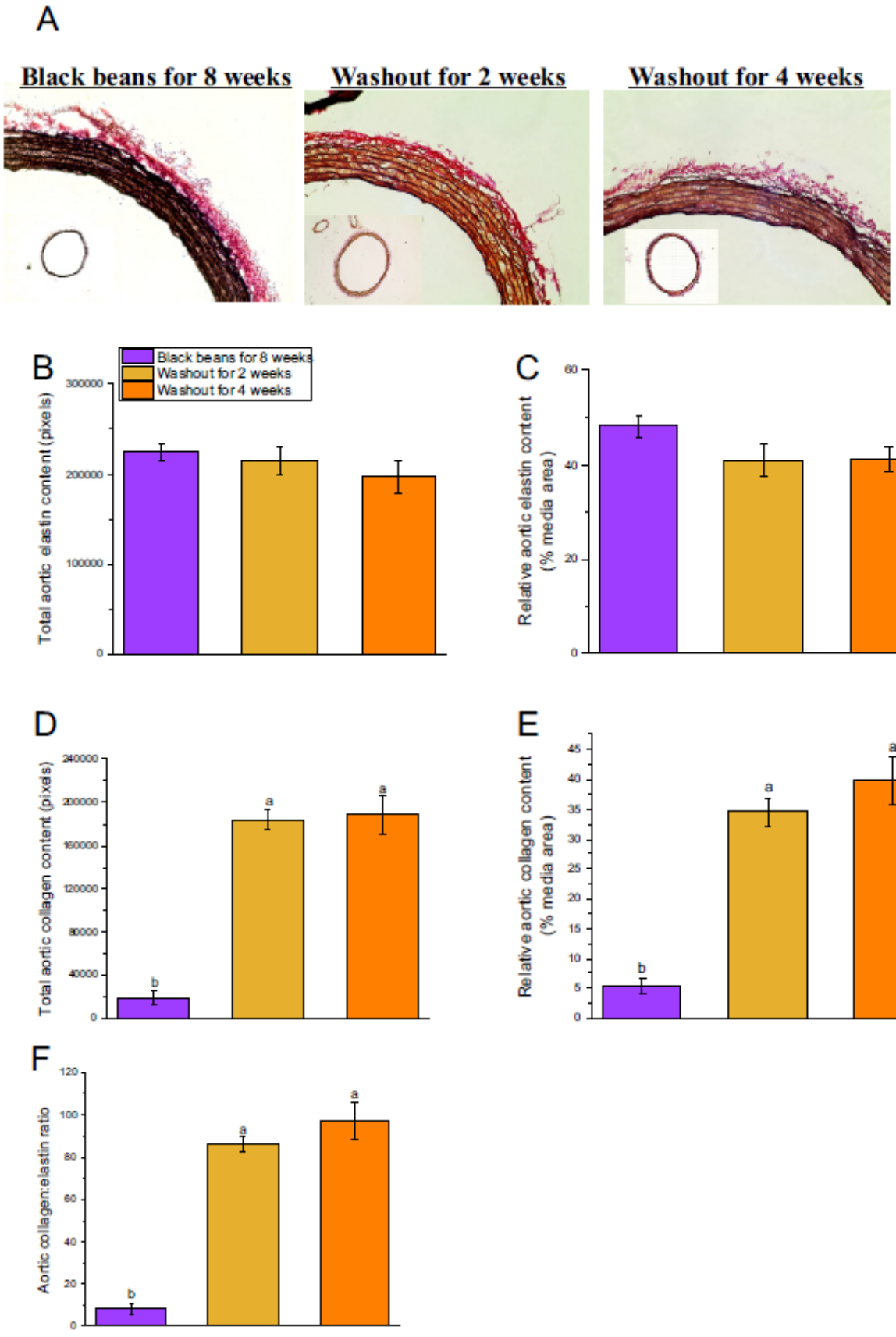

A
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
B
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
Total aortic elastin content (pixels)
300000
200000
100000
0
C
Relative aortic elastin content (% media area)
60
40
20
0
D
Total aortic collagen content (pixels)
240000
200000
160000
120000
80000
40000
0
a
a
b
E
Relative aortic collagen content (% media area)
45
40
35
30
25
20
15
10
5
0
a
a
b
F
Aortic collagen:elastin ratio
120
100
80
60
40
20
0
a
a
b

Figure 6.5 Phase 2: Large-artery elastin and collagen.

(A) Representative cross-sections of aorta stained for elastin (black) and collagen (red); **(B)** Elastin content in aorta as total area; **(C)** Elastin content in aorta as % media area; **(D)** Collagen content in aorta as total area; **(E)** Collagen content in aorta as % media area; **(F)** Aortic collagen:elastin ratio. Aortic cross-sections were stained with an Elastin Stain Kit (Sigma); ImageJ was used to quantify elastin (area of blackness) and collagen (area of redness) content. Data are expressed as mean ± SEM (n=4-7/group). Different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* BB, black bean diet; CTRL, bean-free control diet; NB, navy bean diet; SHR, spontaneously hypertensive rat.

