The Reduction of Hypertension through Dietary Flaxseed Intervention and the Identification of Oxylipins as Therapeutic Targets in Cardiovascular Disease

By

Stephanie P.B. Caligiuri

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

Doctorate of Philosophy

Department of Physiology and Pathophysiology

University of Manitoba

Winnipeg, Manitoba, Canada

R3E 0J9

i. Abstract

Dietary flaxseed is a Manitoban crop rich in the n3 fatty acid alpha-linolenic acid, fibre and antioxidant lignans. Addition of flaxseed to the diet decreased brachial blood pressure in patients with hypertension and peripheral artery disease over one year (n=110). With the addition of flaxseed to standard of care, 21% of patients improved from blood pressure above goal (>140/90 mmHg) to blood pressure within goal (<140/90 mmHg). Dietary flaxseed may have induced these anti-hypertensive effects through the reduction of vascular constriction and inflammation. Healthy older adults, who consumed flaxseed every day for 4 weeks, exhibited significantly decreased concentrations of plasma pro-inflammatory oxylipins such as 5-hydroxyeicosatetraenoic acid and trihydroxyoctadecenoic acid. In patients with hypertension and peripheral artery disease, flaxseed consumption for 1 year significantly decreased plasma oxylipins that are responsible for propagating inflammation and vascular constriction. The oxylipins that decreased in the flaxseed group were all produced by the same enzyme, soluble epoxide hydrolase. Alpha-linolenic acid decreased soluble epoxide hydrolase activity in an activity assay. Thus, flaxseed may exert its anti-hypertensive effects through an inhibition of soluble epoxide hydrolase by alpha-linolenic acid. Flaxseed also induced a significant decrease in central aortic blood pressure without cardiac or arterial elasticity involvement as measured by pulse wave analysis. Some of the same oxylipins that decreased with flaxseed consumption were significantly associated with higher central aortic blood pressure and a higher prevalence of cardiovascular and cerebrovascular events in patients with peripheral artery disease. Every 1 nM increase in plasma 16-hydroxyeicosatetraenoic acid increased the odds of a stroke by 55-fold, thus indicating

the potential of particular oxylipins to act as diagnostic markers or therapeutic targets. New research is currently investigating if dietary flaxseed can lower blood pressure and prevent the need for anti-hypertensive medications in those newly diagnosed with hypertension. The implications of this research may change how standard of care is implemented for patients with hypertension and cardiovascular disease. The goal is to offer patients an additional effective strategy beyond anti-hypertensive medications for the management of hypertension in order to reduce the risk of cardiovascular events and to improve patient care and quality of life.

ii. Acknowledgements

Great science can never be accomplished alone, but is achieved as a collaborative team. There are many people I would like to thank. I would like to thank my lab as they have become my family. I thank them for everything they have taught me and their willingness to help. Because of you my PhD has been a wonderful experience that I will never forget.

Secondly, I would like to acknowledge the Canadian Institutes of Health Research and Research Manitoba for believing in me and the potential I have to make an impact on science and medicine. I would like to acknowledge St. Boniface Hospital Albrechtsen Research Centre and how collectively they have helped me gain experiences and grow as a scientist.

Thirdly, I would to thank my committee. Dr. Carla Taylor has been a mentor to me since my undergraduate years. Dr. Taylor has taught me many things, from sharing personal life experiences to nutrition clinical trial design, and some of the most fundamental skills I have today. Dr. Donald Smyth has challenged me, expected only the best from me, and has helped me to grow as an academic. Dr. Amir Ravandi has inspired and taught me to always reach for the moon for if I miss I will land among the stars.

Fourth, I would like to acknowledge my family and friends. I truly appreciate them listening to presentations they do not understand, supporting me and providing advice. Their guidance, patience, and understanding have been essential to my success and I will always be grateful.

Lastly, I would like to thank my mentor and friend, Grant. I am forever grateful for the number of opportunities you have provided me. You have taught me many things, from personal skills to scientific inquiry. You are my greatest cheerleader, my trainer in my boxer's corner, and my biggest advocate. I owe my success to you.

iii. Contribution of Authors

The manuscripts included within this thesis are multi-authored. Therefore, an outline of contribution by manuscript and author are included below in order of appearance in the thesis.

1) Caligiuri SPB. and Pierce GN**.** A Review of the Relative Efficacy of Dietary, Nutritional Supplements, Lifestyle and Drug Therapies in the Management of Hypertension. Critical Reviews in Food Science and Nutrition. In print.

- Caligiuri: idea inception, literature collection/analysis, data analysis, statistics, writing of the manuscript
- Pierce: intellectual input on study design and editing of manuscript

2) Caligiuri SPB., Edel A., Aliani M., Pierce GN. Flaxseed for hypertension: implications for blood pressure regulation. Curr Hypertens Rep. 2014 Dec;16(12):499.

- Caligiuri: literature collection, writing of sections entitled, "Introduction, flax oil and ALA, anti-hypertensive mechanism of flaxseed, implications for hypertension management, and conclusion. Creation of figures.
- Edel: literature collection, writing of sections entitled, "whole flaxseed, flaxseed protein and peptides, flaxseed lignans, and flaxseed fibre."
- Aliani: editing of manuscript
- Pierce: intellectual inception, intellectual input and editing of manuscript

3) Caligiuri SPB., Aukema H., Ravandi A, Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. Experimental Gerontology. 2014 Nov;59:51-7

- Caligiuri: intellectual inception of idea, oxylipin extraction and analysis, statistical analysis, manuscript preparation
- Aukema: establishment of oxylipin methodology, intellectual input, and manuscript editing
- Ravandi: intellectual input on manuscript and editing of manuscript
- Pierce: study design for clinical trial, inception of clinical trial, intellectual input on manuscript and editing of manuscript

4) Caligiuri SPB., Aukema H., Dibrov E., Guzman R., Ravandi A, Pierce GN. Flaxseed consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via an alpha-linolenic acid induced inhibition of soluble epoxide hydrolase. Hypertension. 2014 Jul;64(1):53-9.

- Caligiuri: intellectual inception of idea, oxylipins extraction and analysis, enzyme activity assay, statistical analysis, manuscript preparation
- Aukema: establishment of oxylipin methodology, intellectual input, and manuscript editing
- Dibrov: performing the inflammatory marker assay
- Guzman: initial clinical trial design and qualified investigator for clinical trial
- Ravandi: intellectual input on direction of study and manuscript editing
- Pierce: inception of clinical trial, designed clinical trial, intellectual input on direction of study and manuscript editing

5) Caligiuri SPB, Aukema H, Ravandi A, Pierce GN. Beyond Conventional Risk Factors: Specific Plasma Oxylipins Increase the Odds of Cardiovascular and Cerebrovascular Events in Patients with Peripheral Artery Disease. In preparation.

- Caligiuri: intellectual inception of idea, oxylipins extraction and analysis, statistical analysis, manuscript preparation
- Aukema: establishment of oxylipin methodology and manuscript editing
- Ravandi: intellectual input on clinical parameters, direction of study, and manuscript editing
- Pierce: study design, intellectual input on analyses, manuscript editing

6) Caligiuri SPB, Rodriguez-Leyva D, Aukema H, Ravandi A, Guzman R, Weighell W, Pierce GN. Dietary flaxseed reduces central aortic blood pressure without cardiac involvement but through changes in plasma oxylipins. Hypertension. In print.

- Caligiuri: intellectual input, data/statistical analysis, figure and table creation, manuscript preparation
- Rodriguez-Leyva: intellectual input and manuscript preparation
- Aukema: establishment of oxylipin methodology and manuscript editing
- Ravandi: intellectual input on clinical parameters, direction of study, and manuscript editing
- Guzman: initial clinical trial design and qualified investigator for clinical trial
- Weighell: Data collection of pulse wave analysis

- Pierce: intellectual inception, study design, intellectual input on analyses, manuscript editing

7) Caligiuri SPB., Penner B., Pierce GN. The HYPERFlax Trial: Determining the anti-HYPERtensive effects of dietary Flaxseed in newly diagnosed Stage 1 hypertensive patients using a parallel, phase II, randomized, controlled, double-blinded clinical trial. Trials. 2014;Jun 18;15:232.

- Caligiuri: intellectual input on study design and manuscript preparation
- Penner: intellectual input on study design and manuscript editing
- Pierce: intellectual input on study design and manuscript editing

8) Caligiuri SPB, Penner B, Austria A, Pierce G. High Blood Pressure Prevalence in Manitoba. In preparation.

- Caligiuri: creation and carrying out hypertension awareness campaign. Responsible for data collection, analysis, and writing.
- Brian Penner: intellectual input on recruitment methods, study design, and writing
- Alejandro Austria: intellectual input on public screening clinics, logistics planning, and assistance with carrying out public screening
- Pierce: intellectual input on recruitment methods, study design, and writing

9) Ethics approvals and Clinical Trial Applications

- Caligiuri : wrote all applications

iv. Table of Contents

v. List of Tables

vi. List of Figures

vii. List of Appendices

viii. List of Abbreviations

$6kPGF_{1\alpha}$ - 6-keto prostaglandin $F_{1\alpha}$,

ACS - acute coronary syndrome

ACE – angiotensin converting enzyme

ALA - Alpha-linolenic acid

ARA - arachidonic acid

AI - augmentation index

BMI - Body Mass Index

BP - brachial blood pressure

cBP - Central blood pressure

cDBP - central diastolic blood pressure

cSBP- central systolic blood pressure

cXBP - central mean blood pressure

CVA - cerebrovascular accident

CVD - cardiovascular disease

DASH – dietary approaches to stop hypertension

DBP - brachial diastolic blood pressure

DHA – docosahexanoic acid

DGLA - dihommo gamma linolenic acid

DiHDPA - dihydroxydocosapentanoic acid

DHET - dihydroxyeicosatrienoic acid

DiHOME - dihydroxyoctadecenoic acid

END – enterodiol

ENL – enterolactone

EPA – eicosapentanoic acid

EpOME - epoxyoctadecenoic acid

HDOHE - hydroxydocosahexanoic acid

HEPE - hydroxyeicosapentanoic acid

HETE - hydroxyeicosatetraenoic acid

HETrE - hydroxyeicosatrienoic acid

HHTrE - hydroxyheptadecatrienoic acid

HODE - hydroxyoctadecadienoic acid

HOTrE - hydroxyoctadecatrienoic acid

HYPERFlax - anti-HYPERtensive effects of dietary Flaxseed

LA - linoleic acid

MUFA – monounsaturated fatty acid

nCPAP – nasal continuous positive airway pressure

oxoODE - oxooctadecadienoic acid

OXoOTrE - oxooctadecatrienoic acid

PAD - peripheral arterial disease

PG - prostaglandin

PWA - pulse wave analysis

PWV- pulse wave velocity

MAP - mean arterial pressure

RCT – randomized controlled trial

SBP - systolic blood pressure

SDG - secoisolariciresinol diglucoside

SECO – secoisolariciresinol

SEM - standard error of the mean

SHR - spontaneously hypertensive rats

TIA - transient ischemic attack

TLC – therapeutic lifestyle changes

TriHOME - trihydroxyoctadecenoic acid

Chapter I – Literature Review

i. Introduction

Hypertension was declared a global crisis by the World Health Organization because it is the number one risk factor for mortality worldwide. Hypertension is a global epidemic in which aging, inflammation, oxidative stress and vascular constriction are implicated. Endogenous bioactive lipids called oxylipins are attractive therapeutic targets due to their role in immunity, inflammation, and/or vascular tone regulation. It is unknown if plasma oxylipins influence hypertension, cardiovascular, or cerebrovascular events. Therefore, an opportunity for scientific discovery exists. In addition, a Manitoban grown crop, flaxseed contains anti-inflammatory n3 fatty acids, and antioxidant lignans, and is a rich source of fibre. These compounds may hold potential to reduce hypertension through influencing inflammation, oxidative stress and vascular tone. Thus, the purpose of the thesis is 4-fold: (1) to characterize plasma oxylipin profiles in healthy normal adults and patients with peripheral artery disease and hypertension, (2) to observe any changes in plasma oxylipins due to dietary flaxseed, (3) to assess the relationship of plasma oxylipins to brachial blood pressure, central aortic blood pressure, and cardiovascular events, (4) to determine if dietary flaxseed can lower blood pressure and prevent the need for anti-hypertensive medications in patients newly diagnosed with hypertension.

ii. Hypertension

Physiology of Blood Pressure

Blood pressure is the force applied to the vessel wall by circulating blood. Blood pressure often refers to arterial pressure which is calculated as cardiac output x peripheral

vascular resistance. Therefore, blood pressure can be affected by heart rate, stroke volume and vascular resistance (1). Blood pressure provides insight into the amount of force the heart is producing in order to circulate blood throughout the body. Systolic blood pressure refers to the force generated by the heart during systole. The force generated during systole is created in order to open the pulmonic and aortic valves to eject blood through the pulmonary artery and aorta, respectively. This force against which the heart must pump is called the afterload. High systolic blood pressure may, therefore, be elevated in any condition in which the valve opening is restricted or the aortic vessel itself is constricted. This can occur, for example, in the presence of aortic coarctation. Systole consists of one-third of the cardiac cycle (1).

Diastolic blood pressure is the force generated during diastole. Diastole consists of two-thirds of the cardiac cycle (1). In diastole of the heart, blood flows from the atria to the ventricles and the myocardium is perfused with blood via the coronary arteries. The force generated during diastole is referred to as the preload. Elevated diastolic blood pressure may result from, for example, elevated blood volume from excess sodium ingestion or renal insufficiency. Excess blood volume increases the preload volume, therefore, causing an increase in stroke volume, cardiac output, and blood pressure (1).

Diagnosis of Hypertension

Chronic high blood pressure, also known as hypertension, is an important condition to recognize as it indicates a physiological imbalance (anatomically or biochemically) that can result in organ damage when left untreated. In Canada, 17.7% of the population aged 12 years and older reported being diagnosed with high blood pressure (2). In Manitoba, 21% of adults aged 20 years and older reported being

diagnosed with hypertension, with 2.5% of adults being newly diagnosed every year (3). The World Health Organization reported the prevalence of those with uncontrolled hypertension increased from 600 million to 1 billion from 1980 to 2008 (4). Whether this is due to a true increase in prevalence and or an improvement in diagnostic procedures was unclear.

The stages for diagnosis of blood pressure are defined in Table 1. According to the Canadian Hypertension Education Program, high blood pressure is deemed as an office ausculatory measurement of ≥140 mmHg and/or ≥90 mmHg, for systolic and diastolic blood pressure, respectively. Using an oscillimetric automated device, high blood pressure is defined as ≥135 mmHg and/or ≥85 mmHg for systolic and diastolic blood pressure, respectively (5). High-normal blood pressure is defined as a systolic blood pressure of 130-139 mmHg or a diastolic blood pressure of 85-89 mmHg using office ausculatory blood pressure measurement only (6). Hypertension can also be divided into Stage 1, Stage 2, and Emergency. Stage 1 is defined as 140/90-159/99 mmHg using an auscultatory method. Stage 2 hypertension is 160/100 – 179/109 mmHg. A hypertensive urgency is defined as a blood pressure ≥180/110 mmHg. A hypertensive emergency is present when blood pressure is ≥180/110 mmHg and symptoms of target organ damage are present, ie: chest or back pain, numbness, vision disturbances, speaking difficulties, and/or shortness of breath (5).

Table 1: Stages of Blood Pressure

Stage	Blood Pressure (mmHg)
Normal	90/60 – 120/80
High-Normal	130/85 – 139/89
Stage 1	140/90 – 159/99
Stage 2	160/100 – 179/109
Urgency or Emergency	≥180/110

Hypertension can be diagnosed several ways (5):

1) Blood pressure is ≥180/110 mmHg at one visit

2) Blood pressure is ≥140/90 mmHg at 4 consecutive visits

3) Blood pressure is ≥135/85 mmHg using ambulatory or home blood pressure monitoring.

Elevated blood pressure readings as detected by any one of the above criteria are enough to constitute a diagnosis of hypertension. Diagnosis of hypertension is preferred using an automated oscillimetric device versus a manual ausculatory method (6). Blood pressure should be measured in both arms as a difference greater than 10 mmHg between arms holds a high possibility for subclavian stenosis. In persons with atherosclerosis, blood pressure may be elevated in one arm and not the other. However, having elevated blood pressure in one arm is adequate for a diagnosis of hypertension (7)

The current standard of care for the measurement of blood pressure is to measure the pressure of the brachial artery. However, technology now exists to measure central aortic blood pressure either directly through a cannulated probe or indirectly using radial pulse wave analysis (8). Central blood pressure refers to blood pressure in the aorta.