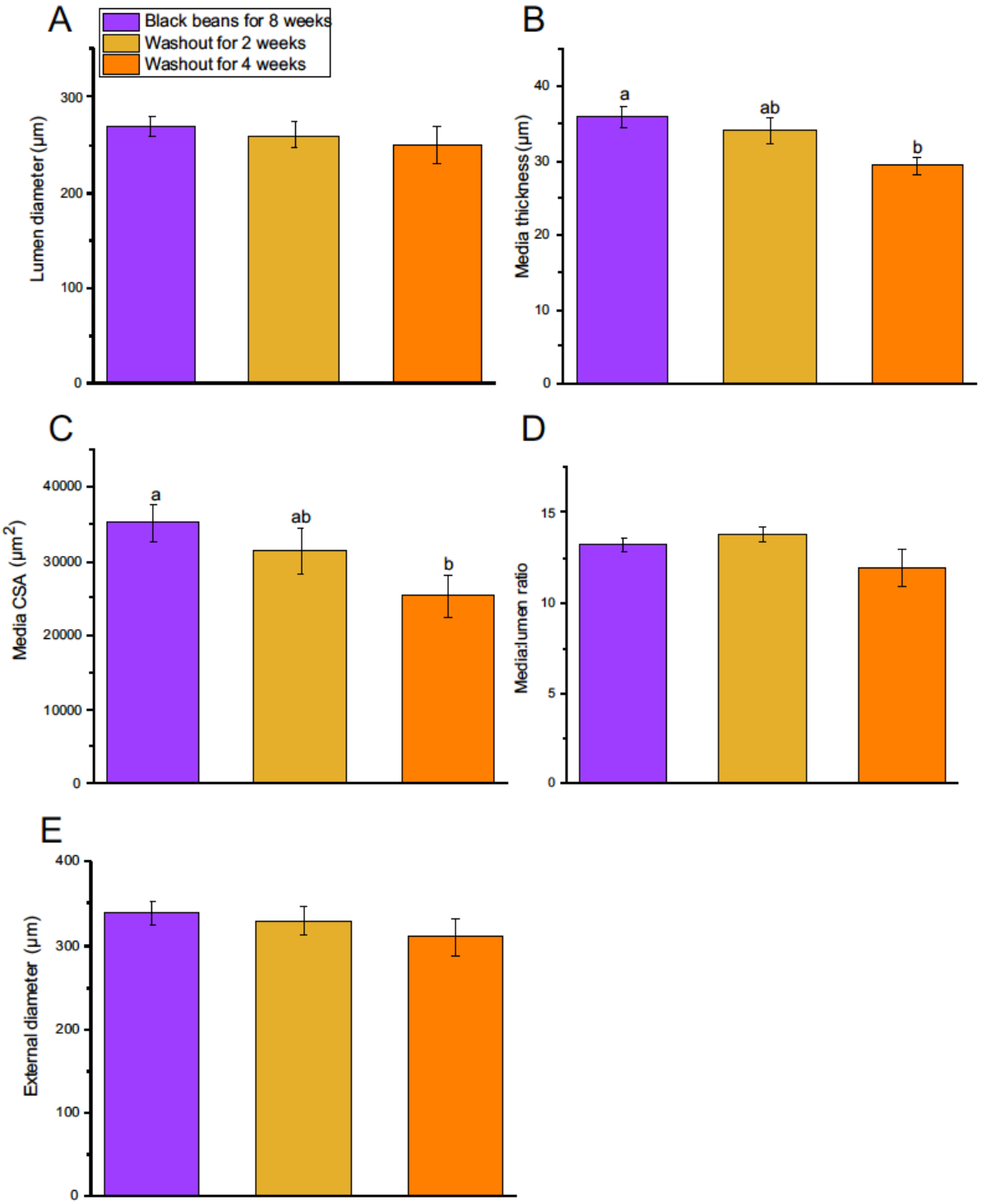

A
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
Lumen diameter (µm)
0
100
200
300
B
Media thickness (µm)
0
10
20
30
40
a
ab
b
C
Media CSA (µm2)
0
10000
20000
30000
40000
a
ab
b
D
Media:lumen ratio
0
5
10
15
E
External diameter (µm)
0
100
200
300
400

Figure 6.6 Phase 2: Small-artery geometry.

(A) Lumen diameter; **(B)** Media thickness; **(C)** Media CSA; **(D)** Media:lumen ratio; **(E)** External diameter. Vascular geometry measurements of mesenteric resistance arteries calculated from data obtained at 45 mmHg on the pressure myograph. Data are expressed as mean ± SEM (n=4-9/group). Different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* CSA, cross-sectional area; SHR, spontaneously hypertensive rat.

Vascular Compliance

The leftward shift of the stress-strain curve in mesenteric resistance arteries of the SHR no longer consuming black beans for 2 or 4 weeks indicates a loss of vascular compliance compared to the SHR after 8 weeks of black bean consumption (**Figure 6.7a**). In concurrence with this observation, the slopes of the stress-strain curve of the SHR that underwent the washout phase were higher than the SHR rats that continuously ate black beans (**Figure 6.7b**).

The switch to a bean-free diet after 8-weeks of continuous black bean feeding increased the slope of the elastic modulus *vs* circumferential stress in SHR (**Figure 6.7c,d**), indicating an increase in wall component stiffness. There were no differences between the two washout periods for either assessment of vascular compliance.

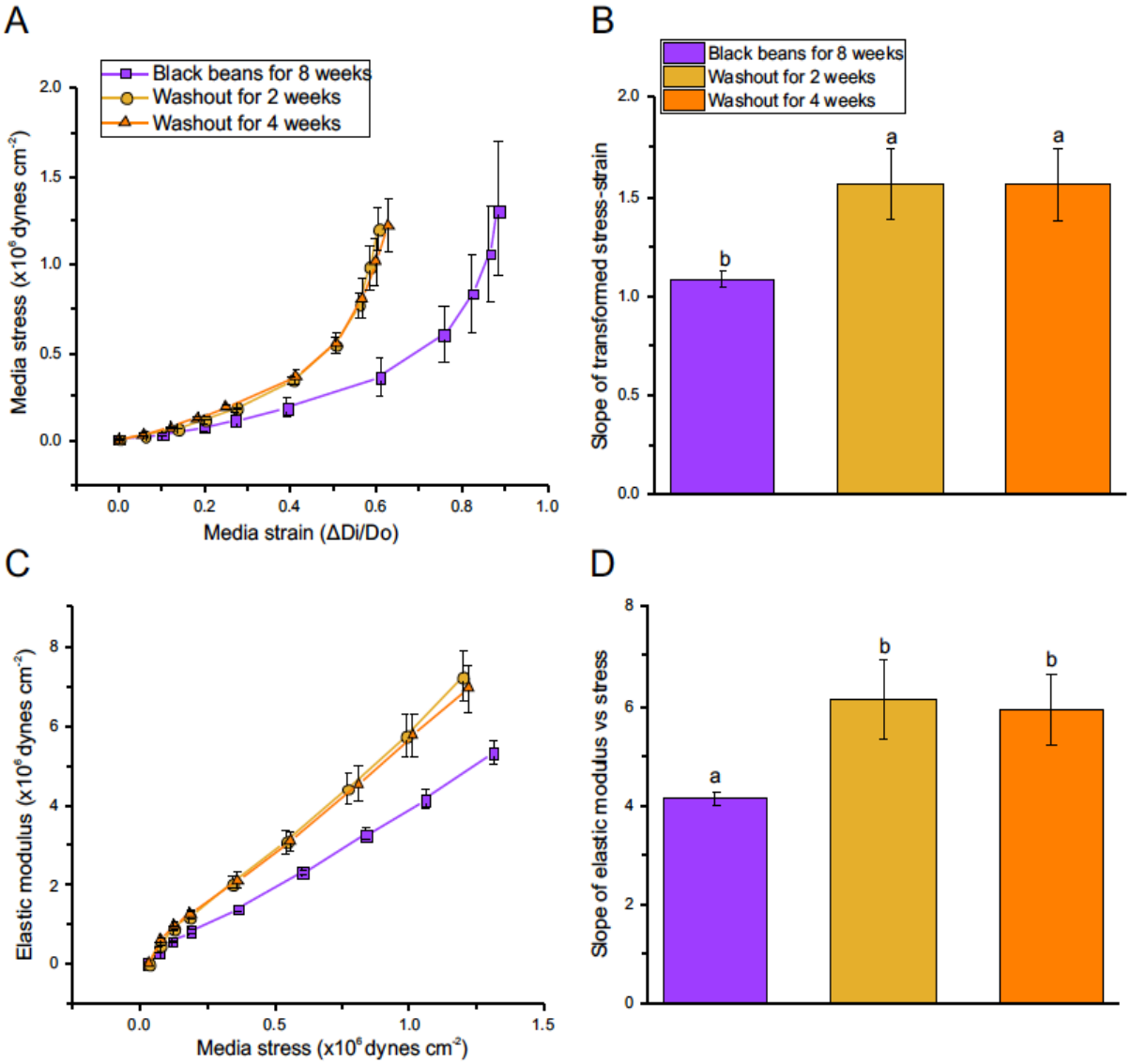

A
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
Media stress (x10⁶ dynes cm⁻²)
Media strain (ΔDi/Do)
B
Black beans for 8 weeks
Washout for 2 weeks
Washout for 4 weeks
Slope of transformed stress-strain
a
a
b
C
Elastic modulus (x10⁶ dynes cm⁻²)
Media stress (x10⁶ dynes cm⁻²)
D
Slope of elastic modulus vs stress
a
b
b

Figure 6.7 Phase 2: Small-artery vascular compliance.

(A) Media stress-strain relationship; **(B)** Slope of transformed media stress-strain relationship; **(C)** Elastic modulus *vs* media stress; **(D)** Slope of elastic modulus *vs* media stress. Vascular compliance measurements of mesenteric resistance arteries were calculated from data obtained at 3, 10, 20, 30, 40, 60, 80, 100, 120, and 140 mmHg on the pressure myograph. The non-linear stress-strain data were transformed using a power transformation calculation (square-root of media stress) determined by Box-Cox analysis (SAS version 9.2). Data are expressed as mean ± SEM (n=4-6/group). Different letters represent significant differences (p<0.05). An absence of letters indicates no statistical differences. *Abbreviations:* SHR, spontaneously hypertensive rat.

6.5 Discussion

Our principal finding was that black beans, but not navy beans, improved vascular compliance and partially attenuated vascular remodelling in SHR, as evidenced by a rightward shift of the stress-strain curve. We also investigated whether the vascular effects from black beans are retained once their consumption has stopped, which produced the novel finding that improvements in vascular function, as determined by arterial compliance and stiffness, and reductions in serum LDL-C levels, were not sustained during the post-black bean washout periods of 2 and 4 weeks. The results from this research indicate that regular long-term consumption of black beans is necessary to achieve and maintain the vascular benefits associated with their consumption.

In the present study, SHR fed black beans, but not navy beans, demonstrated improved compliance of small mesenteric resistance arteries, as depicted by the rightward shift of the stress-strain curve and the subsequent smaller slope change of the stress-strain curve (Kamensky *et al* 2012; Khamdaeng *et al* 2012). This shift of the stress-strain curve is most likely due to the retained engagement of elastic fibres in the vessel wall, indicating preserved compliance with increasing pressure (Aziz *et al* 2016). There were no changes in wall component stiffness (elastic modulus *vs* stress) or small-artery geometry in response to black bean feeding, both of which are factors that determine vascular compliance (Thandapilly *et al* 2012). There is no ready explanation as to why vascular compliance was changed, but not its determinable factors. However, within the aorta, eating black beans resulted in less total collagen content and a reduction in collagen:elastin ratio compared to navy beans. Histological analysis of the mesenteric artery sections needs to be performed to assess changes in the small-artery wall constituents (collagen and elastin) and determine their contributions towards vascular

compliance. However, the changes in large-artery collagen content suggest a propensity for black beans to lower the amount of collagen in blood vessels when compared to navy beans.

Navy beans exhibited negative effects on small and large arteries as evidenced by reduced vascular compliance of small resistance arteries and increased aortic collagen content compared to WKY rats and SHR fed black beans. This is the first study to report negative findings in relation to navy bean consumption. Interestingly, Monk *et al* (2017) reported that a diet containing black beans enhanced colonic barrier integrity and function as well as the microbiotic composition in mice compared to those fed navy beans, and they attributed the improvements in gut health to the unique components of black beans, particularly their polyphenols. It is likely that the phytochemical differences between the two bean types are also responsible for their different vascular effects, given that the nutritional composition of black beans and navy beans is similar (**Table S6.1**). It is unknown at this time if navy beans impair or are just ineffective for improving hypertension-induced structural and functional changes. Studies have shown navy beans to be beneficial for other disease states, such colon cancer (Bobe *et al* 2008; Hangen & Bennink 2002) and obesity (Luhovyy *et al* 2015). Therefore, it is possible that the benefits of navy beans are not preferential to the vasculature but to other metabolic tissues, which may be due to target specificity of their polyphenolic compounds. The distinct biological effects of navy beans and black beans during different disease states may support the inclusion of both beans in the diet for optimal health effects. However, it is important to note that the current study in Chapter 6 did not investigate a mixed diet of black beans and navy beans; therefore, it is unknown if the vascular benefits of black beans would be blunted by navy beans or not. Overall, our results indicate that black beans are the optimal bean choice for improving

vascular function, but further investigation is required to determine if navy beans will have a negative effect on the vasculature.

Black beans had no effect on large-artery geometry compared to navy beans or the bean-free control diet in SHR, but effectively improved small-artery vascular compliance. A similar finding occurred in SHR fed green lentils for 8 weeks (Hanson *et al* 2016). One explanation for the differences in responses between arteries may be due to heterogeneity within the arterial tree. As discussed in Chapter 2, there are established differences between the structural composition and function of large arteries (such as the aorta) and smaller resistance arteries (Marieb & Hoehn 2007). Smaller resistance arteries are composed of greater amounts of smooth muscle (relative to vessel size) compared to large elastic arteries like the aorta (Table 2.1). VSMC are dynamic and can adapt readily in response to local stimuli (Alvim *et al* 2017; van den Akker *et al* 2010). Thus, the changes induced by black beans in smaller arteries but not larger arteries (aorta) may be due to a greater change in VSMC activity within the mesenteric arterial bed. This brings forth the following question: do the bioactive compounds from black beans have selective actions within the vasculature? The answer to this question is unknown at this time; however, elucidating the mechanistic contribution of black beans, and thus, their bioactive compounds, may provide further insight regarding their potentially preferential actions within the small arteries during hypertension. Further exploration could involve investigating cultured SMCs to screen for bioactive compounds that promote the contractile phenotype or alternatively, compounds that prevent the switch from the contractile to the synthetic state.