Researchers are supporting the use of central blood pressure versus brachial for many reasons:

1) Central blood pressure more strongly predicts cardiovascular events versus brachial blood pressure (9).

2) Aortic blood pressure more closely approximates pressures that organs such as the kidneys and brain receive versus brachial (9).

3) Anti-hypertensive medications can differentially affect brachial and central blood pressure, thus differentially affecting the safety and efficacy of anti-hypertensive drugs (9, 10).

Further research is needed in order to determine standards for the methodology and diagnosis of central aortic hypertension.

Regardless of the method for the diagnosis of hypertension, the cause of the patient's hypertension is the most important aspect of diagnosis because causation determines treatment. It is important to determine if hypertension is primary (essential) or secondary (the result of another factor or disease).

Primary and Secondary Hypertension

Primary hypertension is the most common form of hypertension at approximately 80% of diagnosed cases (6). However, the cause of primary hypertension is less clear than secondary. Some causative mechanisms have been hypothesized. The most common cause of primary hypertension is thought to be endothelial dysfunction and inflammation. The endothelial lining of the arteries serves the intrinsic function of producing factors responsible for regulating vascular tone and inflammation. In endothelial dysfunction, an imbalance exists between the endothelial derived vasoconstriction and vasodilatation

factors, thus resulting in vasoconstriction. Endothelial derived vasodilating factors include hydrogen sulfide (11), nitric oxide (12), prostaglandin $I_{2,}$ and epoxyeicosatrienoic acids (13, 14). Endothelial derived vasoconstricting factors include thromboxane A_2 and endothelin-1. This imbalance may be more common in individuals with higher oxidative stress, ie: those eating a diet low in fruits and vegetables, individuals with obesity, those who smoke cigarettes, those with uncontrolled diabetes mellitus, and older individuals. This is likely because oxidative stress will render vasodilators such as nitric oxide inactive, thus resulting in the imbalance (14).

Risk Factors for Hypertension

Older age is one of the most important risk factors for hypertension. The lifetime risk of developing hypertension is 90% (15). The proposed hypothesis behind the relationship of age to hypertension is the concept of inflammaging whereby older individuals may be more likely to have multiple inflammatory related conditions versus younger individuals (16). Inflammaging is thought to also explain the increased prevalence of inflammatory related conditions such as arthritis, pain, atherosclerosis and diabetes with increased age (17). Inflammaging is thought to arise because older individuals may exhibit a decrease in anti-inflammatory protective mediators over time. This is combined with an increase in pro-inflammatory mediators. This influx of pro-inflammatory mediators is potentially due to older adults being exposed to many pathogens over their lifetime and as a result, the immune system develops low grade chronic inflammation or immune hyper-responsiveness as a response mechanism (16). Thus inflammaging may induce endothelial dysfunction and vasoconstriction (14).

A similar cause of primary hypertension has been proposed as the stimulation of the sympathetic nervous system and the infiltration of the vasculature and kidneys with T-cells. In particular, T-17 helper cells that produce interleukin-17 have been implicated in the etiology of essential hypertension (18). Risk factors for developing primary hypertension include being overweight/obese, alcoholism, high salt diet, sedentary lifestyle, older age, African ethnicity, diabetes mellitus and hyperlipidemia (19-23).

In contrast, risk factors for secondary hypertension are less clear. Secondary hypertension is less common than primary hypertension. However, the etiology can often be determined. Secondary hypertension is speculated if patients present with abnormalities such as

1) acute onset or elevation in hypertension, particularly in those < 30 years of age;

2) abdominal bruit;

3) resistance to anti-hypertensive drugs;

4) elevated serum creatinine with angiotensin converting enzyme inhibitors or angiotensin receptor blockers;

5) high risk of atherosclerosis;

6) pulmonary edema;

7) acute hypokalemia;

8) diuretic induced hypokalemia, or

9) adrenal adenomas, pheochromocytoma

Exogenous causes of secondary hypertension are common. For example, chronic use of non-steroidal anti-inflammatory drugs, corticosteroids, oral contraceptives, anti-depressants, cocaine, and decongestants, can all elevate blood pressure and are the most

common causes of secondary hypertension. Other rare causes include Cushing's syndrome, obstructive sleep apnea, pheochromocytoma, aortic coarctation, hyperaldosteronism, and renal artery stenosis (6).

There are several signs through which a differentiation between primary and secondary hypertension can be drawn. For example, primary hypertension is most commonly Stage 1 hypertension, whereas secondary hypertension often causes hypertension in the Stage 2 or hypertensive emergency range. Diastolic and nocturnal hypertension is more common in secondary hypertension. Patients who are asymptomatic, overweight, obese or with a positive family history for hypertension are more likely to have primary hypertension (19, 20).

The consequences of uncontrolled hypertension are macrovascular target end organ damage. This includes retinopathy, blindness, cerebrovascular accidents, dementia, left ventricular hypertrophy, myocardial infarctions, cardiac failure, peripheral arterial disease and renal failure. The World Health Organization declared that hypertension accounts for 57 million disability adjusted life years every year. Hypertension also contributes to 7.5 million or 12.8% of all deaths globally every year. The leading causes of death contributing to this statistic are ischemic heart and cerebrovascular disease (4).

iii. Cardiovascular and Cerebrovascular Disease

Prevalence of Cardiovascular Disease

Ischemic heart disease (IHD) and cerebrovascular accidents (CVAs) are the #1 and #2 causes of death in the world, respectively (24). In 2012, 14.1 million deaths were due to IHD and CVAs (24). Ischemic heart disease and ischemic CVAs refer to a condition or event in which a lack of blood flow and oxygen to the myocardium or brain

results from narrowing of the arteries supplying that tissue. The narrowing of arteries is often due to vasoconstriction or atherosclerotic plaques.

Pathogenesis of Atherosclerosis

Atherosclerosis progression is mediated by many processes including endothelial damage, hyperlipidemia, inflammation, and hyper-responsiveness of the immune system. Atherosclerosis begins with dysfunction or damage to the innermost layer of the artery, the endothelium. Damage to the endothelium can be caused by hypertension (ie: turbulent flow) or endothelial dysfunction (ie: oxidative stress and inflammation caused by cigarette smoking or uncontrolled diabetes mellitus for example). The damaged area of the endothelium becomes susceptible to lipid and platelet entry and accumulation in the subendothelial layers of the vessel wall (ie: the intima). Because lipids are not normally present in the intima, an immune response occurs which causes an infiltration of immune factors. The immune response associated with atherosclerosis may be mediated via modified low density lipoprotein (LDL), heat shock proteins or microbial antigens (25, 26). Immune factors such as monocytes, macrophages and T-cells are recruited. Macrophages will engulf the low density lipoprotein (LDL) that has migrated into the intima. As a result, the macrophages will create foam cells. T-cells will activate the smooth muscle cells to migrate from the media layer to the intima layer in which proliferation occurs. The endothelium is activated by pro-inflammatory stimuli and will express adhesion molecules such as vascular cell adhesion molecule-1. As a result of the damaged endothelium and the migration of lipids, smooth muscle cells, and mediators of immunity to the intima, an atherosclerotic plaque is created. Atherosclerotic plaques can either be stable or unstable. A stable plaque is characterized by having a small lipid core

and a thick fibrotic capsule. An unstable plaque has a larger lipid core and a thin fragile fibrotic capsule. The unstable plaque is susceptible to rupture and thrombus formation. Thrombus formation can lead to acute occlusion of the coronary or cerebral arteries causing acute coronary syndrome or a cerebrovascular accident, respectively (27).

Defining Cardiovascular Disease

IHD is the narrowing or occlusion of coronary arteries which can result in angina pectoris, myocardial infarctions, heart failure, and death (28). Diagnosis of IHD can be achieved primarily through coronary arteriography but can also be detected through an electrocardiogram (EKG) and stress testing (exercise induced stress detection via EKG detection or imaging or dolbutamine induced stress with EKG detection) (29). IHD can either be a result of chronic total occlusion of the coronary arteries or acute coronary syndrome.

Acute coronary syndrome (ACS) can be broken down into three categories:

1) S-T elevation myocardial infarction;
2) non S-T elevation myocardial infarction;
3) unstable angina.

ACS can be diagnosed by a change in serum cardiac biomarkers (primarily cardiac troponin), with one biomarker greater than the 99^{th} percentile upper reference limit in addition to a minimum of one of the following: ischemic symptoms, new ST changes, new left bundle branch block, pathological Q waves, new loss of healthy myocardium, left ventricular wall abnomality, and/or intracoronary thrombus (28). ACS is different from chronic total occlusion. ACS typically results from a thrombosis occurring from a ruptured atherosclerotic plaque (27). Chronic total occlusion occurs

over a longer period of time. The key features of chronic total occlusion are coronary artery calcification, inflammation, and neovascularization. As a result of neovascularization, some patients with chronic total occlusion may be asymptomatic (30). As many as 33% of patients undergoing coronary angiography have at least 1 chronic total occlusion (31).

Cardiovascular disease also includes diseases of the cerebral arteries. An ischemic cerebrovascular accident (CVA) (also known as an ischemic stroke) occurs when there is an impedance of blood flow to the brain as a result of narrowing or blockade (ie: atherosclerosis) of the arteries supplying blood to the brain. A CVA can result in permanent cerebral damage, neurological dysfunction, and/or death (32). Hemorrhagic CVAs are less common accounting for 13% of CVAs compared to ischemic CVAs. Hemorrhagic CVAs occur when a weakened cerebral artery (ie: an aneurysm) ruptures and causes intracrainial bleeding (33). CVAs are diagnosed through magnetic resonance imaging or computed tomography scans (32).

Risk Factors for Cardiovascular Disease

Risk factors for cardiovascular disease include hypertension, cigarette smoking, obesity, sedentary lifestyle, high fat diet, high circulating cholesterol levels, diabetes, family history, and older age (34-36). In particular, patients with peripheral artery disease (PAD) are at an elevated risk for developing cardiovascular events. The survival rate of patients with PAD is 35% less than those with coronary artery disease (37). Therefore, patients living with PAD are an important population to investigate in order to reduce the risk of cardiovascular events. The standard of care to reduce the risk for cardiovascular

disease is to manage the aforementioned risk factors. Of these factors, the number one risk factor for cardiovascular disease is hypertension. Hypertension alone contributes to approximately 54% of CVAs and 47% of IHD every year (38). Therefore, the primary goal for physicians is to manage hypertension in order to reduce the risk of IHD and CVAs. The optimal blood pressure goal for most patients is below 140/90 mmHg. If patients are diagnosed with diabetes mellitus, the blood pressure target is 130/80 mmHg due to being at higher risk for heart disease. If an individual is over the age of 80, the target systolic blood pressure is 150 mmHg, primarily due to maintaining cranial blood perfusion and to avoid postural hypotension (5).

Because hypertension is the leading cause for mortality worldwide, many different categories of treatments have been investigated including pharmaceutical, diet and functional foods, natural health products and lifestyle. In the next chapter, many of the available treatments for hypertension are summarized and compared.

Chapter I Section i-iii References

1. Pappano A. The cardiovascular system. In: Koeppen B, Stanton B, eds. Berne & Levy Physiology. 6th ed. Canada: Mosby Elsevier, 2010. 289-414.

2. Statistics Canada. High blood pressure, by age group and sex. 2015;2016.

3. Robitaille C, Dai S, Waters C, *et al*. Diagnosed hypertension in canada: Incidence, prevalence and associated mortality. CMAJ. 2012;184:E49-56.

4. World Health Organization. Global health observatory data - raised blood pressure. 2016.

5. Hypertension Canada. CHEP guidelines. 2015. http://guidelines.hypertension.ca/ retrieved April 01 2015.

6. Daskalopoulou SS, Rabi DM, Zarnke KB, *et al*. The 2015 canadian hypertension education program recommendations for blood pressure measurement, diagnosis, assessment of risk, prevention, and treatment of hypertension. Can J Cardiol. 2015;31:549-68.

7. Clark CE, Taylor RS, Shore AC, Ukoumunne OC, Campbell JL. Association of a difference in systolic blood pressure between arms with vascular disease and mortality: A systematic review and meta-analysis. Lancet. 2012;379:905-14.

8. Ding FH, Fan WX, Zhang RY, Zhang Q, Li Y, Wang JG. Validation of the noninvasive assessment of central blood pressure by the SphygmoCor and omron devices against the invasive catheter measurement. Am J Hypertens. 2011;24:1306-11.

9. Roman MJ, Devereux RB, Kizer JR, *et al*. Central pressure more strongly relates to vascular disease and outcome than does brachial pressure: The strong heart study. Hypertension. 2007;50:197-203.

10. McEniery CM, Cockcroft JR, Roman MJ, Franklin SS, Wilkinson IB. Central blood pressure: Current evidence and clinical importance. Eur Heart J. 2014;35:1719-25.

11. van Goor H, van den Born JC, Hillebrands JL, Joles JA. Hydrogen sulfide in hypertension. Curr Opin Nephrol Hypertens. 2016;25:107-13.

12. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. FASEB J. 1989;3:2007-18.

13. Montezano AC, Touyz RM. Molecular mechanisms of Hypertension—Reactive oxygen species and antioxidants: A basic science update for the clinician. Canadian Journal of Cardiology. 2012 (cited 2012/6//];28:288-95.

14. Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. Acta Cardiol. 2000;55:221-32.

15. Vasan RS, Beiser A, Seshadri S, *et al*. Residual lifetime risk for developing hypertension in middle-aged women and men: The framingham heart study. JAMA. 2002;287:1003-10.

16. Franceschi C, Capri M, Monti D, *et al*. Inflammaging and anti-inflammaging: A systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev. 2007;128:92-105.

17. Statistics Canada. 2013.

18. Madhur MS, Lob HE, McCann LA, *et al*. Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. Hypertension. 2010;55:500-7.

19. Flynn JT. Differentiation between primary and secondary hypertension in children using ambulatory blood pressure monitoring. Pediatrics. 2002;110:89-93.

20. Seeman T, Palyzova D, Dusek J, Janda J. Reduced nocturnal blood pressure dip and sustained nighttime hypertension are specific markers of secondary hypertension. J Pediatr. 2005;147:366-71.

21. Staessen JA, Wang J, Bianchi G, Birkenhager WH. Essential hypertension. Lancet. 2003;361:1629-41.

22. Carnethon MR, Evans NS, Church TS, *et al*. Joint associations of physical activity and aerobic fitness on the development of incident hypertension: Coronary artery risk development in young adults. Hypertension. 2010;56:49-55.

23. de Simone G, Devereux RB, Chinali M, *et al*. Risk factors for arterial hypertension in adults with initial optimal blood pressure: The strong heart study. Hypertension. 2006;47:162-7.

24. The World Health Organization. Top 10 causes of death. fact sheet N°310. 2014.

25. Deniset JF, Pierce GN. Heat shock proteins: Mediators of atherosclerotic development. Curr Drug Targets. 2015;16:816-26.

26. Hirono S, Pierce GN. Dissemination of chlamydia pneumoniae to the vessel wall in atherosclerosis. Mol Cell Biochem. 2003;246:91-5.

27. Falk E. Pathogenesis of atherosclerosis. J Am Coll Cardiol. 2006;47:C7-C12.

28. Thygesen K, Alpert JS, Jaffe AS, *et al*. Third universal definition of myocardial infarction. Circulation. 2012;126:2020-35.

29. Fihn SD, Gardin JM, Abrams J, *et al*. 2012 ACCF/AHA/ACP/AATS/PCNA/SCAI/STS guideline for the diagnosis and management of patients with stable ischemic heart disease: A report of the american college of cardiology Foundation/American heart association task force on practice guidelines, and

the american college of physicians, american association for thoracic surgery, preventive cardiovascular nurses association, society for cardiovascular angiography and interventions, and society of thoracic surgeons. Circulation. 2012;126:e354-471.

30. Stone GW, Kandzari DE, Mehran R, *et al*. Percutaneous recanalization of chronically occluded coronary arteries: A consensus document: Part I. Circulation. 2005;112:2364-72.

31. Kahn JK. Angiographic suitability for catheter revascularization of total coronary occlusions in patients from a community hospital setting. Am Heart J. 1993;126:561-4.

32. Sacco RL, Kasner SE, Broderick JP, *et al*. An updated definition of stroke for the 21st century: A statement for healthcare professionals from the american heart Association/American stroke association. Stroke. 2013;44:2064-89.

33. American Heart Association. Hemorrhagic strokes. 2015.

34. Kannel WB, Dannenberg AL, Abbott RD. Unrecognized myocardial infarction and hypertension: The framingham study. Am Heart J. 1985;109:581-5.

35. Kannel WB. Lipids, diabetes, and coronary heart disease: Insights from the framingham study. Am Heart J. 1985;110:1100-7.

36. Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: A 26-year follow-up of participants in the framingham heart study. Circulation. 1983;67:968-77.

37. Welten GM, Schouten O, Hoeks SE, *et al*. Long-term prognosis of patients with peripheral arterial disease: A comparison in patients with coronary artery disease. J Am Coll Cardiol. 2008;51:1588-96.

38. Lawes CM, Vander Hoorn S, Rodgers A, International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. Lancet. 2008;371:1513-8.

iv. Hypertension Treatments

A Review of the Relative Efficacy of Dietary, Nutritional Supplements, Lifestyle and Drug Therapies in the Management of Hypertension

In Press in Critical Reviews in Food Science and Nutrition

Stephanie P.B. Caligiuri and Grant N. Pierce

Abstract

Despite advancements in hypertensive therapies, the prevalence of hypertension and associated morbidities are still immense. Physicians are in great need for updated information on novel and effective anti-hypertensive therapies. Therefore, the study objective was to provide comprehensive information on the efficacy of available anti-hypertensive therapies. Anti-hypertensive therapies were divided into four general approaches: diet, nutritional supplements, lifestyle modification, and conventional anti-hypertensive medications. A search of PubMed and Google Scholar resulted in an analysis of 30 anti-hypertensive therapies from meta-analyses and randomized-controlled trials (RCTs). The studies were analyzed using the American Heart Association/American College of Cardiology classification system. Calculated average blood pressure reductions were: (systolic/diastolic) 6/4 mmHg, 4/2 mmHg, 5/3 mmHg and 9/5 mmHg for dietary, nutritional supplements, lifestyle, and medications, respectively. The results demonstrate that dietary, nutritional supplement and lifestyle strategies have a solid level of evidence to support their efficacy as anti-hypertensive strategies. These strategies can be as effective as medications and, in some cases, even

more effective. Dissemination of this information to physicians/dietitians can help facilitate an important shift in hypertension management.

Introduction

In 2013, the World Health Organization deemed high blood pressure as a global crisis. Hypertension can lead to dementia, cerebrovascular disease, retinopathy, myocardial infarctions, renal failure, and peripheral arterial disease (1). The number of adults with hypertension exceeds 40% worldwide. Despite advancements in anti-hypertensive therapies in the last 60 years, the prevalence of hypertension and these associated diseases is at an all-time high and present estimates predict even higher rates for the future (1). The problem is further compounded by a staggeringly low adherence to anti-hypertensive medications (18.8% of patients) (2). One potential reason for this low adherence may be due to current treatments resembling a "one size fits all" strategy that may not work for every individual. Drug treatment may not be the only answer for some patients. Lifestyle and dietary modification may be a viable alternative for many hypertensive patients. Current hypertension management guidelines recommend the use of lifestyle and dietary strategies as a first approach for the management of hypertension (3, 4). However, comprehensive and up to date information on all hypertension management strategies is not always available to physicians. Therefore, the purpose of this review is to provide an in depth analysis of dietary/natural health product and lifestyle interventions available, how these strategies compare to anti-hypertensive medications, and the strength of the data available. This study, therefore, provides a comparison of the effects of novel and well-known dietary strategies, supplements, lifestyle interventions and pharmaceuticals on blood pressure control. The purpose of this research is to provide a resource to physicians/dietitians and in earnest to start a change in

how we approach medicine in order to improve the standard of care for the management of hypertension.

Methods

Google Scholar and PubMed Databases were utilized to create a targeted search (last search performed January 3 2016). The following key words were used: blood pressure AND meta-analysis OR randomized controlled trial, AND diet OR nutrition OR exercise OR cigarette smoking OR alcohol reduction, OR sodium, OR drugs OR vitamins OR minerals OR calcium OR magnesium OR potassium OR bioactive peptides. Meta-analyses with the largest number of trials or of highest quality were included in the current review. The literature search was limited to full articles, reports in English, clinical research, meta-analyses, and randomized, controlled studies with the exception of the cessation of cigarette smoking trial conducted by Lee et al (2001) (5). This exception was allowed because a randomized controlled trial cannot be ethically conducted on cessation of cigarette smoking. However, cessation of cigarette smoking is recommended to patients in order to reduce their risk of cardiovascular disease. Therefore, the impact of this on blood pressure may not be realized by all physicians and is necessary to include in order to provide a comprehensive review. The search was limited to adults aged 18 years and older.

The findings were graded for level of evidence and class of recommendation based on the American College of Cardiology and American Heart Association guidelines for assessment of recommendations (6). Studies were graded for level of evidence: A (multiple populations evaluated), B (limited populations evaluated), or C (very limited populations evaluated) and class of recommendation: I (should be recommended), II (reasonable/may (to) recommend), or III (no benefit or harmful). The

studies were assessed for quality according to the fundamentals of the Cochrane Collaboration, and this was taken into consideration for grading the level of evidence and recommendation. The assessment of recommendations was also based on number of trials, type of trials, number of different populations, and presence of conflicting findings (6).

Data Extraction and Quality Assessment

The study type was first identified as meta-analysis, randomized controlled, or observational. Next, the participant characteristics were identified: sample size, stage of blood pressure, age and gender if available. The intervention type and dose were then obtained, followed by intervention duration. Next, the change in blood pressure was recorded. If available, the change in blood pressure only for those that were hypertensive at baseline was also recorded. Any adverse effects reported from the trials were recorded. Lastly, the studies were evaluated for level of evidence and class of recommendation based on the American Heart Association and American College of Cardiology Criteria and additionally assessed for quality according to the Cochrane Collaboration (Higgins et al. 2013).

Statistical Analysis

The average change in blood pressure for therapies with an A level of evidence and Class I or IIA recommendation were grouped by therapy category. Means and standard errors were calculated. The reductions in blood pressure by therapy category were compared using the advanced mixed model procedure. Unstructured, Huynh-Feldt, and compound symmetry models were run and selected based on the Akaike and Schwarz' Bayesian criteria. Least squared means were compared and multiple comparisons were corrected for using the Bonferroni correction with a p-value of 0.008.

The purpose of the review is to evaluate populations (normotensive, pre-hypertensive, hypertensive) that have undergone a dietary/natural health product, lifestyle change, or pharmaceutical treatment that can affect blood pressure, with an appropriate comparator or control for the intervention, with an outcome of blood pressure in meta-analyses or randomized controlled trials. Limitations and potential bias of the methodology employed for this review may have been skewed towards reporting more effective versus non-effective therapies.

Results

A. Dietary Interventions/Functional Foods

Several studies have been conducted to understand the blood pressure lowering effects of functional foods or diets. Table 2 outlines the study design, outcomes, adverse effects, level of evidence and class of recommendation for several dietary therapies. The observed reductions due to dietary therapy in systolic blood pressure ranged from 1.1 to 15 mmHg and diastolic blood pressure from 0.6 to 8.6 mmHg.

Table 2: Observed Reductions in Blood Pressure with Diets or Functional Foods

Dietary Intervention	Study Type	Participant Characteristics	Intervention	Duration	Results	Potential Adverse Effects	Level of Evidence	Class of Recommendation
Food Derived Bioactive Peptides (7)	Meta-analysis of RCTs	n=15 trials, 826 participants Mixed normotensive and hypertensive individuals. Most were >50 years old with more males than females.	100-400 mL of fermented milk or hydrolyzed protein powder that contained 2.6 mg – 1500 mg of bioactive peptides.	Average: 7.4 weeks Range: 4-12 weeks	SBP: - 5.3 (-7.1, -3.1) DBP: -2.4 (-3.8, -1.0)	No observed adverse effects	A	I
Garlic (8)	Meta-analysis of RCTs	n=11 studies, 565 patients Normotensive and hypertensive participants	Garlic powder, aged garlic extract, or distilled garlic oil Dose: 12.3-2400 mg /day	12-23 weeks	Total patients: SBP: -4.6 ± 2.8 DBP: -2.44 (-4.97, 0.08) Hypertensive patients: SBP: -8.4 ± 2.8 DBP: -7.3 ± 1.5	Garlic odour	A	I
DASH Diet with and without sodium reduction (9).	RCT - crossover	n= 412 average age = 48 years SBP:135 mmHg (120-159) DBP: 86 mm Hg (80-95)	Control Western diet and DASH diet with low (1.5 g/day), medium (2.5 g /day), and high sodium intake (3.5 g/day)	30 days/diet	DASH diet at a high (typical) sodium intake: SBP: -5.9 (-8.0, -3.7) DBP: -2.9 (-4.3, -1.5) DASH diet then changing from high to medium sodium intake: SBP: -1.3 (-2.6, 0.0) DBP: -0.6 (-1.5, 0.2) DASH diet then changing from medium to low sodium intake:	No adverse effects observed	A	I

					SBP: -1.7 (-3.0, -0.4) DBP: -1.0 (-1.9, -0.1) DASH diet then changing from high to low sodium intake: SBP: -3.0 (-1.7, -4.3) DBP: -1.6 (-0.8, -2.5)			
Dietary Sodium Reduction (10).	Meta-analysis of RCTs	56 RCTs n=3505 Normotensive and hypertensive participants Median age: 38 (8-73 years)	Dietary sodium reduction as ensured by decreased urinary sodium excretion.	Median: 14 days Range: 4-1095 days	Normotensive: SBP: -1.2 (-1.6, -0.8) DBP: -0.6 (-1.0. -0.03) Hypertensive: SBP:-5.4 (-6.3, -4.5) DBP: -3.5 (-4.1, 2.9)	Increase in renin-angiotensin activity	A	I
Fibre (11).	Meta-analysis including RCTs	n= 24 trials, 1404 participants Average age = 42 years (23-63 years) Normotensive (n=1083) and hypertensive (n=321)	Insoluble and/or soluble fibre supplementation average dose 11.5 g/day (3.5-42.6 g/day)	2-24 weeks	All: SBP: -1.1 (-2.5, 0.23) DBP: -1.26 (-2.0, -0.48) Hypertensive: SBP: -2.4 (-5.3, 0.45) DBP: -1.8 (-3.5, -0.14)	Large doses of fibre may cause flatulence, bloating, or diarrhea. However these disappear with time.	A	I
Flaxseed (12)	Meta-analysis of RCTs	N=15 trials, 1302 participants 14 trials – average baseline blood pressure was normotensive	Ground flaxseed (28-60 g/day) Or Flax oil containing 1.2-15 g of alpha-linolenic acid /day	4 weeks – 12 months	Total of all interventions: SBP: -2.9 (-5.4, -0.33) DBP: -2.4 (-3.8, -0.99) Ground flaxseed: SBP: -1.8 (-2.0, -1.6) DBP: -1.3 (-2.4, -0.11) Flax oil:	Bloating, flatulence, diarrhea may occur with ground flaxseed that disappears with time	B	I for ground flaxseed IIB for flax oil IIB for lignans

		1 trial – average baseline blood pressure was hypertensive	Or Flaxseed lignans (360-600 mg secoisolariciresinol diglucoside).		SBP: -4.6 (-11.9, 2.6) DBP: -4.1 (-6.8, -1.4) Lignans: SBP: 0.28 (-3.5, 4.0) DBP: -1.8 (-4.3, 0.72)			
Cocoa Powder OR Black or Green Tea (13)	Meta-analysis of RCTs	Cocoa : 5 RCTs n=173 Tea: 5 RCTs n=343 Normontension or Stage 1 Hypertension	Cocoa: 46-100 g/day of dark chocolate OR flavanol containing milk chocolate Tea:900-1500 mL of black tea or one green tea packet daily	Cocoa: average 2 weeks Tea: average 4 weeks	Cocoa: SBP: -4.7 (-7.6, -1.8) DBP: -2.8 (-4.8, -0.8) Tea: SBP: +0.4 (-1.3, +2.2) DBP: -0.6 (-1.5, +0.4)	No observed adverse effects	B	Cocoa – I Tea- III no effect
Diet rich in protein or monounsaturated fatty acids (14).	Randomized, crossover trial	N=164 adults with pre-hypertension or stage 1 hypertension	Diet: Carbohydrate: DASH diet with 3% greater amount of carbohydrate that replaced 3% of protein Protein:25% of calories as protein versus 15% in other diets MUFA:37% of calories as fat, with 21% as MUFA and 10% as PUFA.	6 weeks per diet	All participants: Change from Baseline: DASH+3% Carbohydrate: SBP:-8.2 (-9.6, -6.8) DBP: -4.1 (-5.0, -3.3) Protein diet: SBP: -9.5 (-10.9, -8.2) DBP: -5.2 (-6.1, -4.4) MUFA diet: SBP:-9.3 (-10.6, -8.0) DBP: -4.8 (-5.6, -4.0) Stage 1 Hypertension: Change from Baseline: DASH +3% Carbohydrate	No adverse effects observed	B	I

					SBP: -12.9 (-16.6, -9.2) DBP: -6.3 (-8.4, -4.3) Protein Diet: SBP:-16.1 (-19.7, -12.5) DBP: -8.6 (-10.9, -6.4) MUFA Diet: SBP: -15.8 (-19.4, -12.3) DBP: -8.2 (-10.4, -6.0)			
Soy Nuts (15).	Randomized, Crossover Trial	N=60 Post-menopausal women (n=12 hypertensive)	Control: Therapeutic Lifestyle Changes (TLC) diet alone Experimental: TLC diet of similar energy, fat, and protein content With ½ cup of unsalted soy nuts that replaced 25 g of non-soy protein.	8 weeks	Normotensive: SBP: -5.2% DBP:-2.9% Hypertensive: SBP: -9.9% DBP: - 6.8%	No observed adverse effects observed	B	IIA
Mediterranean Diet (16).	Meta-analysis of prospective, cross-sectional, and RCTs	Studies that included blood pressure measurement: N=14 RCTs, 3,060 subjects	A diet that is rich in monounsatured fatty acids, primarily from olive oil, frequent consumption of fruits, vegetables, whole grains, and low-fat dairy products. Less frequent	4 weeks – 5 years	SBP: -2.35 (-3.51, -1.18) DBP: -1.58 (-2.02, -1.13)	No adverse effects were reported	B	IIA

			consumption of poultry, nuts, legumes and red meat. Lastly, moderate daily consumption of alcohol.					
Nitrates or Beetroot Juice (17).	Meta-analysis	n=16 trials, 254 participants Male and female participants (age >18 years). Majority of participants were young physically active healthy men.	Inorganic nitrate: 15.5±9.2 mmol/dose Beetroot juice: 12.0±13.2 mmol/dose (140 – 500 mL/day)	2 hours-15 days	SBP: -4.4 (-5.9, -2.8) DBP: -1.1 (-2.2, 0.1)	Red coloured urine or stools from beetroot pigment	B	IIB

*grading is based on several individual clinical trials investigating different populations; but one clinical trial was chosen to present in the table. Other clinical trials used to create the grading are mentioned in the text.

Food Derived Bioactive Peptides

Food derived bioactive peptides are created by the hydrolysis of protein isolates. Of the 15 clinical trials included in the meta-analysis, 8 provided fermented milk as the source of bioactive peptides (7). Of 15 randomized clinical trials, the average reduction in blood pressure was 5.3/2.4 mmHg in a mixed population of normotensive and hypertensive individuals (7). The trial with the largest sample size (n=144) was conducted by Sano et al. (2005) (18). In this trial, a casein hydrolysate was provided to patients with high-normal or Stage 1 hypertension. The treatment group exhibited a significant reduction in systolic (5.9 mmHg) and diastolic (3.2 mmHg) blood pressure versus control over a 12 week period (18). The mechanism of action was reported to be through the ability of bioactive peptides to escape digestion, enter the circulation intact and inhibit angiotensin converting enzyme (ACE). Bioactive peptides are thought to lower the concentration of angiotensin II and thereby reduce its hypertensive effects (19). Only one trial found no significant change with whey peptides (20). This meta-analysis assessed and included trials with double blinding, randomization, controls, adequate quality and sensitivity. The research pertaining to food-derived bioactive peptides and blood pressure reduction achieved an A Level of Evidence and Class I Recommendation due to the meta-analysis of 15 RCTs in different populations and the consistent, significant reduction of blood pressure.