Interestingly, Phase 2 demonstrated changes in both small- and large-artery geometry with the cessation of black bean intake. However, the small mesenteric arteries showed decreased media thickness and CSA, without changes in lumen diameter, whereas aortae showed

increases in media thickness, CSA, and media:lumen ratio, without changes in lumen diameter, after 4 weeks of bean-free diet compared to 8 weeks of black bean feeding. The differences in media geometry between the two artery types suggest local differences in VMSC activity, phenotype modulation, and/or remodelling proteins (e.g., elastin, collagen). The increase in aortic media thickness and area in SHR after the washout phases are likely due to the higher amounts of collagen present in the aorta. There is no ready explanation for the loss of media thickness or area in the small arteries; however, performing histological analysis of artery sections as well as immunoblotting of arterial tissue may elucidate the changes specifically occurring within the artery walls, such as fewer VSMCs, modulation of VSMC phenotype (i.e. markers for specific phenotypes such as smooth muscle α-actin (contractile) and vimentin (synthetic) (Beamish *et al* 2010)), changes in VSMC size, or alterations in ECM composition.

Phase 2 of our study has provided novel information regarding the retention of biological effects induced by black beans. Improvements to vascular compliance of mesenteric resistance arteries induced by 8 weeks of black bean consumption were lost after halting black bean consumption for 2 weeks; thus, at this point it is speculated that the bioactive compounds from long-term consumption of beans are retained within tissues and exert their effects for less than 2 weeks. To this author's knowledge, retention of bioactive compounds, such as polyphenols, has not been measured in vascular tissues. This is likely because the kinetics of phytochemical penetration and elimination at the tissue and cellular level is unknown (Manach *et al* 2004). Alternatively, the changes in endogenous metabolites that promote better vascular functionality upon the consumption of black beans (as previously proposed in Chapter 5) may be swiftly lost once black beans are no longer consumed. It was novel, yet unsurprising, that that the positive effects on small artery function were reversed following the cessation of black bean

consumption. VSMC are dynamic in nature, allowing for the immediate responses required for regulating BP and blood flow (Alvim *et al* 2017). They are also susceptible to phenotype switching, wherein they can become proliferative and promote vascular dysfunction (Rajendran *et al* 2013; Yamin & Morgan 2012). Without the continual intake of black beans and their bioactive compounds, the endogenous pathways attenuating small-artery stiffness are no longer favourably regulated. Thus, VSMC adapt to the new vascular environment which favours stiffness.

Anthocyanins are the most probable phytochemical compounds responsible for the observed effects in our study simply because they are present in black beans and not in white beans (McClean *et al* 2018; Singh & Basu 2012), as indicated by the contrasting seed coat colours of the beans. Additionally, anthocyanins, as constituents of whole grape powder, were speculated to be responsible for improving arterial compliance in mesenteric resistance arteries of SHR (Thandapilly *et al* 2012). Studies have reported anthocyanin levels in tissues such as the liver, eyes, and brain obtained from animal models (Kalt *et al* 2008; Pojer *et al* 2013). It has not yet been determined whether anthocyanins can enter the vascular wall *in vivo*; however, anthocyanin metabolites can enter human vascular endothelial cells *in vitro* (Pojer *et al* 2013). This evidence, in addition to the ability of anthocyanins to cross endothelial cells of the blood-brain barrier (Kalt *et al* 2008), suggest anthocyanins may be able to enter vascular cells *in vivo* and thereby accumulate in blood vessels. However, it is unknown how long the anthocyanins remain in the tissues. While it was not investigated in our study, we believe it is important in the future to determine the presence and abundance of phytochemical compounds, such as anthocyanins, within target vascular tissues before, during, and after bean consumption to elucidate the absorption, accumulation, and retention of such compounds.

PWV, a marker of arterial stiffness, was measured at the femoral artery, and was reduced after 8 weeks of black bean feeding compared to baseline (in Phase 2), and this reduction was maintained during the four-week washout period. The change in peak flow velocity and mean flow velocity potentially indicate a change in the CSA of the femoral artery; however, the morphology of the femoral artery was not assessed in our study. The reduction in PWV between baseline and 8 weeks in SHR fed black beans in Phase 2 was not observed in Phase 1, even though the black beans, Doppler method, and trained personnel did not differ between the studies. However, it is important to note that in both phases black bean treatment modulated the compliance of small mesenteric resistance arteries. Therefore, it is possible some unknown factor such as the signaling pathway(s) in the femoral arteries was more affected by, or more sensitive to, black beans in Phase 2 than in Phase 1. Studies have shown that rats can be classified as responders and non-responders to stimuli (Fais *et al* 2012; Reid *et al* 2010), although whether this response can be specific to vascular beds is unknown to this author's knowledge. To confirm such a hypothesis for our data, an additional study with a larger sample size (e.g., n=150 (Fais *et al* 2012)) would have to be conducted.

There was a positive effect of navy beans, but not black beans, on body composition in SHR during Phase 1. Navy beans increased lean mass after 8 weeks, which is considered a favourable direction for changes in body composition (Churchward-Venne *et al* 2013). Also in the current study, navy beans incorporated into the diet at 30% w/w were sufficient to prevent changes in fat mass, but did not reduce fat mass. Thompson *et al* (2017) reported that at a higher inclusion rate (60% w/w) of beans in the diet, obesity-resistant and obesity-sensitive rats had reduced visceral adipose mass after 25 days, independent of weight loss and feed intake. Thus, it is possible that given a higher dose of beans in the diet, greater changes in fat mass could have

occurred in our study. It is noteworthy to mention that lean mass was not measured by Thompson *et al* (2017); therefore, it is unknown how a greater intake of beans would affect lean mass. Maintaining and increasing lean mass is important since skeletal muscle and bone loss (both components of lean mass) occur with age and increase the risk for osteoporosis, falls, fractures, physical disabilities, and mortality (Boutari & Mantzoros 2017; Demontiero *et al* 2012). Thus, the ability of beans to prevent lean mass loss over 8 weeks could be beneficial for preventing age-related declines in muscle and bone mass. It is important to note that the current study did not measure bone or muscle mass separate of total lean mass; therefore, it is not certain if the beans act directly on these individual components. Interestingly, black beans did not elicit this effect on body composition in Phase 1, but did in Phase 2. There is no ready explanation for the discrepancy in the effects of black beans on body composition between the two phases. Overall, the ability of light- and dark-coloured beans to positively affect both whole body lean mass and fat mass may add to the evidence supporting dietary pulses as part of a healthy diet for body weight loss and maintenance since decreasing fat and increasing or preserving muscle mass is recommended as part of a healthy weight loss strategy (Josse *et al* 2011). Furthermore, beans may be beneficial for preventing age-related muscle and bone loss; however, further investigation is required to test this hypothesis.

As previously mentioned, favourable metabolic changes occurred in response to black bean feeding in Phase 2 where black beans increased lean mass without changing fat mass after 8 weeks, but cessation of black bean consumption reversed the favorable changes. Indeed, the lack of change in fat mass in Phase 2, and the increased lean mass of SHR with black bean consumption is highly desirable. Interestingly, when the consumption of black beans ceased, the increase in lean mass was halted while both fat mass and LDL-C increased. The loss of these

positive metabolic effects suggests that black beans need to be continually consumed to maintain their beneficial effects on body composition and LDL-C.

It bears mentioning that the design of Phase 2 did not include age-matched control animals to properly discern the effects of the bean washout from that of ageing. However, based on the literature, the vascular changes observed by the 25-29 weeks old SHR in the current study are most likely due to the change in diet, and are not related to age. Chen *et al* (1998) noted that blood pressure and the left ventricle weight of SHRs plateaus between 16 and 40 weeks old. The progression to left ventricular hypertrophy is usually preceded by structural changes to resistance and large arteries (Kahan & Berfeldt 2005); therefore, it would be expected that the plateau in blood pressure and left ventricular weight would also be accompanied by a plateau for other vascular properties, such as compliance and stiffness.

Overall, the unique results of this study suggest that not only could black beans be used as part of a potential therapeutic dietary strategy to combat the remodelling and diminished compliance of blood vessels underlying hypertension, but also that adhering to regular consumption of black beans, without discontinuation, is necessary to maintain their vascular benefits. Therefore, the results of this study suggest regular incorporation of black beans in the diet may be beneficial for improving vascular health and attenuating progressive damage from hypertension. Further investigation is warranted to identify the mechanism(s) behind the observed vascular improvements.

Acknowledgements

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Mehrabadi for aorta sectioning, You Liang for statistical consultation, and the St. Boniface Hospital Foundation for providing infrastructure support.

6.6 Supplementary Material

Table S6.1 Proximate composition of beans[1] and experimental diets.

Proximate analysis (g/100 g DW)	Analytical method	Control diet	Navy bean diet	Black bean diet	Navy bean powder	Black bean powder
Crude protein	AOAC 990.3	16.8	17.8	16.5	24.0	22.8
Crude fibre	AOCS Ba6a-05	1.04	3.51	3.04	5.4	4.9
Non-fibre carbohydrate	n/a	65.7	64.4	64.0	64.8	66.7
Fat	AOCS Am 5-04	6.89	7.04	7.76	2.48	2.18
Ash	AOAC 923.03	2.39	3.19	3.25	2.40	2.45
Energy (kcal/g)	n/a	3.92	3.92	3.92	3.77	3.77

Determinations based on dry weight of material. Proximate analysis was conducted by Central Testing Labs Ltd. (Winnipeg, MB, Canada). Proximate analyses without an analytical method were determined by calculation. [1]Analysis conducted on lyophilized, cooked and milled bean powders as described in the Methods. *Abbreviations:* AOAC, Association of Official Analytical Chemists; AOCS, American Oil Chemist Society; DW, dry weight.

6.7 Literature Cited

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Chapter 7: Conclusions

7.1 General Discussion

This dissertation used pre-clinical and clinical research approaches to determine the efficacy of dry beans for improving vascular function within acute and longer timeframes, in healthy humans and hypertensive rats, respectively. There were four overall objectives: (1) to determine the ability of different bean varieties to modify postprandial vascular and metabolic responses in healthy humans; (2) to investigate alterations in endogenous metabolites of healthy human adults in response to the consumption of different bean varieties; (3) to determine the long-term effects of black and navy beans on vascular function and structure in an animal model of essential hypertension; and (4) to determine whether eating beans regularly is necessary to maintain the vascular improvements obtained by their long-term consumption. In addition to these objectives, the overarching aim of this dissertation was to determine if the seed coat colour of beans is indicative of their physiological potency *in vivo* (i.e. vascular functionality) for acute and long-term feeding periods.

There were five main findings of this dissertation with respect to the proposed objectives: (1) darker-coloured beans (red kidney beans and black beans) induced positive postprandial vascular responses in healthy adults, as evidenced by relaxation of blood vessels within a few hours of consumption; (2) each variety of bean induced a distinct response in relation to certain vascular and metabolic parameters in healthy adults; (3) darker-coloured beans altered circulating levels of endogenous metabolites at time points associated with the postprandial vascular responses; (4) black beans improved vascular compliance and partially attenuated vascular remodelling in SHR; and (5) improvements in vascular compliance and remodelling were not sustained after black bean consumption was discontinued, indicating regular

consumption of black beans is necessary to maintain the vascular benefits associated with their consumption.

In addition to the above findings that are directly related to the main hypotheses and objectives, the following observations are novel and add to the current state of knowledge in the field: (1) in healthy adults, total vascular resistance was lowered within 2 hours of consuming pinto beans, but this effect was not demonstrated by other bean types; (2) BP was lowered within 2 hours of consuming red kidney beans, but not other bean types, in healthy adults; (3) navy beans had no effect on postprandial vascular or metabolic parameters in healthy adults; (4) in healthy adults, black bean intake lowered serum short-chain acylcarnitines (putatively identified), an indicator of improved fatty acid metabolism in skeletal muscle which may be linked to improved blood flow; (5) in healthy adults, black bean intake lowered serum oxoglutaric acid which may result in modulation of short-chain acylcarnitines via regulation of BCAA metabolism; (6) navy beans exhibited a negative effect on vascular compliance and aortic collagen content in SHR; (7) navy beans exhibited a positive effect on body composition (increase in lean mass, but not fat mass) in SHR; and (8) black bean feeding induced favourable changes in certain metabolic parameters (whole body composition, LDL-cholesterol) in SHR which were not sustained following the discontinuation of black bean intake.