Garlic

A meta-analysis of 11 randomized, controlled, clinical trials was conducted to determine the effect of garlic supplementation on blood pressure. The participants included in the meta-analysis presented with normotension, pre-hypertension, stage 1 or stage 2 hypertension. Reductions in systolic/diastolic blood pressure (4.6/2.4 mmHg)

were observed by 5 weeks and greater reductions were observed in participants with hypertension (8.4/7.3 mmHg) (8). The anti-hypertensive properties of garlic have been attributed to allicin, however, this has yet to be proven (8). Garlic supplementation also lowered serum cholesterol and triglycerides in rats (21) and humans (22). Therefore, the capacity of garlic to lower blood pressure may also involve a reduction of atherosclerosis. Three of the 11 clinical trials favoured the control over intervention group; however these three trials were performed in a normotensive population. All trials conducted in a hypertensive population observed a consistent reduction in blood pressure. This meta-analysis included randomized, controlled, and double-blinded trials only and the studies included were independently assessed for quality according to the Cochrane Collaboration guidelines. Research regarding garlic supplementation and hypertension achieved an A Level of Evidence and Class I Recommendation due to the various populations investigated and the consistent, significant reductions in blood pressure.

DASH Diet With and Without Sodium Reduction

A large randomized, controlled, crossover trial was conducted to determine the effect of the DASH (Dietary Approaches to Stop Hypertension) Diet with and without dietary sodium reduction. The DASH diet encourages the consumption of fruits, vegetables, whole grains, low fat dairy products, and limiting red meat and sugar intake. The DASH diet contains less total fat, saturated fat and cholesterol than a typical western diet. It also contains more potassium, calcium, magnesium, dietary fibre and protein versus a western diet. The study population included participants with normotension, pre-hypertension or Stage 1 hypertension. The DASH diet alone resulted in a reduction of systolic/diastolic blood pressure of 5.9/2.9 mmHg (9). When dietary sodium reduction

was added to the DASH diet, further reductions in blood pressure were observed (9). The largest reduction in blood pressure was observed when the DASH diet was combined with dietary sodium reduction (3.5 reduced to 1.5 g/day). The reduction of dietary sodium may cause a decrease in blood volume, cardiac output and, therefore, mean arterial pressure (23). Because the DASH diet is high in calcium, magnesium, potassium and fibre, these nutrients may have led to reductions in blood pressure as well (9). Many clinical trials have reported similar outcomes after adopting the DASH diet (9, 24-26). The study reported in Table 1 is the largest clinical trial investigating the impact of the DASH diet alone on blood pressure. We assessed the quality of the DASH clinical trial using the Cochrane Collaboration guidelines. The trial meets the quality criteria except for the lack of blinding in the study. Blinding in a DASH trial, however, is impractical. Because the DASH diet has been studied in several clinical trials with consistent findings, it received an A Level of Evidence and Class I Recommendation.

Dietary Sodium Reduction

Results from an expansive meta-analysis of 56 clinical trials concluded that dietary sodium reduction significantly lowered blood pressure. The average length of follow-up included 14 days. Reductions in systolic/diastolic blood pressure in normotensive participants were 1.2/0.6 mmHg and 5.4/3.5 mmHg in participants with hypertension. The dietary sodium reduction was assessed by urinary sodium excretion. The average reduction in urinary sodium was 118 mmol/d. Blood pressure reduction was larger in individuals with hypertension and when the subjects were of an older age (10). A more recent meta-analysis performed on mostly blinded studies with longer term dietary sodium reduction (≥4 weeks) have also concluded similar results (27). As

mentioned above, the potential mechanism of action may be a decrease in blood volume, cardiac output and, therefore, mean arterial pressure (23). Interestingly, a low sodium diet for the short term can increase the renin-angiotensin system activity and perhaps circulating triglycerides and cholesterol (28). Despite this, dietary sodium reduction results in favourable responses for blood pressure reduction. Due to this finding, if a physician instructs a patient to reduce their dietary sodium intake, follow-up on lipid profiles are important. The trial by Midgley et al. (1996), had a large number of quality assessment criteria but it included mostly unblinded studies. However, in another meta-analysis performed by He at al. (2013) on mostly blinded studies, similar results were observed (27). Therefore, the impact of dietary sodium reduction on the lowering of blood pressure achieved an A Level of Evidence and Class I Recommendation due to the 2 above mentioned analyses concluding the same findings with sample sizes greater than 3000 participants.

Fibre

A meta-analysis of 24 trials concluded that dietary fibre supplementation resulted in a modest reduction in blood pressure (2.4/1.8 mmHg) (11). The trial with the largest sample size (n=201) was performed by Fehily et al (1986) (29). The intervention included the addition of 12 g of dietary soluble fibre for 4 weeks. The average systolic/diastolic blood pressure at baseline was 132/80 mm Hg. The average drop in systolic blood pressure was 0.4 mmHg whereas diastolic blood pressure increased by 0.2 mm Hg. Therefore, adding fibre to the diet may not lower blood pressure in normotensive individuals.

The clinical trial with the highest baseline blood pressure was performed by Nami et al. The average baseline systolic/diastolic blood pressure was 157/99 mm Hg. The intervention included 3.5 g of soluble fibre for 2 weeks (n=16). The average reduction in systolic and diastolic blood pressure was 14.3 ± 6 mm Hg (mean ± SE) and 5.4 ± 1.5 mm Hg, respectively (30). Dietary fibre was more effective in patients older than 40 years of age. Those younger than 40 years old exhibited an average decrease of 0.2 mm Hg in systolic blood pressure whereas individuals older than 40 years exhibited a decrease of 3.0 mm Hg (11). It was speculated that fibre may improve insulin resistance and, therefore, improve vascular endothelial health (11). However, this has yet to be proven. The only trials that observed a slight increase in blood pressure with fibre supplementation were performed in normotensive populations. This meta-analysis performed quality assessment and scored the trials based on blinding (open, single, and double) (11). The findings in hypertensive populations were consistent. Dietary fibre was provided an A Level of Evidence and a Class I Recommendation.

Flaxseed

A meta-analysis including 15 trials concluded that components of flaxseed significantly lowered blood pressure in primarily normotensive populations (2.9/2.4 mmHg). Interventions lasting longer than 12 weeks were more effective at lowering blood pressure (3.1/2.6 mmHg) versus interventions less than 12 weeks (1.6/1.7 mmHg). Ground flaxseed appeared to be more effective than flax oil and flax lignans (Table 1). It is important to note that all but one trial were performed in individuals with normotension or pre-hypertension.

The trial investigating the impact of ground flaxseed on hypertension was a randomized, controlled, double-blinded clinical (31) (n=110). The majority (75%) of participants were hypertensive and were already on anti-hypertensive medications. Despite this, the study population still had an average systolic blood pressure of 143 mmHg and a diastolic blood pressure of 78 mmHg. After 6 months of consuming flaxseed, the flaxseed group exhibited a decrease in systolic and diastolic blood pressure of 9.4 and 6.7 mmHg, respectively, versus the control group. In those that were hypertensive at baseline (~158 mmHg systolic and 84 mmHg diastolic pressure), the drop in systolic blood pressure was 15 mmHg and the diastolic blood pressure was reduced by 6.1 mmHg. After 1 year, the reduction in blood pressure was not as large. This could have been due to a lack of compliance or desensitization but this has yet to be determined (31).

The proposed mechanism of action of flaxseed is in part attributed to the capacity of the alpha-linolenic acid content to inhibit the activity of soluble epoxide hydrolase and thereby decrease the concentration of pro-inflammatory and vasoconstrictive oxylipins (32). Further discussion of the capacity of alpha-linolenic acid and flaxseed to reduce blood pressure is found elsewhere (33).

In terms of consistency, six of the 15 clinical trials favoured the control over flaxseed intervention. The average baseline blood pressures of these 6 clinical trials were in the pre-hypertensive range and all but one trial involved flax oil or flax lignans as the intervention. By comparison, interventions with ground flaxseed resulted in more consistent reductions in blood pressure. The meta-analysis was assessed for quality and bias according to the Cochrane collaboration checklist. Because the majority of clinical

trials were conducted in individuals with normotension, the research regarding flaxseed and blood pressure reduction received a B Level of Evidence. Due to the presence of conflicting findings for flax oil and flax lignans, the recommendation is Class IIB. Due to the consistent findings regarding ground flaxseed, the recommendation is I.

Cocoa

A meta-analysis of 5 RCTs concluded that cocoa in dark chocolate or flavanol containing chocolate milk significantly lowered systolic/diastolic blood pressure (4.7/2.8 mmHg) (13). The largest clinical trial in the meta-analysis was conducted by Fraga et al (2005) in healthy normotensive participants (n=28). Milk chocolate (105 g/day) lowered systolic blood pressure by 6 mmHg and diastolic blood pressure by 5 mmHg after 2 weeks. The study with the highest baseline blood pressure was conducted by Taubert et al (2003) (13). This study provided 100 g of dark chocolate/day to participants with stage 1 hypertension. The average reduction in systolic and diastolic blood pressure was 4.7 mmHg and 1.6 mmHg, respectively, after 2 weeks of consumption in the cocoa group. Cocoa may lower blood pressure through the capacity of its rich polyphenol content to induce a nitric oxide-mediated arterial vasodilation (34, 35). The studies included in this meta-analysis were independently assessed for quality and sensitivity by two reviewers and performed based on the Jadad quality scoring; however the meta-analysis was performed on mostly unblinded studies. This research achieved a B Level of Evidence and a Class I Recommendation because different populations were assessed and there was an absence of conflicting findings.

Tea

Based on the meta-analysis performed by Taubert et al, tea, primarily black tea, was not associated with a significant reduction in blood pressure versus control (13). Intake up to 1.5 L/day did not result in significant blood pressure reductions. Similar to cocoa, black tea contains polyphenols. This may, therefore, argue against the contribution of polyphenols in cocoa to blood pressure reduction. However, the polyphenol composition of tea may be quite different than cocoa suggesting that some specific polyphenols may be more effective at blood pressure reduction than others. Alternatively, other constituents within tea may have induced vasoconstrictive effects that counteracted the vasodilatory actions of the polyphenols. The studies included in this meta-analysis were independently assessed for quality and sensitivity by two reviewers and performed based on the Jadad quality scoring; however the meta-analysis was performed on mostly unblinded studies. This research was given a B Level of Evidence and a Class III-No Effect Recommendation.

Protein or Monounsaturated Fatty Acids

A randomized controlled crossover trial was conducted to determine the impact of a high protein (25% of calories) or a high monounsaturated fatty acid (MUFA) diet (21% of energy from MUFA) on blood pressure (14). The source of protein for the high protein diet was mostly from vegetable sources (ie: soy, beans, legumes, nuts, and seeds). The control diet was very similar to the DASH diet except with 3% greater energy distribution from carbohydrates. The three diets were isocaloric and designed to have similar levels of cholesterol, sodium, potassium, magnesium and calcium. The study population included participants with pre-hypertension or stage 1 hypertension. The majority of participants were overweight/obese and were never smokers. Higher consumption of protein or

MUFA lowered systolic/diastolic blood pressures (9.5/5.2 mmHg and 9.3/4.8 mmHg, respectively). Those with higher baseline blood pressure exhibited greater reductions in blood pressure (16.1/8.6 mmHg and 15.8/8.2 mmHg, respectively). Because a high sugar intake can increase blood pressure, it was hypothesized that the replacement of carbohydrates itself likely contributed to the reduction of blood pressure (14). According to the Cochrane Collaboration guidelines, this trial was well designed, randomized and controlled but not blinded. Due to the need for additional studies to confirm these results in other populations, this research achieved a B Level of Evidence and a Class I Recommendation.

Soy Nuts

The replacement of 25 g of dietary protein with ½ a cup of unsalted soy nuts per day combined with a therapeutic lifestyle changes (TLC) diet resulted in significant percent reductions in systolic and diastolic blood pressure in post-menopausal women (-10% and -7%, respectively) (15). However, the reductions in blood pressure were variable (15). Therefore, it is important to perform further research to determine which characteristics allow for an individual to respond better to soy nuts and the TLC diet. The TLC diet was created to help individuals manage high cholesterol levels. The TLC diet provides guidelines for the percent of energy from macronutrients with a focus on eating whole grains, fruits, vegetables, and limiting sources of dietary cholesterol and saturated fat. There was no confirmed mechanism of action for the hypotensive effects of soy nuts. Soy nuts are rich in potassium, fibre and calcium. All may have contributed. L-arginine is also present in soy nuts and may have acted as a precursor to the vasodilator nitric oxide but this has yet to be confirmed. The isoflavones in soy nuts may not be responsible for

the blood pressure reduction because clinical trials with isolated isoflavone tablets have not resulted in blood pressure lowering (36). Because the data surrounding soy nuts and blood pressure is based on one clinical trial and the trial was not blinded, the research on soy nuts received a B Level of Evidence and Class IIA Recommendation.

Mediterranean Diet

The Mediterranean diet has been associated with cardioprotection (37) and the reduction of cardiovascular events (38). However, the cardioprotection may not be blood pressure related, as a current meta-analysis observed a modest decrease in blood pressure of 2.4 mmHg and 1.6 mmHg for systolic and diastolic blood pressure (16). However, it is important to note that the meta-analysis included populations that were not hypertensive. In the large clinical trial published by Estruch (2013), those participants that were hypertensive responded better in terms of prevention of cardiovascular outcomes versus normotensive subjects (38). The proposed mechanism of action may be due to the high content of monounsaturated fatty acids and polyphenols in the diet that can lower cholesterol, inflammation, and atherosclerosis (37, 39). The meta-analysis was assessed by the original authors using detailed quality criteria. The current research on the Mediterranean diet for blood pressure reduction was provided a B Level of Evidence and a Class IIA recommendation due to blood pressure outcomes always being secondary outcome measures and due to the presence of conflicting findings.

Inorganic Nitrates/Beetroot Juice

A meta-analysis of 16 clinical trials concluded that nitrate and beetroot juice supplementation resulted in an average drop in systolic and diastolic blood pressure of 4.4 and 1.1 mmHg, respectively (17). Blood pressure reduction occurred as early as 2

hours after ingestion. The proposed mechanism of action was through the ability of beetroot juice or nitrates to increase the production of nitric oxide and cyclic guanosine monophosphate which ultimately causes vasodilation (17).

It is important to note that all studies but one were conducted in normotensive individuals and some trials were performed with athletes. The one exception was the study conducted by Gilchrist et al. (2013) with patients living with type 2 diabetes and hypertension. The participants were on an average of two anti-hypertensive medications with office systolic and diastolic blood pressures of 143 mmHg and 81 mmHg, respectively. The beetroot juice group did not have a significant difference in blood pressure compared to the control group after 2 weeks (40). This meta-analysis was performed according to the Cochrane Risk of Bias Tool. Due to the conflicting evidence and the lack of hypertensive populations studied, the research surrounding nitrates/beetroot juice and blood pressure reduction received a B Level of Evidence and a Class IIB Recommendation.

B. Supplements and Nutraceuticals

Many trials have investigated the impact of isolated vitamins, minerals, or nutraceuticals on blood pressure. Table 3 outlines the study design, outcomes, adverse effects, level of evidence and class of recommendation for several supplement therapies. The observed reductions due to supplements and nutraceuticals on systolic blood pressure ranged from 1.3 to 16.6 mmHg and diastolic blood pressure from 0.4 to 10.3 mmHg.