The studies in this thesis assessed whether the seed coat colour of beans is indicative of their physiological potency for improving vascular function. It was determined that beans with dark seed coat colours (black and dark red) showed the greatest beneficial effect on vascular function (Chapters 4 and 6). Furthermore, Chapter 4 discerned that the different coloured bean types have distinct biological responses, likely related to the specific pigment compounds present in the seed coat. This notion of plant colour being a marker of physiological functionality is not

new. For example, in the early 1900s, Harry Steenbock, known for his work using UV light irradiation to increase vitamin D content of foods (Mowery *et al* 2004), determined only yellow-coloured vegetables (corn, carrots, sweet potato) eliminated symptoms of vitamin A deficiency in rats (Buttriss *et al* 2017). These yellow colours were later attributed to the pigment compounds (carotenoids) within the vegetables (Khoo *et al* 2011). Lycopene is another phytochemical compound for which its content and bioactivity are positively correlated with the degree of red colour in plants, such as tomatoes (Brandt *et al* 2006). Overall, the colour of fruits and vegetables is known to reflect their varying phytochemical profiles based on the presence of pigmentation compounds (Luo *et al* 2015), and this is also relevant to the common dry bean (Pitura & Arntfield 2019). Studies have reported greater antioxidant activity in darker-coloured beans due to their greater total phenolic content compared to lighter-coloured beans; however, *in vivo* functionality has been rarely examined. The current research (Chapters 4 & 6) is the first to report bean seed coat colour as a visual marker for vascular functionality.

Key studies have shown pulses to be effective in improving vascular function of subjects with established hypertension or arterial disease when consumed over many weeks (Hanson *et al* 2014; Hanson *et al* 2016; Zahradka *et al* 2013). However, prior to this study, their ability to modulate vascular function before arterial disease is established had not been investigated. Thus, the earliest point of intervention to investigate was the postprandial vascular response. In addition, these responses were tested in healthy adults to ascertain the direction (positive or negative) of postprandial modulation by beans, and thus, their contribution to vascular health. Many foods consumed as part of the Western diet pattern are known to induce negative postprandial vascular responses in healthy individuals (Jackson 2000; Jakulj *et al* 2007; Rudolph *et al* 2007; Vogel *et al* 1997), and thus contribute over time to vascular dysfunction and disease

(Oikonomou *et al* 2018). Less research focus has been placed on foods which may have positive effects on the postprandial vascular response. This was the first randomized controlled crossover study to report the novel postprandial vasorelaxant abilities of darker-coloured beans, but not lighter-coloured beans. As discussed in Chapter 4, since the contrasting seed coat colours indicate varying bioactive compound profiles (Pitura & Arntfield 2019), bioactive compounds, such as anthocyanins in the darker-coloured beans, were likely responsible for the observed vasorelaxant effects. Anthocyanins have demonstrated both endothelium-dependent and endothelium-independent mechanisms for improving endothelial function and vasorelaxation *in vitro* (Thilavech *et al* 2017). However, neither the bioactive compounds nor their potential mechanisms of action were investigated in Chapter 4. Since anthocyanins are difficult compounds to detect due to their unstable nature (Manach *et al* 2004), the next study (Chapter 5) explored the effects of darker-coloured beans on circulating endogenous metabolites, representing changes to endogenous pathways, using an untargetted metabolomics approach.

Based on the literature, untargetted metabolomics is the best approach to use when testing an exploratory hypothesis where target compounds are unknown (Scalbert *et al* 2009). The results from Chapter 4 did not confirm which compounds were the effectors for vasorelaxation; thus, we were unable to determine which endogenous pathways would be targetted by these compounds. Utilizing the untargetted approach, 47 circulating endogenous metabolites were changed in response to darker-coloured beans, and black beans potentially decreased levels of short-chain acylcarnitine compounds that may influence blood flow and vasorelaxation (Chapter 5). However, absolute identities were not confirmed and require further exploration. Thus, while mechanism of action was not elucidated, we concluded that darker-coloured beans did elicit changes in endogenous metabolites that occurred concomitantly with vasorelaxation.

In Chapter 4, it is shown that postprandial BP was lower only after the consumption of red kidney beans or pinto beans. Originally, it was suggested that a hypotensive effect might be induced if a longer feeding period was provided for each of the four bean types. This was proven null in Chapter 6, when black beans and navy beans failed to reduce BP in SHR after 8 weeks. Long-term feeding was not conducted with red kidney beans or pinto beans in Chapter 6, therefore, comparisons cannot be made between the bean types for long-term BP-lowering effects. However, the BP results of the postprandial study (Chapter 4), in addition to the lack of long-term effect on BP with other bean types (Chapter 6), suggests that red kidney beans or pinto beans may be the most effective beans for BP-lowering. Further investigation of red kidney beans and pinto beans is necessary to validate this conclusion. The distinct biological response of different varieties within a particular pulse type was demonstrated with lentils by Hanson *et al* (2016). When comparing between red and green lentils, green lentils yielded a greater improvement in arterial compliance than red lentils, and it was hypothesized that bioactive compounds unique to green lentils were responsible (Hanson *et al* 2016). Our findings suggest a similar possibility for beans. Since the bean types in Chapter 4 did not vary much in nutrient content (Table S4.1), it is likely that red kidney beans and pinto beans contain bioactive compounds that are effective for modulating endogenous metabolites or pathways involved in BP regulation that are not present in the other two bean types. However, given the limitations of compound identities in the current metabolomics analysis of Chapter 5, further investigation using the *m/z* ratios and mass spectra information of the compounds, with the addition of authentic reference compounds, is required to accurately determine which compounds and/or pathways are modulated.

The discussions summarized from Chapters 4 and 6 postulate anthocyanins to be the effector compounds responsible for vasorelaxation and improved arterial compliance of small arteries, respectively. However, no mechanism of action has been confirmed for the vascular protective effects of anthocyanins *in vivo* thus far. Thilavech *et al* (2017) reported that the mechanism of action for cyanidin-3-rutinoside, an anthocyanin found in blackberries and raspberries, to induce vasorelaxation of aortic rings was dose-dependent. At a lower dose (1-100 nM) the mechanism was endothelium-dependent, whereas at a higher dose (≥0.3 μM) the mechanism was endothelium-independent and suggestive of VSMC modulation. Furthermore, the primary mechanism for vasorelaxation is proposed to be up-regulation of eNOS (Thilavech *et al* 2017). We are unable to postulate which type of pathway (endothelium-dependent or -independent) is most likely affected by darker-coloured beans in the current studies (Chapters 4 and 6) using the information reported by Thilavech *et al* (2017), as the amount and type of anthocyanins reaching the vascular cells from consumption of the darker-coloured beans is unknown. However, in Chapter 4, postprandial serum NOx levels were unchanged, indicating the bean bioactive compounds may act via mechanisms that do not depend upon NO. A caveat of the serum NOx measurements is that the levels are low and that commercial NOx kits do not discriminate the sources of nitrates and nitrites including those from diet, or artificial formation (e.g. during sample preparation or from laboratory supplies) (Csonka *et al* 2015; Kim-Shapiro & Gladwin 2015). Moreover, Loader (2017) reported total aortic eNOS protein levels unchanged in SHR fed a black bean diet, also suggesting that an alternative mechanism may responsible for vascular improvements with chronic black bean consumption. It is important to note that levels of phosphorylated eNOS were not reported by Loader (2017), which would be a better indicator of eNOS activity. The potential alternative vasorelaxation mechanisms include, but are not

limited to, adrenergic receptors (Fok *et al* 2012; Leblanc & Tabrizchi 2018), changes in specific vasoactive compounds (e.g., endothelin-1, prostaglandin, ACE), modulation of VSMC phenotype (promoting the contractile state or preventing the synthetic state), activation of K^+ channels, and/or inhibition of Ca^{2+} channels (Luna-Vázquez *et al* 2013; Ng *et al* 2013; Schinzari *et al* 2018). However, evidence is limited regarding the modulation of these molecules and their pathways by anthocyanins. Nevertheless, these proposed mechanisms offer areas of exploration for future studies to investigate the vascular mechanisms of darker-coloured beans and their respective phytochemicals. Additionally, cyanidin-3-rutinoside could be used as a reference compound to compare peaks within the metabolomics profile of black beans to determine if this compound is present in black beans. If cyanidin-3-rutoniside is present in black beans, this could help direct the investigation of the vasorelaxant responses detected in the present study.

The common conception surrounding phytochemicals, particularly polyphenols (which include anthocyanins), is that their biological and functional properties are greatly dependent on their bioavailability in the host (Cardona *et al* 2013). However, the bioavailability of polyphenols is considered to be relatively low (D'Archivio *et al* 2010). The results from Chapters 4 and 5 of this dissertation demonstrate that within a few hours of consumption, darker-coloured beans induced vascular relaxation and altered endogenous metabolites, respectively. These actions induced by darker-coloured beans indicate that low bioavailability should not be a ceiling against the functional potential for phytochemical compounds. Bioactive compounds are extensively modified as they are metabolized by the host, generating several metabolites of the parent compound. Consequently, the metabolites that reach tissues and cells are chemically, biologically, and may be functionally different from the parent compound (D'Archivio *et al*

2010). Therefore, the bioavailability of the parent compound does not necessarily dictate the bioavailability of its metabolites, which are likely the effectors at the cellular level.

Chapter 6 addressed an important knowledge gap in the literature: is frequent consumption of beans required to maintain their benefits? We determined that within two weeks of discontinuing the consumption of black beans, the previously observed vascular improvements in hypertensive rats returned to the pre-bean consumption state, thus, indicating that regular consumption of black beans is required to maintain their function in the vasculature. The frequency of consumption of foods containing bioactive compounds for maintenance of physiological functions is likely controlled by at least two factors: (1) the amount of bioactive compounds retained within the body; and (2) the dynamic nature of the vascular wall. Polyphenol concentrations have been detected in various animal tissues, ranging from 30 to 3,000 ng aglycone equivalents/g tissue; however, these concentrations depend on the dose administered, the tissue matrix, and the time of tissue sampling (Manach *et al* 2004). Vascular remodelling is a dynamic process of structural changes in the vessel wall as an adaptive response to long-term changes in haemodynamic conditions, such as those that occur with hypertension (Renna *et al* 2013). Due to this dynamic nature, therapies to attenuate hypertension are recommended to be continued indefinitely unless adverse events arise (van der Wardt *et al* 2017). Overall, evidence from Chapter 6 and that of the literature strongly suggest regular and frequent consumption of black beans is required for maintenance of the biological effects exerted by their bioactive compounds.

7.2 Summary and Implications

In summary, these findings present evidence for including darker-coloured beans regularly in the diet as a dietary strategy to maintain blood vessel health. Darker-coloured beans

proved efficacious for improving vascular function at two points of intervention along the vascular health spectrum: (1) for the postprandial response in healthy individuals before the onset of arterial disease (Chapter 4); and (2) at the hypertensive stage when pathological changes in the vasculature have occurred (Chapter 6).

This research indicates there is strong potential for using darker-coloured beans to protect against the development of arterial disease as evidenced by their ability to modulate the postprandial vascular response in healthy adults. The postprandial vascular response is an important factor for maintaining vascular health. For example, foods that induce poor postprandial vascular responses, such as those of the Western diet (Jackson 2000; Jakulj *et al* 2007; Rudolph *et al* 2007; Vogel *et al* 1997), are often implicated as dietary contributors towards CVD and its risk factors (Oikonomou *et al* 2018). In contrast, food and food products rich in polyphenols are recommended as part of a cardioprotective diet (Habauzit & Morand 2012) and have been shown to induce positive postprandial vascular responses (Alqurashi *et al* 2016; Habauzit & Morand 2012; Rendeiro *et al* 2016). This dissertation has shown darker-coloured beans induce positive postprandial responses. Therefore, in theory, if incorporated regularly into the diet, darker-coloured beans could potentially off-set the effects of foods that induce negative postprandial vascular responses. However, further investigation into this possibility would be required to confirm this hypothesis.

Not only did darker-coloured beans induce acute effects, as seen with improved postprandial vascular responses, they also demonstrated important effects with long-term consumption for ameliorating hypertension-induced functional changes. Presently there is no cure for hypertension or arterial stiffness, and it is projected that 90% of adults will develop hypertension in their lifetime (Whelton *et al* 2017). Additionally, the similar structural changes

occurring in both hypertension and arterial stiffness, albeit independently of each other, suggest a high likelihood that most adults may also develop arterial stiffness. Furthermore, current pharmacological treatments aim to reduce clinical symptoms, and not the underlying structural changes. This research demonstrates black beans to be efficacious in improving vascular compliance of small resistance arteries during hypertension. Further studies are required to determine what structural changes occur within the arteries as a result of bean consumption, and thus, establish a mechanistic pathway by which black beans attenuate hypertension-induced stiffness. However, at present, black beans present a potential therapeutic agent for the vascular dysfunction underlying hypertension.