Table 3: Observed Reductions in Blood Pressure with Vitamin/Mineral Supplements or Neutraceuticals

Intervention	Study Type	Participant Characteristics	Intervention	Duration	Results	Potential Adverse Effects	Level of Evidence	Class of Recommendation
Long chain omega 3s Eicosapentanoic and Docosahexanoic acid (41)	Random Effects Meta-analysis of RCTs	n=70 RCTs with 93 data points (4489 participants)	Capsules (marine oil or ethyl esters) or food sources of EPA/DHA Dose of EPA+DHA: 0.1 – 15.0 g/day	21 days – 365 days	Normotensive: SBP: -1.3 (-2.1, -0.46) DBP: -0.62 (-1.2, -0.02) Hypertensive: SBP: -4.5 (-6.1, -2.8) DBP: -3.1 (-4.4, -1.7)	Fish aftertaste Belching Increased bleeding time with higher doses	A	I
Vitamin C (42).	Meta-analysis of RCTs	N=29 trials, 1407 participants Average age range: 22-74 years Normotensive and hypertensive participants	Supplement of 60-4000 mg/day Median dose: 500 mg/day	2-26 weeks Median : 8 weeks	Total participants: SBP: -3.84 (-5.29, -2.38) DBP: -1.48 (-2.86, -0.10) Hypertensive participants: SBP: -4.85 (-7.50, -2.20) DBP: -1.67 (-4.05, 0.72)	No adverse effects should be observed below the tolerable upper intake level of 2,000 mg/day for those 19 years and older. In rare cases, diarrhea, nausea, and abdominal cramps may occur.	A	IIA
Potassium (43).	Meta-analyses of RCTs	33 RCT trials n=2609 Participants were either normotensive or hypertensive.	Intervention: 48-200 mmol/day Median dose: 75 mmol/day (2.9 g/day) from supplements or diet	4 days – 3 years, median : 5 weeks	Total Participants: SBP: -3.11 (-1.91, -4.31) DBP: -1.97 (-0.52, -3.42)	Rare cases included: flatulence, upset stomach, or diarrhea.	A	IIA

		Average baseline SBP:105-187 median 147 mmHg DBP:63-105, median 95 mmHg	Control: Usual diet, placebo tablet, or lower dose of potassium supplementation					
Coenzyme Q10 (44)	Meta-analysis of 12 trials (3 RCTs 1 open label, 8 crossover)	RCT: n=120 Crossover: n=18 Open-label: n=214	RCT: 100-120 mg/day Crossover: 100 mg/day Open-label: 30-225 mg/day	RCT: 8-12 weeks Crossover: 10 weeks Open-label: 1-56 weeks	RCT: SBP: -16.6 (-20.6, -12.6) DBP: -8.2 (-10.2, -6.2) Crossover: SBP: -11.0 DBP: -8.0 Open-Label: SBP: -13.5 (-17.1. -9.8) DBP: - 10.3 (-12.3, -8.4)	Very rare mild side effects such as nausea, upset stomach, or diarrhea	B	IIA
Calcium (45).	Meta-analysis including RCTs	n= 13 trials, 485 participants Average age = 45 years (16-86 years) SBP ≥140 DBP ≥85 Average BP: 148/91. Essential hypertension	Oral calcium (citrate, lactogluconate, carbonate, or oyster shell electrolysate) supplementation Mean dose 1.1 g/day (0.4 – 2.0 g/day)	8-15 weeks	SBP: -2.5 (-4.5, -0.6) DBP: -0.8 (-2.1, 0.4) Higher Baseline SBP >145: SBP: -2.50 DBP: -0.38	No observed side effects below the daily tolerable upper intake level of 2.5 g/day for > 1 year of age.	B	IIB
Magnesium (46).	Meta-analysis including	n=12 trials, 544 participants	Oral magnesium (oxide, pidolate,	8-26 weeks	SBP: -1.3 (-4.0, 1.5) DBP: -2.2 (-3.4, -0.9)	No observed side effects below the tolerable upper	B	IIB

	RCTs	SBP ≥140 DBP ≥85 Average BP: 148/91. Essential HTN	lactate, citrate, aspartate, hydrochloride) Mean dose: 17 mmol (10-40 mmol/day).		SBP >150 mmHg: SBP: -2.22 (-9.04, 4.61) DBP: -2.93 (-6.19, 0.34)	intake level of 350 mg/day (14.4 mmol) for > 9 years of age		

Long Chain Omega-3 Fatty Acids

A meta-analysis on the effect of long chain omega 3 fatty acids included 70 randomized controlled trials (n=4489) and concluded supplementation on average lowered blood pressure by 4.5/3.1 mmHg in those with hypertension (41). The weighted group mean difference was greater with capsule supplementation versus food, with eicosapentanoic acid (EPA) alone versus docosahexanoic acid (DHA) alone, and in hypertensive versus normotensive subjects. The greatest blood pressure reduction was observed with 3 to < 4 g/day of EPA and DHA.

The study with the largest number of hypertensive patients (n=156) was conducted by Bonaa et al. (1990) (47). In a double-blinded randomized controlled trial, the baseline blood pressure of the fish oil group was 145/95 mmHg. The majority of the participants were male (60%) and non-smokers (60%) with an average age of 49 years. The intervention was 6 grams of fish oil (85% EPA and DHA) or a control with corn oil in gel capsules consumed daily for 10 weeks. A reduction of 4.6 mm Hg and 3.0 mmHg was observed in systolic and diastolic blood pressure, respectively, in the fish oil group. The reduction in blood pressure was less pronounced in individuals consuming ≥3 servings of fish per week or had a baseline plasma phospholipid n3 concentration above 175 mg/L (48). The increasing n3:n6 ratio was thought to be important (47) as well as the effects of n3 fatty acids on reducing inflammation and preventing pressure overload-induced cardiac dysfunction (49).

Another meta-analysis conducted in 1993 by Morris et al. concluded similar findings with 31 clinical trials (50). A dose dependent relationship was observed between the quantity of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) and blood pressure reduction. A dose of <3 g of EPA and DHA per day resulted in a minor decrease

in systolic (1.3 mm Hg) and diastolic (0.7 mm Hg) blood pressure. A dose of 3.3 – 7.0 g/day resulted in a 2.9 and 1.6 mmHg drop in systolic and diastolic blood pressure, respectively. The largest dose of 15 g/day resulted in a reduction of 8.1 and 5.8 mmHg in systolic and diastolic blood pressures, respectively. The blood pressure reduction from EPA and DHA was observed as early as 3-4 weeks. The reduction in blood pressure was greater in people with hypertension, diabetes, high cholesterol or cardiovascular disease versus healthy individuals.

The studies included in the meta-analysis by Miller et al (2014) were assessed for quality and majority were double blinded (41). Only 2 clinical trials found more favourable effects with the control group versus omega 3 fatty acids in hypertensive patients. Therefore, due to the high quality of evidence and very low abundance of conflicting results, an A level of evidence and Class I recommendation has been recommended. It must also be recognized that doses of up to 15 g/day of EPA and DHA is extremely large (up to 50 mL/day) and likely will have adverse effects (51).

Vitamin C

A meta-analysis of 29 RCTs concluded that vitamin C can lower blood pressure in patients with hypertension by 4.9 mmHg and 1.7 mmHg for systolic and diastolic blood pressure, respectively. Vitamin C supplementation was more effective in individuals less than 50 years of age (42). The proposed mechanism of action is through the ability of vitamin C to improve endothelial function and to preserve the bioactivity of nitric oxide and therefore increase vasodilation (52, 53). The trials within the meta-analysis were randomized and controlled. Majority of the clinical trials included in the meta-analysis were double blinded. The meta-analysis was assessed for quality and

performed in compliance with adherence to conventional trial standards. The research surrounding vitamin C supplementation and blood pressure was provided an A Level of Evidence but a Class IIA recommendation due to the presence of 3 trials showing a slight increase in diastolic blood pressure with vitamin C supplementation in hypertensive patients.

Potassium Supplementation

A meta-analysis performed by Whelton et al included 33 randomized controlled trials and concluded that potassium supplementation through capsules and/or foods resulted in a moderate decrease in blood pressure of 3.1 mmHg and 2.0 mmHg in systolic and diastolic blood pressure, respectively (43). The recommended intake of potassium is 4.7 g/day (120 mmol/day) by the Institute of Medicine. The average intake for Americans over the age of 2 years old is 2.64 g/day. It is recommended to eat foods that are rich in potassium as these foods also contain other nutrients such as fibre and vitamin C. Potassium supplementation was significantly more effective in lowering systolic blood pressure in African Americans (-5.6 mmHg) versus Caucasians (-2.0 mmHg). Potassium supplementation was also more effective in interventions lasting 4-11 weeks versus shorter and longer durations and in those with greater urinary sodium excretion during follow-up (43). The hypertension status, sample size, net change in urinary potassium, and net change in urinary sodium during the trial did not significantly influence the effect of potassium on blood pressure. The proposed mechanism of action for the blood pressure lowering effects of potassium include the ability to act as a diuretic, promotion of vasorelaxation, and inhibition of the renin-angiotensin system (54). An alternate meta-analysis performed by Dickinson et al (2006) observed a lack of statistical significance

for blood pressure reduction and potassium supplementation. This meta-analysis in particular include 6 RCTs (n=483 participants) with 8-16 weeks of follow up included studies with oral potassium supplementation and in populations with raised blood pressure. The difference in findings between the two meta-analyses is likely due to the number and type of trials selected.

The trials within the meta-analysis performed by Whelton et al. were assessed for quality and the meta-analysis was performed according to strict criteria to reduce bias and increase specificity. The data surrounding potassium supplementation has been given an A Level of Evidence but because another potassium supplementation meta-analysis found no significant improvement (55) a Class IIA recommendation has been achieved.

Coenzyme Q10

A meta-analysis of 12 clinical trials concluded that coenzyme Q10 supplementation resulted in a significant reduction of blood pressure (11-17 mmHg in systolic blood pressure and 8-10 mmHg in diastolic blood pressure). The clinical trial with the largest sample size (n=109) and longest duration (56 weeks) was conducted by Langsjoen et al. (1994). It was an open-label study design with 225 mg/day as the median dose of coenzyme Q10. The participants had essential hypertension (159/94 mmHg) but were already given anti-hypertensive medications. The average reduction in systolic/diastolic blood pressure was 11.4/9.0 mmHg after 56 weeks. In addition, more than 50% of participants ceased taking at least one of their anti-hypertensive medications. The proposed mechanism of action was through the antioxidant capacity of Coenzyme Q10. Reactive oxygen species react with nitric oxide to form peroxynitrite, a dangerous free radical species (56). Coenzyme Q10 may prevent the “de-activation” of nitric oxide

and, therefore, increase its vasodilating activity in individuals with hypertension and oxidative stress. Coenzyme Q10 as a result induces vasorelaxation at the endothelium and vascular smooth muscle level (57). The meta-analysis included blinded and open studies and did not perform a quality assessment. Therefore, the research pertaining to Coenzyme Q10 and blood pressure reduction has been provided a B Level of Evidence and Class IIA Recommendation due to the presence of underpowered studies. Although this meta-analysis reports significant blood pressure lowering effects with coenzyme Q10 supplementation, larger trials that are more tightly controlled are needed.

Calcium

A meta-analysis of randomized controlled trials concluded that calcium supplementation can result in a modest reduction in systolic blood pressure (2.50 mmHg) and a non-significant reduction in diastolic blood pressure (45). The blood pressure outcomes were similar between calcium supplementation doses of 0.4-2 g/day (45). As a result, a lower dose may be more feasible and just as effective. The trial with the largest sample size (n=90) included mostly males (82% of the population) with essential hypertension and an average blood pressure of 143/82 mmHg (58). Baseline mean dietary calcium intake was 1.41 g/day in the treatment group and 1.27 g/day in the control group. The intervention included 1 g/d calcium in the form of calcium citrate (powdered form). The compliant participants in the calcium group did not exhibit a significant decrease in systolic blood pressure but did observe a decrease in diastolic blood pressure (5.1 mmHg) after 12 weeks (58). It is important to note that the baseline calcium intake was already above the daily requirement for men of that age. Therefore, calcium supplementation may not lower systolic blood pressure in individuals already meeting the daily requirement for

calcium. However, this has yet to be determined. The mechanism of action for blood pressure reduction by dietary calcium is unknown. The studies within the meta-analysis were assessed for quality and the authors concluded that better quality controlled clinical trials are required to assess the impact of calcium supplementation on blood pressure. Therefore, dietary supplementation with calcium achieved a B Level of Evidence and Class IIB Recommendation due to the presence of conflicting findings.

Magnesium

In a meta-analysis of 12 clinical trials, magnesium supplementation lowered both systolic and diastolic blood pressure (1.3/2.2 mmHg) (46). However, in the hypertensive subgroup, the decrease in blood pressure was not significant due to variability in response (46). The trial with the largest sample size that included both men and women was performed by Borrello et al (1996). This trial consisted of 83 men and women with an average baseline blood pressure of 156/93 mm Hg. The intervention group was provided 10 mmol/day of magnesium oxide. Those taking magnesium exhibited a change of -6.7 mm Hg (-10.0, -3.40) in systolic blood pressure and -5.7 mm Hg (-8.05, 3.35) in diastolic blood pressure. However, many clinical trials with similar designs have conflicting results. Some trials have even shown an increase in blood pressure with magnesium supplementation (46). The studies within the meta-analysis were assessed for quality and the authors concluded that better quality controlled clinical trials are required to assess the impact of magnesium supplementation on blood pressure. As a result, magnesium received a B Level of Evidence and a Class IIB level of recommendation.

C. Lifestyle Interventions

Lifestyle interventions may be very useful for hypertension management. Lifestyle modifications resulted in blood pressure reductions that ranged from 0.8 to 5.0 mmHg for systolic blood pressure and 1.3 to 3.6 mmHg for diastolic blood pressure as outlined in **Table 4.**

Table 4: Lifestyle Modifications Associated with Blood Pressure Reduction

Lifestyle Modification	Study Type	Participant Characteristics	Intervention	Duration	Results (mmHg)	Potential Adverse Effects	Level of Evidence	Class of Recommendation
Weight Loss (59).	Meta-analysis of RCTs	n=25 trials, 4874 participants Mean initial body weight: 88.3 kg Mean initial BMI: 30.7 kg/m^2	Weight loss: diet, physical activity or a combination of both	Mean total duration : 67 weeks	Mean body weight change: -5.8% initial body weight (-6.03, -4.25) SBP: -4.44 (-5.93, -2.95) DBP: -3.57 (-4.88, -2.25) Average duration until maximal BP reduction was achieved: 35 weeks	No observed adverse effects	A	I
Aerobic Exercise (60).	Meta-analysis of RCTs	n=54 trials, 2419 participants	Supervised or unsupervised aerobic activity	3 weeks-2 years Median: 12 weeks	SBP: -3.84 (-4.97, -2.72) DBP: -2.58 (-3.35, -1.81) Hypertensive: SBP: -4.94 (-7.17, -2.70) DBP: -3.73 (-5.69, -1.77)	Must be approved by physician	A	I
Reduction of Alcohol Consumption (61).	Meta-analysis of RCTs	n=15 trials, 2234 participants Age: 27-57 years Primarily males (12 of 15 trials were 100% male) Average alcohol consumption: 3-6 drinks/day	Reduction of alcohol consumption (average: 67% reduction in alcohol consumption)	Average: 8 weeks (1-104 weeks)	SBP: -3.31 (-2.52, -4.10) DBP: -2.04 (-1.49, -2.58) Hypertensive: SBP: -3.9 (-5.04, -2.76) DBP: -2.41 (-3.25, -1.57)	No observed adverse effects	B	I
Stress Reduction (62).	Meta-analysis of RCTs Mixed pre-hyperrtensive and hypertensive	n=23 trials, 960 participants	Various stress reduction techniques including: Biofeedback, progressive muscle relaxation,	≥8 weeks	Biofeedback: SBP: -0.8 (-4.1, 2.6) DBP: -2.0 (-5.1, 1.2) Progressive Muscle Relaxation: SBP: -1.9 (-6.8, 3.1) DBP: -1.4 (-4.3, 1.4)	No observed adverse effects	A	I for Transcendental Meditation Programs IIB for biofeedback, progressive

			stress management training, and transcendental meditation programs		Stress Management Training: SBP:-2.3 (-5.0, 0.50) DBP: -1.3 (-5.4, 2.7) Transcendental Meditation Program: SBP: -5.0 (-7.6, -2.3) DBP: -2.8 (-5.0, -0.5)			muscle relaxation, and stress management training
Nasal continuous positive airway pressure (nCPAP) (63).	RCT	N=118 men with obstructive sleep apnea with pre-hypertension (133.7/85.1 mmHg)	Effective nocturnal nCPAP Control: sub-therapeutic nocturnal nCPAP	1 month	(mean ± SD) 24-hour Mean arterial pressure: -2.5 ± 0.8 Versus control: Systolic: -3.4 (-6.3, -0.6) Diastolic: -3.3 (-5.3, -1.2)	Rhinitis, nasal congestion, claustrophobia	B	IIA
Resistance Training (64).	Meta-analysis of RCTs	N=9 trials, 351 participants Sedentary normotensive or hypertensive individuals	Resistance training (static, circuit, or conventional training)	6-26 weeks Median: 25.5 weeks	All: SBP: -6.0 (-10.4, -1.6) DBP: -4.7 (-8.1, -1.4) Hypertensive: No subgroup average change in blood pressure was recorded. Unweighted average of three trials: SBP: -0.1 DBP: -3.5	Must be approved by a physician.	B	IIB
Cessation of Cigarette Smoking (5).	Observational	N=8170 healthy males that at baseline did not have hypertension. Baseline: n= 708 individuals that	Followed 8170 and compared blood pressure outcomes in those who quit smoking or continued to	4 years	Change in Blood Pressure Over 4 Years: Currently Smoking: SBP: +3.8 (3.5-4.0) DBP: +2.9 (2.7-3.1) Currently Non-Smoking:	Increased risk of hypertension over time	B	III Harm

		quit smoking over 4 years	smoke		SBP: +4.9 (4.4, 5.3) * DBP: +3.8 (3.5-4.2) * Quit smoking: < 1 year: SBP: +3.6 (2.3, 4.8) DBP: +3.1 (2.2, 4.1) 1-3 years: SBP: +5.2 (4.3, 6.2) * DBP: +4.1 (3.4, 4.8) * >3 years: SBP: +5.6 (3.9, 7.2) DBP: +4.2 (2.9, 5.4) *Significantly different versus currently smoking group Relative Risk of Hypertension: Currently Non-Smoking : 0.9 Quit Smoking: <1 year: 0.6 1-3 years: 1.5 > 3 years: 3.5			

Weight Loss

A meta-analysis consisting of 25 randomized controlled trials concluded weight loss can result in a significant reduction in blood pressure (59). The average maximal reduction in blood pressure was achieved after 35 weeks of weight loss intervention for overweight or obese individuals. For every 5.8% decrease in weight, a 4.4 mmHg drop in systolic and a 3.6 mmHg decrease in diastolic blood pressure was observed. Greater reductions in blood pressure were observed in individuals not on anti-hypertensive medications, individuals that lost >5 kg of body weight, individuals of Asian ethnicity, and those that combined both physical activity and diet for weight loss. However, gender, whether hypertensive or normotensive, age, or the initial body mass index, did not have as large an impact on weight loss and blood pressure (59). The mechanism of action may be due to a decrease in the activity or concentration of the hypertensive mediators, renin and aldosterone (65). The studies included in the meta-analysis were chosen based on select criteria of quality, and a funnel plot was used to assess for bias. The studies were not double blinded as this is impractical for a weight loss trial. This research received an A Level of Evidence and a Class I Recommendation.

Aerobic Exercise

A meta-analysis of 54 clinical trials concluded that aerobic exercise significantly lowered systolic/diastolic blood pressure on average by 4.9/3.7 mmHg in individuals with hypertension (60). Blood pressure reduction was slightly better (approximately 0.35 mmHg) when exercise was supervised or participants were not provided anti-hypertensive medications. Aerobic exercise lowered blood pressure more effectively in individuals of Asian ethnicity (6.2 mmHg) or African ethnicity (11.0 mmHg) versus individuals of Caucasian ethnicity (3.4 mmHg) (60). However, the type of aerobic

activity, baseline BMI, duration of study, exercise frequency or intensity did not have a significant impact on the magnitude of blood pressure reductions (60). This meta-analysis (60) observed significant reductions in blood pressure independent of weight loss. The potential mechanism of action may be due to the ability of aerobic exercise to improve vascular compliance (66) and insulin resistance (67, 68). The studies included in the meta-analysis were chosen based on select quality criteria. The meta-analysis was assessed for bias. The trials were not double-blinded as this is impractical. This research received an A Level of Evidence and a Class I Recommendation due to the large number of clinical trials executed and the consistent findings.

Reduction of Alcohol Consumption

A meta-analysis of 15 randomized controlled clinical trials concluded that a reduction in alcohol intake in individuals consuming on average 3-6 drinks/day at baseline lowered systolic and diastolic blood pressure by 3.9 and 2.4 mmHg, respectively (61). The majority of participants were male and half of the studies included individuals with diagnosed hypertension. Eight of the 15 clinical trials used a low alcohol beer substitute and the remaining clinical trials utilized counselling to help reduce alcohol consumption. Pre-treatment blood pressure and the % reduction in alcohol consumption explained the majority of the variance observed with the blood pressure response to alcohol consumption. Whether the participants were provided substitute low alcohol beer or counselling did not significantly impact the reduction in blood pressure. In addition, study length, age, or the presence of anti-hypertensive medications did not have a significant impact on blood pressure reduction with alcohol restriction (61). It is important to note that it is unclear if these findings translate to women as the studies were

primarily performed with males. There is no clear mechanism behind alcohol intake reduction and blood pressure reduction but it is known that heavy drinking is associated with hypertension, reduction of alcohol intake lowers blood pressure, and when an individual returns to heavy drinking, blood pressure increases once again (69). The meta-analysis included studies of good quality and specificity, and was scored based on quality for inclusion. Due to the research being conducted primarily in men, the research pertaining to reducing alcohol intake and blood pressure received a B Level of Evidence and a Class I Recommendation.

Stress Reduction

A meta-analysis was performed to determine the effect of four stress-reducing techniques on blood pressure reduction (62). Of the four techniques, transcendental meditation resulted in a significant reduction of blood pressure (systolic: 5.0 mmHg; diastolic: 2.8 mmHg) (62). Transcendental meditation allows one's mind to settle to a silent and peaceful level of consciousness, to meditate quietly, sitting, with the eyes closed for 15-20 minutes twice a day in order to reduce stress and achieve pure mind awareness. This meditative practice requires a certified teacher in Maharishi Vedic Education (62). In this meta-analysis, highlighted as of major importance was a randomized, single-blinded, controlled study that was conducted in 103 participants with stable coronary heart disease. The transcendental meditation group received introductory lectures, personal interviews, and group meetings included with the meditation. The control group attended the same number of meetings about health education. The treatment group exhibited a significant reduction in systolic blood pressure of 3.4 ± 2.0 mmHg versus control, and a non-significant difference in diastolic blood pressure. In

addition, an improvement in insulin resistance and heart rate variability was observed (70). The meta-analysis included clinical studies that met strict quality criteria. This research received an A Level of Evidence and a Class I Recommendation for transcendental meditation and Class IIB for biofeedback, progressive muscle relaxation and stress management training.

Nasal Continuous Positive Airway Pressure (nCPAP)

Sleep apnea is associated with hypertension (71). This association may be due to the stressful response that occurs when the pharynx collapses and asphyxia occurs followed by sudden awakening during sleep. nCPAP is a treatment strategy for individuals with sleep apnea. In a randomized, double-blinded, controlled, clinical trial the control group with sleep apnea was provided a subtherapeutic nCPAP in which the pressure provided was too low to keep the pharynx open. Those in the therapeutic group exhibited a significant decrease in systolic/diastolic blood pressures versus control (3.4/3.3 mmHg). Individuals with more severe sleep apnea or better compliance exhibited larger decreases in blood pressure. The mechanism of action may have been through the reduction of stressful awakening responses associated with sleep apnea (71). According to the Cochrane Collaboration, this research meets the criteria for a well designed high quality study. However, due to the conclusions being based on one single clinical trial performed with men only, a B Level of Evidence and a Class IIA Recommendation has been recommended.

Resistance Training

In a meta-analysis of 9 trials, resistance training resulted in blood pressure reduction of 6.0/4.7 mmHg over an average of 25.5 weeks in a mixed population of

normotensive and hypertensive individuals (64). Three trials were performed in hypertensive individuals (64). The average reduction in blood pressure was 0.1/3.5 mmHg. Two of the three trials observed an increase in systolic blood pressure (64). Blumenthal et al. (2004) performed the trial with the largest sample size in hypertensive individuals. The control group included those who were on the wait list for the exercise program. Both the control and treatment group exhibited reductions in blood pressure over 4 months. The reduction in blood pressure versus control was 1.0/1.2 mmHg with great variability. The meta-analysis included high quality studies that met the quality criteria and was assessed for bias. However, the authors conclude that more trials need to be performed with hypertensive patients. Therefore, the evidence for resistance training in hypertensive individuals has been given a B Level of Evidence and a Class IIB recommendation.

Cessation of Cigarette Smoking

Despite the adverse effects of cigarette smoking on cardiovascular disease, epidemiological studies have reported that those who smoke cigarettes have a lower blood pressure than non-smokers. Therefore, cigarette smoking does not appear to be a risk factor for hypertension (72). In contrast, cessation of smoking cigarettes increases the risk of hypertension in healthy men. Those that quit smoking increased their odds of developing hypertension up to 3.5 fold (5). The hypothesis for this response could be the stress involved with quitting smoking, the loss of a coping mechanism, and weight gain associated with cessation of smoking. Counseling/treatment for hypertension prevention or management is essential when cessation of cigarette smoking begins. Cessation of cigarette smoking should be recommended to prevent its other adverse effects. The

clinical trial was observational and comparative. As a result, cessation of smoking was provided a B Level of Evidence due to results coming from an observational trial in men only and a Class III-Harm Recommendation.

D. Anti-Hypertensive Medications

Anti-hypertensive medications have been thoroughly researched in large sample sizes of hypertensive participants to assess efficacy as well as safety. The average blood pressure reduction with a standard dose of anti-hypertensive medication is 8.5 to 10.3 mmHg for systolic blood pressure and 4.4 to 6.7 mmHg for diastolic blood pressure as outlined in **Table 5**. The mechanisms of action have been thoroughly assessed and proven for anti-hypertensive medications. As a result, anti-hypertensive medications as a whole have achieved an A Level of Evidence and Class I Recommendation. However, unlike many of the lifestyle or dietary interventions, there are more side effects associated with anti-hypertensive medications as illustrated throughout Tables 1-3. The meta-analysis included only randomized, blinded, controlled trials.

Table 5: Observed Effects on Blood Pressure by Anti-Hypertensive Pharmaceuticals

Pharmaceutical	Study Type	Participant Characteristics *	Intervention	Duration	Results (mmHg)	Potential Adverse Effects	Level of Evidence	Class of Recommendation
Thiazides (73).	Meta-analysis of RCT	n=7138 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)	Standard Dose	24 hours	SBP: -8.8 (-8.3, -9.4) DBP: -4.4 (-4.0, -4.8)	Dizziness, impotence, nausea, muscle cramps	A	I
ß-blockers (73).	Meta-analysis of RCT	n=7890 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)	Standard Dose	24 hours	SBP: -9.2 (-8.6, -9.2) DBP: -6.7 (-6.2, -7.1)	Feeling cold, fatigue, nausea	A	I
Angiotensin Converting Enzyme Inhibitor (73).	Meta-analysis of RCT	n=14062 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)	Standard Dose	24 hours	SBP: -8.5 (-7.9, -9.0) DBP: -4.7 (-4.4, -5.0)	Cough	A	I
Angiotensin II Receptor Blocker (73).	Meta-analysis of RCT	n=17940 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)	Standard Dose	24 hours	SBP: -10.3 (-9.9, -10.8) DBP: -5.7 (-5.4, -6.0)	No reported adverse effect	A	I
Calcium Channel Blocker (73).	Meta-analysis of RCT	n=11974 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)	Standard Dose	24 hours	SBP: -8.8 (-8.3, -9.2) DBP: -5.9 (-5.6, -6.2)	Flushing, ankle edema, dizziness	A	I

*Baseline blood pressure was only indicated for the entire meta-analysis population and therefore is the same for the different anti-hypertensive drug classes. Copyright permission to partially include a table previously published by Law et al 2003 was obtained.

Thiazides

Thiazides lower blood pressure by inhibiting the Na^+/Cl^- cotransporter in the distal convoluted tubule. This induces an increase in sodium and water excretion, decreases in blood volume and pre-load, and as a result, blood pressure decreases (74). A standard dose of a thiazide lowered systolic blood pressure on average by 8.8 mmHg and diastolic blood pressure by 4.4 mmHg within 24 hours (73). Thiazides had an incidence of 9.9% for side effects in this meta-analysis (73).

β-*Blockers*

β-blockers lower blood pressure by inhibiting the binding of catecholamines to β-adrenergic receptors and thereby inhibiting sympathetic stimulation of the heart (Couch and Krummel. 2008). A standard dose of β-blockers can lower systolic blood pressure by 9.2 mmHg and diastolic blood pressure by 6.7 mmHg within 24 hours, with a 7.5% incidence of side effects (73).

Angiotensin-Converting Enzyme (ACE) Inhibitors

ACE inhibitors lower blood pressure by reducing the effect of the renin-angiotensin-aldosterone system on blood pressure. ACE inhibitors bind to ACE and thereby reduce the action of ACE and its ability to convert angiotensin I to angiotensin II (75). ACE inhibitors lowered systolic and diastolic blood pressure within 24 hours by 8.5 and 4.7 mmHg, respectively, with a 3.9% incidence of side effects (73).

Angiotensin II Receptor Blocker

Angiotensin II receptor blockers act in a similar manner as ACE inhibitors in that they reduce the effects of the renin-angiotensin-aldosterone system on blood pressure. Instead of binding to ACE to reduce ACE activity, angiotensin II receptor blockers bind to the angiotensin

receptor and therefore prevent the action of angiotensin II (75). Angiotensin receptor blockers lower blood pressure within 24 hours with an average decrease of 10.3 mmHg for systolic blood pressure and 5.7 mmHg for diastolic blood pressure. In this study, Angiotensin II receptor blockers had a reported 0% incidence of side effects (73).

Calcium Channel Blocker

L-type calcium channel blockers decrease calcium entry into the vascular smooth muscle cells, cardiomyocytes, and cardiac nodes. By decreasing calcium entry, calcium channel blockers cause vasorelaxation, decrease heart rate, lower cardiac generated force and reduce conduction velocity (Pappano. 2010). Through these actions, calcium channel blockers lower systolic blood pressure on average by 8.8 mmHg and diastolic blood pressure by 5.9 mmHg with an incidence of side effects of 8.3% (73).

E. Comparison of Dietary, Nutritional Supplements, Lifestyle and Pharmaceutical Therapies

Figure 1 illustrates the magnitude of blood pressure reduction observed in participants with hypertension. Dietary (6/4 mmHg) and pharmaceutical therapies (9/7 mmHg) both result in substantial reductions in systolic/diastolic blood pressure. However, dietary therapies may vary in their ability to lower blood pressure more so than pharmaceuticals. Supplements (4/2 mmHg) and lifestyle interventions (5/3 mmHg) are also very effective in reducing systolic/diastolic blood pressure. When compared statistically, on average, pharmaceutical therapies resulted in significantly greater reductions in systolic blood pressure versus the other therapies. However, the therapies were not statistically different for diastolic blood pressure.

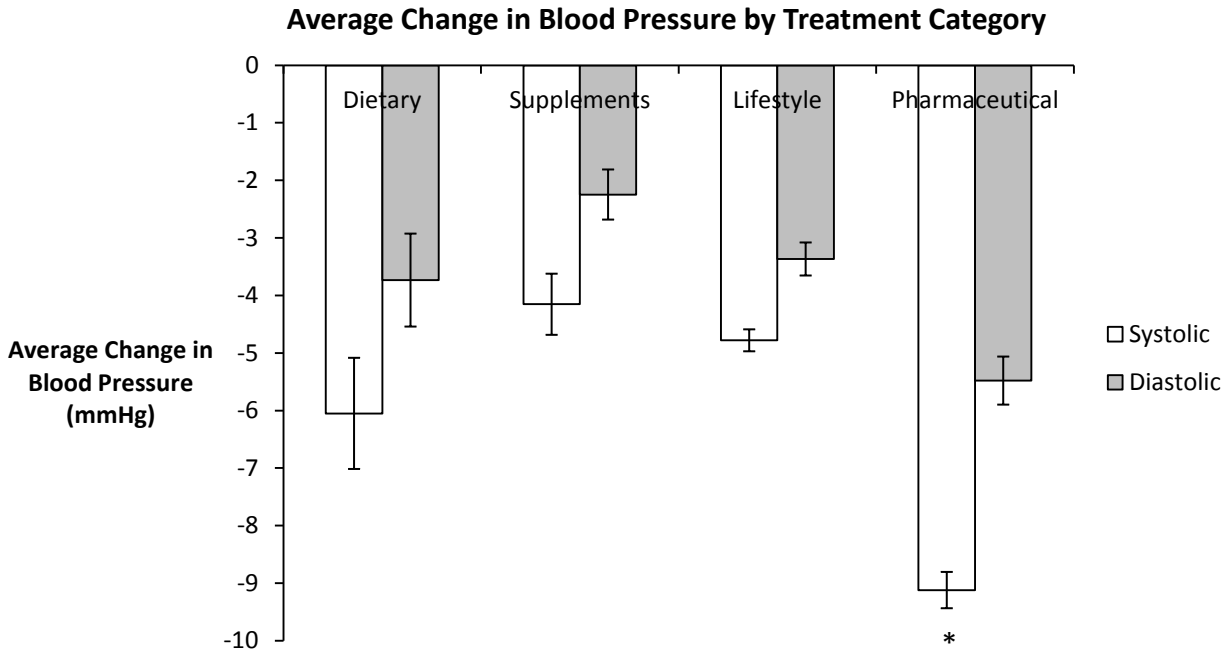


Figure 1: Magnitude of blood pressure reduction in patients with hypertension by category of therapy

This figure contains data from Studies that have an A Level of Evidence and a Class I or IIA Recommendation Level. Bars represent mean ± standard error. The standard error represents the variability among the different therapies within that category. The averages are unweighted. Least squared means were calculated for the mixed model procedure, followed by a post hoc least squared means comparison with a bonferroni correction. * - systolic blood pressure reduction for pharmaceuticals was significantly greater than other therapies $p<0.008$.