While the mechanism(s) of action has not been elucidated for the vascular improvements induced by darker-coloured beans, it is postulated based on the research of this dissertation that the bioactive compounds in the darker-coloured beans positively modulate endogenous compounds and pathways related to vascular function. Therefore, this thesis not only describes the potency of darker-coloured beans as a dietary strategy for vascular health, but it also highlights the areas that can further be investigated, as described in Section 7.4, to contribute to the growing knowledge base surrounding functional foods, such as beans, and their vascular benefits.

The novel findings from this research have contributed to the current state of knowledge by demonstrating the following: (1) the potential of seed coat colour of beans to be a visual marker for their function in the vasculature; (2) the ability of darker-coloured beans to induce positive postprandial vascular responses in healthy adults; (3) the ability of black beans to improve vascular compliance during hypertension; and (4) the requirement for regular consumption of black beans to maintain their function in the vasculature. The larger contribution

of this study to the field of nutrition is the discovery that darker-coloured beans, particularly black beans, improve vascular function with and without the onset of arterial disease. Furthermore, this thesis provides the foundation for future clinical studies to explore the potential of darker-coloured beans as a dietary strategy for reducing the risks of developing CVD.

7.3 Strengths and Limitations

Strengths

- Randomized, controlled, crossover design for Chapter 4 – controlled potential confounding factors and reduced inter-individual variability
- Minimum one-week separation between study visits for Chapter 4 – provided an adequate washout period to reduce confounding differences due to overlap of food article consumption
- Realistic sample serving size for Chapter 4 – ¾ cup of beans is the serving size recommended by Eating Well with Canada's Food Guide 2007 for one serving of Meat & Alternatives
- Single-blinded study in Chapter 5 – researcher was blind to the sample identities while performing sample analysis using HPLC-QTOF-MS, data processing, and statistical analysis
- Multiple databases utilized in Chapter 5 – suggested identities of metabolites were compared across multiple databases for the most accurate relative identities
- Combination approaches in Chapter 6 – *in vivo* and *ex vivo* approaches were used to measure arterial stiffness parameters of large and small arteries in SHR

Limitations

- Small sample size for Chapter 4 –a larger sample size would have allowed analysis of the data for sex differences
- Food article consumption for Chapter 4 – beans were consumed in isolation and not as part of meal which would be the typical consumption pattern
- Lack of mixed bean control for Chapter 4 – comparing against a mixed bean control (equal parts of all four bean types) would have helped to determine if the benefits of darker-coloured beans are inhibited by the presence of the other beans in the diet matrix
- Single metabolomics method for Chapter 5 – utilizing multiple analytical methods would enhance the detection of endogenous metabolites and increase the confidence of suggested identities; however, this would be extremely costly
- Missing data points for Chapter 5 – statistical analysis by repeated measures ANOVA requires data for all food articles for all participants at all time points; due to some missing samples (blood collections at certain time points or not enough serum for triplicate samples) this statistical approach was not feasible
- Tail-cuff method for blood pressure measurements of Chapter 6 – this method can produce variable results and is stressful for the animal, possibly blunting diet-related effects. Proper training familiarizes the animals with the procedure; however, it is extremely time consuming and the hyperactivity of SHR limits their compliance to the procedure
- Exclusion of female rats in Chapter 6 – the effects of navy and white beans on hypertension-induced vascular changes in age-matched female SHR were not explored. Therefore, it is unknown if black beans produce the same results for vascular and

metabolic parameters in females, thereby limiting the translatability of the results for both sexes

- Age-matched controls for Phase 2 of Chapter 6 – it would have strengthened the study to include two groups of age-matched SHR for comparison against the two washout groups: (1) bean-free control diet for 10 weeks (equivalent to the 2-week washout group's end-point), (2) black bean diet for 10 weeks; (3) bean-free control diet for 12 weeks (equivalent to the 4-week washout group's end-point); and (4) black bean diet for 12 weeks
- Small sample size for Phase 2 of Chapter 6 – a larger sample size would have strengthened the power of the results

7.4 Future Directions

- Postprandial vascular responses of darker-coloured beans *vs* high-fat meal and investigate the attenuation effects of black beans consumed concurrently with a high-fat meal
- Foods containing darker-coloured beans *vs* navy beans *vs* white rice fed to participants with peripheral artery disease (long-term, crossover study)
- Exploration into non-NO vasorelaxation pathways for mechanism(s) of action by darker-coloured beans
- Optimize untargetted metabolomics method for detecting known (flavonoids, phenolic acids, etc.) and unknown metabolites in beans, and in serum after uptake
- Profile the metabolites within the four bean varieties, measure their uptake in humans, and investigate associations between bean metabolite peaks, endogenous metabolite levels, and postprandial vascular function

- Performing secondary analysis of "*.d" files from metabolomics using different software to compare suggested identities between software programs to refine a prioritized list of compounds of interest for verification against authentic standards
- Morphometry of SHR vessel sections for mesenteric and femoral arteries and staining for collagen and elastin to determine changes in vessel wall structural components (follow-up from Chapter 6)
- Explore black beans *vs* red kidney beans *vs* black beans + red kidney beans *vs* bean-free control diet in SHR for antihypertensive effects of darker-coloured beans
- Determine the minimum retention time of black bean vascular improvements in SHR; if method for detecting known compounds from beans is optimized, these metabolites can be measured from time-course blood samples to determine their clearance rate
- Determine if a dose of black beans that is less than 30% w/w in the diet of SHR can induce the beneficial vascular effects observed in Chapter 6. This research would increase the translatability of preclinical results for clinical outcomes in humans by determining if doses of black beans realistic for human consumption (e.g. ¾ cup daily serving such as in Chapter 4) are effective for improving vascular function in hypertension
- Explore the efficacy of black beans for attenuating hypertension-induced vascular dysfunction in pre- and post-menopausal female SHR compared to age-matched males. This research would increase the translatability of preclinical results for both sexes in the population.
- Measure bean metabolites and/or endogenous metabolite levels in vascular tissues of SHR

7.5 Literature Cited

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