Discussion

Anti-hypertensive medications are often the first line therapies for hypertension. This is because, as observed in Table 5, anti-hypertensive medications are very effective at lowering blood pressure. Diet, supplements, and lifestyle modifications are also encouraged in standard of care practice. However, the Hypertension Canada guidelines (3) contain only limited information on the variety of dietary and lifestyle changes available. The guidelines are limited to increasing exercise, weight loss, alcohol and sodium reduction, adopting the DASH diet, and stress management (3). However, there are novel anti-hypertensive therapies that have sufficient levels of evidence to argue for their use in hypertension management. Unfortunately, they are not routinely employed. Their lack of adoption as conventional hypertension management strategies may be because they are not yet included in the different hypertension guidelines. As a result, potentially significant strategies to control hypertension are not introduced to physicians and are not prescribed as a standard of care practice. The data in the present study demonstrate that many other effective therapies exist and, therefore, should be considered and suggested to hypertensive patients as potential avenues for the control of hypertension. It is important to note that many interventions require improvement in terms of level of evidence. The therapies with a B Level of Evidence require further testing in other populations in order to determine if they should be implemented in standard of care.

This review provides a comparative analysis for drugs, dietary, nutritional supplements, and lifestyle therapies as effective anti-hypertensive strategies. Figure 1 illustrates that dietary, nutritional supplements, and lifestyle interventions are very

effective therapies. Dietary interventions may be just as effective as medications and in some cases more effective. However, it is important to note that the response of blood pressure to the different dietary interventions appears to be more variable than drug therapy. This variable efficacy is, in turn, probably a result of the different mechanisms through which the dietary interventions work to produce changes in blood pressure. Lifestyle changes and supplements are also effective therapies and are likely best when combined with another therapy.

There are strengths and weaknesses to each category of hypertension therapy. Dietary, nutritional supplements and lifestyle therapies take much longer to lower blood pressure, ranging from 2 hours to 35 weeks (Table 2). In contrast anti-hypertensive medications can be effective by 24 hours (Table 5) (73). Compliancy issues are a challenge in all four interventions. High adherence to anti-hypertensive medications is alarmingly low at 18.8% (2). By contrast, 78% completed a 1 year clinical trial with daily flaxseed ingestion (31). The DASH diet had 95% of participants complete the 90 day trial (9). Completion of a 24 month exercise or exercise+diet program was 84% and 80%, respectively (76). It appears when participants are a part of a nutrition or lifestyle program that adherence is higher than anti-hypertensive medications. In addition, anti-hypertensive medications are known to have more serious side effects than dietary and lifestyle therapies (Tables 2-5). All of these considerations provide the rationale for a patient-specific approach in order to understand which category of therapy or combination is best. It is obvious that there will not be 100% adherence to any given anti-hypertensive strategy. However, each person may be more receptive to certain strategies over others. It may be important for physicians to thoroughly discuss the options

available as identified in this paper. Once an option is identified which is optimal for a specific patient, a healthcare professional can discuss its implementation in detail. What is most important is to find which strategy that will allow the highest adherence for the patient without concern that, for example, only a pharmaceutical direction will produce the desired results.

Particularly in view of the non-compliancy exhibited for drug therapy, a combination of several therapies may provide the best strategy for blood pressure management for many people. Combining dietary or lifestyle changes with anti-hypertensive medications has already been proven to be very effective and more effective than one strategy alone (31, 33, 76). By providing an overview of many novel and well established anti-hypertensive therapies, as accomplished in the present paper, it will be possible to create a patient specific, effective hypertension management strategy. Future research can combine many of these effective strategies to create a new lifestyle and diet plan that will directly test the efficacy of combining different therapies.

Combination therapies are not only important in individuals with Stage 2 hypertension but particularly those that have high-normal or Stage 1 hypertension that do not have macrovascular target organ damage or other cardiovascular disease risk factors. The Canadian and European guidelines state that patients with Stage 1 hypertension without macrovascular target organ damage should adopt dietary and lifestyle changes rather than be prescribed anti-hypertensive medications (3, 4). However, many patients in this category are prescribed anti-hypertensive medications perhaps because a specific protocol for dietary and lifestyle management of hypertension is currently lacking. Most importantly, this review may provide information on expectations for blood pressure

reduction. For example, if a physician instructs their patient to reduce dietary sodium, on average the patient may exhibit a drop of 5/4 mmHg. If the patient must decrease their blood pressure by more than 5/4 mmHg, another intervention may need to be suggested by the physician.

Providing very specific treatment options to patients on the type of intervention (i.e. flaxseed or garlic), the dose (30 g or 12 mg/day) and the amount of time one must remain compliant before they can expect significant changes in blood pressure are currently needed. Prescriptions for dietary or lifestyle therapies should be as specific as they are for pharmaceuticals. The present study has provided initial guidelines in this regard. It is essential that this foundation of information is added to and updated as new research is published. This review hopes to start in earnest a wave of information in order for physicians to give specific advice to patients on what and how much to ingest rather than vague general diet and lifestyle recommendations. Specific instructions will help patients become more successful in the management of hypertension (77). This review has identified and assessed many anti-hypertensive therapies in order to advance standard of care practice. If standard of care can include several of these strategies, it is anticipated that patient adherence will increase, management of hypertension will improve, and patient risk for associated morbidity and mortality will decline.

Chapter I Section iv References

1. World Health Organization. A global brief on hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization, 2013.

2. Mazzaglia G, Ambrosioni E, Alacqua M, *et al*. Adherence to antihypertensive medications and cardiovascular morbidity among newly diagnosed hypertensive patients. Circulation. 2009;120:1598-605.

3. Hypertension Canada. The 2016 CHEP recommendations: 2016. http://guidelines.hypertension.ca/ Retrieved April 01 2016

4. 2013 ESH/ESC guidelines for themanagement of arterial hypertension. The Task force for the management ofarterial hypertension of the European Society of Hypertension (ESH) and of the European society of cardiology (ESC). 2013.

5. Lee DH, Ha MH, Kim JR, Jacobs DR,Jr. Effects of smoking cessation on changes in blood pressure and incidence of hypertension: A 4-year follow-up study. Hypertension. 2001;37:194-8.

6. Greenland P, Alpert JS, Beller GA, *et al*. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: A report of the american college of cardiology Foundation/American heart association task force on practice guidelines. Circulation. 2010;122:e584-636.

7. Pripp AH. Effect of peptides derived from food proteins on blood pressure: A meta-analysis of randomized controlled trials. Food Nutr Res. 2008;52:10.3402/fnr.v52i0.1641. Epub 2008 Jan 18.

8. Ried K, Frank OR, Stocks NP, Fakler P, Sullivan T. Effect of garlic on blood pressure: A systematic review and meta-analysis. BMC Cardiovasc Disord. 2008;8:13,2261-8-13.

9. Sacks FM, Svetkey LP, Vollmer WM, *et al*. Effects on blood pressure of reduced dietary sodium and the dietary approaches to stop hypertension (DASH) diet. DASH-sodium collaborative research group. N Engl J Med. 2001;344:3-10.

10. Midgley JP, Matthew AG, Greenwood CM, Logan AG. Effect of reduced dietary sodium on blood pressure: A meta-analysis of randomized controlled trials. JAMA. 1996;275:1590-7.

11. Streppel MT, Arends LR, van 't Veer P, Grobbee DE, Geleijnse JM. Dietary fiber and blood pressure: A meta-analysis of randomized placebo-controlled trials. Archives of Internal Medicine. 2005;165:150-6.

12. Khalesi S, Irwin C, Schubert M. Flaxseed consumption may reduce blood pressure: A systematic review and meta-analysis of controlled trials. J Nutr. 2015;145:758-65.

13. Taubert D, Roesen R, Schomig E. Effect of cocoa and tea intake on blood pressure: A meta-analysis. Arch Intern Med. 2007;167:626-34.

14. Appel LJ, Sacks FM, Carey VJ, *et al*. Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids: Results of the OmniHeart randomized trial. JAMA. 2005;294:2455-64.

15. Welty FK, Lee KS, Lew NS, Zhou JR. Effect of soy nuts on blood pressure and lipid levels in hypertensive, prehypertensive, and normotensive postmenopausal women. Arch Intern Med. 2007;167:1060-7.

16. Kastorini CM, Milionis HJ, Esposito K, Giugliano D, Goudevenos JA, Panagiotakos DB. The effect of mediterranean diet on metabolic syndrome and its components: A

meta-analysis of 50 studies and 534,906 individuals. J Am Coll Cardiol. 2011;57:1299-313.

17. Siervo M, Lara J, Ogbonmwan I, Mathers JC. Inorganic nitrate and beetroot juice supplementation reduces blood pressure in adults: A systematic review and meta-analysis. J Nutr. 2013;143:818-26.

18. Sano J, Ohki K, Higuchi T, *et al*. Effect of casein hydrolysate, prepared with protease derived from aspergillus oryzae, on subjects with high-normal blood pressure or mild hypertension. J Med Food. 2005;8:423-30.

19. Fuglsang A, Rattray FP, Nilsson D, Nyborg NC. Lactic acid bacteria: Inhibition of angiotensin converting enzyme in vitro and in vivo. Antonie Van Leeuwenhoek. 2003;83:27-34.

20. Lee YM, Skurk T, Hennig M, Hauner H. Effect of a milk drink supplemented with whey peptides on blood pressure in patients with mild hypertension. Eur J Nutr. 2007;46:21-7.

21. Ali M, Al-Qattan KK, Al-Enezi F, Khanafer RM, Mustafa T. Effect of allicin from garlic powder on serum lipids and blood pressure in rats fed with a high cholesterol diet. Prostaglandins Leukot Essent Fatty Acids. 2000;62:253-9.

22. Steiner M, Khan AH, Holbert D, Lin RI. A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged garlic extract and placebo administration on blood lipids. Am J Clin Nutr. 1996;64:866-70.

23. Kawasaki T, Delea CS, Bartter FC, Smith H. The effect of high-sodium and low-sodium intakes on blood pressure and other related variables in human subjects with idiopathic hypertension. Am J Med. 1978;64:193-8.

24. Moore TJ, Conlin PR, Ard J, Svetkey LP. DASH (dietary approaches to stop hypertension) diet is effective treatment for stage 1 isolated systolic hypertension. Hypertension. 2001;38:155-8.

25. Lopes HF, Martin KL, Nashar K, Morrow JD, Goodfriend TL, Egan BM. DASH diet lowers blood pressure and lipid-induced oxidative stress in obesity. Hypertension. 2003;41:422-30.

26. Appel LJ, Champagne CM, Harsha DW, *et al*. Effects of comprehensive lifestyle modification on blood pressure control: Main results of the PREMIER clinical trial. JAMA. 2003;289:2083-93.

27. He FJ, Li J, Macgregor GA. Effect of longer term modest salt reduction on blood pressure: Cochrane systematic review and meta-analysis of randomised trials. BMJ. 2013;346:f1325.

28. Graudal NA, Hubeck-Graudal T, Jurgens G. Effects of low-sodium diet vs. high-sodium diet on blood pressure, renin, aldosterone, catecholamines, cholesterol, and triglyceride (cochrane review). Am J Hypertens. 2012;25:1-15.

29. Fehily AM, Burr ML, Butland BK, Eastham RD. A randomised controlled trial to investigate the effect of a high fibre diet on blood pressure and plasma fibrinogen. J Epidemiol Community Health. 1986;40:334-7.

30. Nami R, Gallo V, Pavese G, Panza F, Gennari C. Antihypertensive activity of a vegetable fibre preparation: A preliminary, double-blind, placebo-controlled study. Eur J Clin Nutr. 1995;49 Suppl 3:S201-6.

31. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013.

32. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, Pierce GN. Flaxseed consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via an alpha-linolenic acid-induced inhibition of soluble epoxide hydrolase. Hypertension. 2014.

33. Caligiuri SP, Edel AL, Aliani M, Pierce GN. Flaxseed for hypertension: Implications for blood pressure regulation. Curr Hypertens Rep. 2014;16:499,014-0499-8.

34. Fisher ND, Hughes M, Gerhard-Herman M, Hollenberg NK. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. J Hypertens. 2003;21:2281-6.

35. Karim M, McCormick K, Kappagoda CT. Effects of cocoa extracts on endothelium-dependent relaxation. J Nutr. 2000;130:2105S-8S.

36. Hodgson JM, Puddey IB, Beilin LJ, *et al*. Effects of isoflavonoids on blood pressure in subjects with high-normal ambulatory blood pressure levels: A randomized controlled trial. Am J Hypertens. 1999;12:47-53.

37. Nadtochiy SM, Redman EK. Mediterranean diet and cardioprotection: The role of nitrite, polyunsaturated fatty acids, and polyphenols. Nutrition. 2011;27:733-44.

38. Estruch R, Ros E, Salas-Salvado J, *et al*. Primary prevention of cardiovascular disease with a mediterranean diet. N Engl J Med. 2013;368:1279-90.

39. Massaro M, Carluccio MA, De Caterina R. Direct vascular antiatherogenic effects of oleic acid: A clue to the cardioprotective effects of the mediterranean diet. Cardiologia. 1999;44:507-13.

40. Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, Benjamin N. Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. Free Radic Biol Med. 2013;60:89-97.

41. Miller PE, Van Elswyk M, Alexander DD. Long-chain omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid and blood pressure: A meta-analysis of randomized controlled trials. Am J Hypertens. 2014;27:885-96.

42. Juraschek SP, Guallar E, Appel LJ, Miller ER,3rd. Effects of vitamin C supplementation on blood pressure: A meta-analysis of randomized controlled trials. Am J Clin Nutr. 2012;95:1079-88.

43. Whelton PK, He J, Cutler JA, *et al*. Effects of oral potassium on blood pressure. meta-analysis of randomized controlled clinical trials. JAMA. 1997;277:1624-32.

44. Rosenfeldt FL, Haas SJ, Krum H, *et al*. Coenzyme Q10 in the treatment of hypertension: A meta-analysis of the clinical trials. J Hum Hypertens. 2007;21:297-306.

45. Dickinson HO, Nicolson DJ, Cook JV, *et al*. Calcium supplementation for the management of primary hypertension in adults. Cochrane Database Syst Rev. 2006;(2):CD004639.

46. Dickinson HO, Nicolson DJ, Campbell F, *et al*. Magnesium supplementation for the management of essential hypertension in adults. Cochrane Database Syst Rev. 2006;(3):CD004640.

47. Bonaa KH, Bjerve KS, Straume B, Gram IT, Thelle D. Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the tromso study. N Engl J Med. 1990;322:795-801.

48. Bonaa KH, Bjerve KS, Straume B, Gram IT, Thelle D. Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the tromso study. N Engl J Med. 1990;322:795-801.

49. Duda MK, O'Shea KM, Tintinu A, *et al*. Fish oil, but not flaxseed oil, decreases inflammation and prevents pressure overload-induced cardiac dysfunction. Cardiovasc Res. 2009;81:319-27.

50. Morris MC, Sacks F, Rosner B. Does fish oil lower blood pressure? A meta-analysis of controlled trials. Circulation. 1993;88:523-33.

51. Knapp HR, FitzGerald GA. The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty acid supplements in essential hypertension. N Engl J Med. 1989;320:1037-43.

52. McNulty PH, Robertson BJ, Tulli MA, *et al*. Effect of hyperoxia and vitamin C on coronary blood flow in patients with ischemic heart disease. J Appl Physiol (1985). 2007;102:2040-5.

53. Huang A, Vita JA, Venema RC, Keaney JF,Jr. Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. J Biol Chem. 2000;275:17399-406.

54. Treasure J, Ploth D. Role of dietary potassium in the treatment of hypertension. Hypertension. 1983;5:864-72.

55. Dickinson HO, Nicolson DJ, Campbell F, Beyer FR, Mason J. Potassium supplementation for the management of primary hypertension in adults. Cochrane Database Syst Rev. 2006;(3):CD004641.

56. Grunfeld S, Hamilton CA, Mesaros S, *et al*. Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. Hypertension. 1995;26:854-7.

57. Digiesi V, Cantini F, Bisi G, Guarino G, Oradei A, Littarru G. Mechanism of action of coenzyme Q10 in essential hypertension. Curr Ther Res. 1992;51:668-72.

58. Grobbee DE, Hofman A. Effect of calcium supplementation on diastolic blood pressure in young people with mild hypertension. Lancet. 1986;2:703-7.

59. Neter JE, Stam BE, Kok FJ, Grobbee DE, Geleijnse JM. Influence of weight reduction on blood pressure: A meta-analysis of randomized controlled trials. Hypertension. 2003;42:878-84.

60. Whelton SP, Chin A, Xin X, He J. Effect of aerobic exercise on blood pressure: A meta-analysis of randomized, controlled trials. Ann Intern Med. 2002;136:493-503.

61. Xin X, He J, Frontini MG, Ogden LG, Motsamai OI, Whelton PK. Effects of alcohol reduction on blood pressure: A meta-analysis of randomized controlled trials. Hypertension. 2001;38:1112-7.

62. Rainforth MV, Schneider RH, Nidich SI, Gaylord-King C, Salerno JW, Anderson JW. Stress reduction programs in patients with elevated blood pressure: A systematic review and meta-analysis. Curr Hypertens Rep. 2007;9:520-8.

63. Pepperell JC, Ramdassingh-Dow S, Crosthwaite N, *et al*. Ambulatory blood pressure after therapeutic and subtherapeutic nasal continuous positive airway pressure for obstructive sleep apnoea: A randomised parallel trial. Lancet. 2002;359:204-10.

64. Cornelissen VA, Fagard RH. Effect of resistance training on resting blood pressure: A meta-analysis of randomized controlled trials. J Hypertens. 2005;23:251-9.

65. Tuck ML, Sowers J, Dornfeld L, Kledzik G, Maxwell M. The effect of weight reduction on blood pressure, plasma renin activity, and plasma aldosterone levels in obese patients. N Engl J Med. 1981;304:930-3.

66. Cameron JD, Dart AM. Exercise training increases total systemic arterial compliance in humans. Am J Physiol. 1994;266:H693-701.

67. Brown MD, Moore GE, Korytkowski MT, McCole SD, Hagberg JM. Improvement of insulin sensitivity by short-term exercise training in hypertensive african american women. Hypertension. 1997;30:1549-53.

68. He J, Klag MJ, Caballero B, Appel LJ, Charleston J, Whelton PK. Plasma insulin levels and incidence of hypertension in african americans and whites. Arch Intern Med. 1999;159:498-503.

69. Saunders JB, Beevers DG, Paton A. Alcohol-induced hypertension. Lancet. 1981;2:653-6.

70. Paul-Labrador M, Polk D, Dwyer JH, *et al*. Effects of a randomized controlled trial of transcendental meditation on components of the metabolic syndrome in subjects with coronary heart disease. Arch Intern Med. 2006;166:1218-24.

71. Peppard PE, Young T, Palta M, Skatrud J. Prospective study of the association between sleep-disordered breathing and hypertension. N Engl J Med. 2000;342:1378-84.

72. Green MS, Jucha E, Luz Y. Blood pressure in smokers and nonsmokers: Epidemiologic findings. Am Heart J. 1986;111:932-40.

73. Law MR, Wald NJ, Morris JK, Jordan RE. Value of low dose combination treatment with blood pressure lowering drugs: Analysis of 354 randomised trials. BMJ. 2003;326:1427.

74. Couch S, Krummel D. Medical nutrition therapy for hypertension. In: Mahan K, Escott-Stump S, eds. Krause's Food and Nutrition Therapy. 2008. 874.

75. Stanton B, Koeppen B. The renal system. In: Berne & Levy Physiology Sixth Edition. 2010. 576.

76. Wing RR, Venditti E, Jakicic JM, Polley BA, Lang W. Lifestyle intervention in overweight individuals with a family history of diabetes. Diabetes Care. 1998;21:350-9.

77. Strecher VJ, Seijts GH, Kok GJ, *et al*. Goal setting as a strategy for health behavior change. Health Educ Q. 1995;22:190-200.

Acknowledgements
This study was indirectly supported by the St Boniface Hospital Foundation.

Author Contributions

This review was written by SPB Caligiuri and GN Pierce.

Funding and Sponsorship

SPBC is a recipient of the Canadian Institutes of Health Research Patient Oriented Research Doctoral Scholarship and the Research Manitoba Doctoral Schoalarship. This study was supported through grants from the Canadian Institutes of Health Research, the Agri-Food Research Development Initiative and the Western Grains Research Foundation.

Declaration of Interest

The authors declare no conflict of interest.

Abbreviations

ACE – angiotensin converting enzyme

DASH – dietary approaches to stop hypertension

DHA – docosahexanoic acid

EPA – eicosapentanoic acid

MUFA – monounsaturated fatty acid

nCPAP – nasal continuous positive airway pressure

RCT – randomized controlled trial

TLC – therapeutic lifestyle changes

V. Flaxseed and Hypertension

Of the investigated therapies for hypertension, dietary flaxseed is of particular interest because it contains a combination of potential anti-hypertensive bioactive components, ie: n3 fatty acids, fibre, antioxidant lignans and L-arginine. In addition, flaxseed is a Manitoban grown crop that is highly accessible worldwide. Dietary flaxseed may, therefore, be a valuable anti-hypertensive therapy. The next section summarizes the evidence available for flaxseed as an anti-hypertensive therapy and the potential implications it may have on hypertension management.

Flaxseed for Hypertension: Implications for Blood Pressure Regulation

Parts of the Manuscript Published in: Current Hypertension Reports. 2014 Dec;16(12):499 are included below.

Stephanie P. B. Caligiuri[1-3*], Andrea L. Edel[1-3*], Michel Aliani[1,4] and Grant N. Pierce[1-3]

[1]Canadian Centre for Agri-food Research in Health and Medicine (CCARM) and [2]the Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre, and [3]the Departments of Physiology, and [4]Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada

Running Title: Flaxseed as an Anti-Hypertensive Therapy

Abstract

Hypertension is the single largest risk factor attributed to mortality in the world. Medications are the primary treatment for hypertension, however, adherence to drug regimens is low (~50%). Low adherence may be a contributing factor leading to uncontrolled blood pressure in patients. An effective alternative or complement to medications in managing hypertension is through lifestyle modifications. A recent, randomized controlled year-long trial observed impressive reductions in blood pressure in patients with hypertension consuming flaxseed daily. Therefore, attention has been garnered for flaxseed as a potentially valuable strategy for the management of hypertension. This review will highlight the recent data for flaxseed and its extracts in blood pressure regulation in both animal models and clinical trials. Insight into the proposed anti-hypertensive mechanism of flaxseed and the implications of flaxseed as a potential global anti-hypertensive therapy will be discussed.

Keywords dietary flaxseed; anti-hypertensive therapy; blood pressure regulation; alpha-linolenic acid; lignans; enterolignans; enterolactone; enterodiol

Abbreviations

ALA - Alpha-linolenic acid, BP- blood pressure, DBP - diastolic blood pressure, END - enterodiol, ENL - enterolactone, MAP - mean arterial pressure, SBP - systolic blood pressure, SDG - secoisolariciresinol diglucoside, SECO - secoisolariciresinol, SHR - spontaneously hypertensive rats

Introduction

Hypertension accounts for approximately half of the nearly 17 million cardiovascular deaths worldwide making it the leading risk factor attributed to death globally (1). Approximately 40% of adults aged 25+ years have hypertension worldwide (1). It is not surprising, therefore, that the cost of heart disease globally from 2011-2015 is estimated at $3.76 trillion USD (1).

In view of these alarming statistics, the control of hypertension should be of paramount medical and economic importance. The most common treatment strategy for hypertension includes medications. However, a large proportion of newly diagnosed hypertensive patients do not adhere to their routine of anti-hypertensive medication. Of 18,806 study patients, only 18.8% of patients were high adherers, with 32.3% and 48.9% being intermediate and low adherers, respectively (2). This could explain why up to 73% of patients in Europe and North America do not have control of their blood pressure (BP) (3). Non-adherence was associated with a higher risk for developing cardiovascular events (2). Obstacles to anti-hypertensive drug compliancy, therefore, exist for many patients.

A dietary therapeutic strategy may be preferable and just as effective in managing hypertension. The emphasis on lifestyle modifications that include nutritional strategies has increased recently due to changes in the hypertension management guidelines (4,5). According to Canadian and European guidelines, individuals with Grade or Stage 1 hypertension without macrovascular target organ damage or other risk factors should not be prescribed anti-hypertensive medication (4,5). Rather these patients are encouraged to adopt lifestyle changes. Even patients with Grade or Stage 2 hypertension with risk factors are encouraged to make lifestyle changes for several weeks before determining

the dosage of medication and if medication is still necessary (4,5). American guidelines also recommend that lifestyle interventions begin before prescribing medication (6).

Lifestyle changes to manage hypertension include weight loss, aerobic exercise, cessation of smoking, adopting the Dietary Approaches to Stop Hypertension diet, and reducing alcohol and sodium intake. However, in the last decade, a greater research emphasis has been placed on functional foods that may influence BP. Functional foods are foods that provide health benefits beyond basic nutritional qualities. One functional food that may help patients manage hypertension is flaxseed.

Flaxseed, *Linum usitatissimum*, is an oilseed crop that produces pale blue flowers and fruit capsules filled with small, brown seeds. Flaxseed is one of the richest plant sources of the omega-3 (n-3) fatty acid, alpha-linolenic acid (ALA; C18:3n-3) (7). Oils comprise about 41% of the overall seed weight with 57% of that oil representing ALA. Flaxseed is also a major source of the lignan secoisolariciresinol diglucoside (SDG), a potent antioxidant (8). SDG constitutes 34-38% of the overall lignans in flaxseed (9). Matairesinol, lariciresinol and pinoresinol are also present in much smaller amounts (10). Conversion of SDG in the colon by gut microbiota yields the unconjugated derivative secoisolariciresinol (SECO) which is further converted into the primary metabolites enterolactone (ENL) and enterodiol (END). These compounds structurally mimic oestrogen and can competitively bind to the oestrogen receptor (11). Proteins, another major constituent in flaxseed, comprise 20% of the overall seed composition. Flaxseed is also a prominent source of both soluble and insoluble fibres. Flax oil is devoid of fibre and very low in lignans (12).

The health-related actions of dietary flaxseed may be due to four bioactive ingredients: ALA, lignans, fibre or peptides or it may be due to a synergistic effect of these components together (12). The bioavailability of ALA is optimal when ingested as flaxseed oil but poor when ingested as a whole seed. Milled flaxseed provides the best option for the bioavailability for each of these bioactive components and is well tolerated (13,14).

Anti-Hypertensive Effects Of Dietary Flaxseed In Animals

a) Whole flaxseed

Only one study has used whole flaxseed as an anti-hypertensive strategy. In spontaneously hypertensive rats (SHR), there was a moderate but insignificant decrease in systolic blood pressure (SBP) compared to control (15). More studies using ground flaxseed are needed in different hypertensive animal models.

b) Flax oil and ALA

One of the first studies to examine flax oil as an anti-hypertensive strategy observed a significant decrease in systolic blood pressure (SBP) within hours of ingestion versus a high oleic sunflower oil control in spontanously hypertensive rats (16). Subsequent work using a variety of dietary interventions that contained oils enriched in ALA have shown a consistent pattern of BP reduction. This was also found even in the offspring from mothers fed ALA (16).

c) Flaxseed lignans

Dietary supplementation with SDG has induced a significant lowering of SBP, but only when administered in the presence of a high fat diet in Sprague-Dawley rats (17). However, when SDG was delivered intravenously (18), substantial decreases in SBP (15-

40%), diastolic BP (DBP) (24-48%) and mean arterial pressure (MAP) (22-43%) were observed as early as 15 minutes after injection and were maintained for 4 hours in Sprague-Dawley rats. The mechanism of action was suggested to involve guanylate cyclase activation and angiotensin I inhibition (18,19). Intravenous SDG may represent a useful pharmacological therapy, however, the anti-hypertensive effects from consuming flaxseed lignans will likely not proceed via SDG itself but rather through its metabolites. SDG is not present in circulation in the conjugated form, but rather is converted by gut micro flora to its aglycone, SECO (20). SECO is bioavailable, but may be further metabolized to the enterolignans, END and ENL, which are the physiological form of lignans circulating as a result of flaxseed ingestion (20).

d) Flaxseed protein and peptides

Recently, a flax protein hydrolysate and an isolated fraction (KCl-F1) reduced SBP 2-8 h post oral gavage in spontaneously hypertensive rats (21). These outcomes mimicked those of the anti-hypertensive captopril. The maximal reduction in SBP for all 3 treatments occurred after 4 hours (-27 mm Hg), was maintained until 8 hours, but was ineffective after 24 hours. The beneficial action of these interventions was attributed to low molecular weight peptides which are abundant in arginine (22).

Anti-Hypertensive Effects Of Dietary Flaxseed in Clinical Trials

a) Whole flaxseed

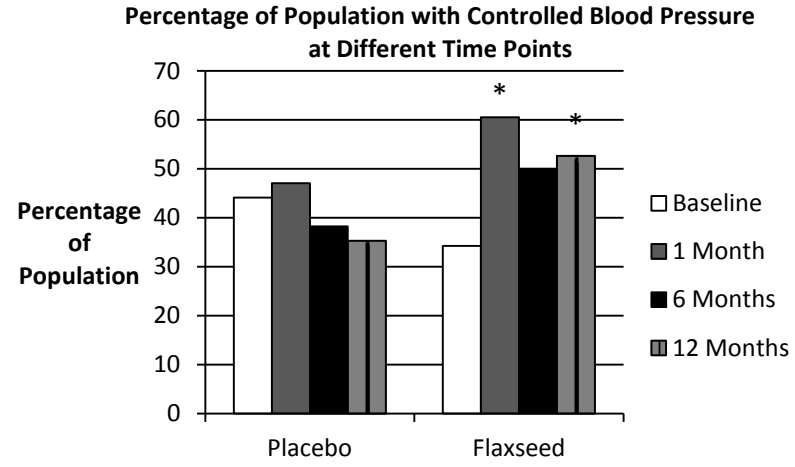
Flaxseed and components of flaxseed have been associated with BP reduction in both observational studies and randomized controlled trials. The most tightly controlled and longest study, the FLAX-PAD Trial, was a randomized, double-blinded, controlled year-long trial investigating the effects of flaxseed consumption on patients with

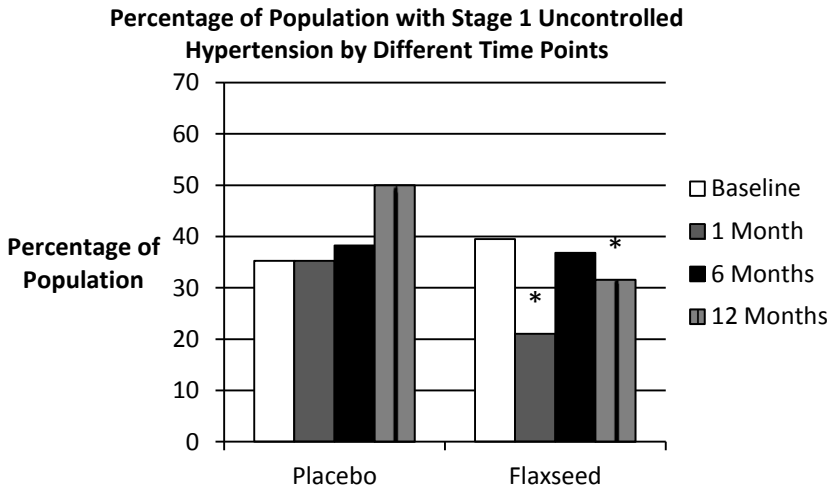
peripheral arterial disease (75% were hypertensive). Patients consumed either 30 g of ground flaxseed or control food products every day for 1 year (n=110). Ground flaxseed was chosen as the dietary supplement because it provides optimal bioavailability of all four potential anti-hypertensive bioactives (ALA, lignans, fibre and peptides). Patients in the flax group exhibited a significant reduction in SBP and DBP of 10 mm Hg and 7 mm Hg, respectively, over 6 months. In those with diagnosed hypertension at baseline, the reduction in SBP was even greater (15 mm Hg) (23). It is important to note that the drop in BP was greater than the average decrease observed with the standard dose of anti-hypertensive medications (24).

Ultimately, dietary flaxseed changed the distribution of patients within the different categories of hypertension. The percentage of patients in the flax group with uncontrolled high BP decreased from 65% to 48% by one year. By comparison, in the control group, the percentage of patients with uncontrolled hypertension increased from 57% to 65% (Figure 2). Therefore, after one year of standard of care, the control group exhibited a worsening of BP control whereas the flaxseed group improved. The decrease in BP was less striking at one year but this may have been due to a decrease in dietary compliancy.

The anti-hypertensive effect of flaxseed in the FLAX-PAD Trial was observed in the presence of standard anti-hypertensive medication. Thus, flaxseed does not inhibit the anti-hypertensive action of conventional therapy. However, it is not clear yet if it can replace anti-hypertensive medication or be effective in patients without peripheral arterial disease. Further work is on-going to determine if dietary flaxseed can independently

lower BP and reduce the need for anti-hypertensive medication in patients with Stage 1 hypertension (25).





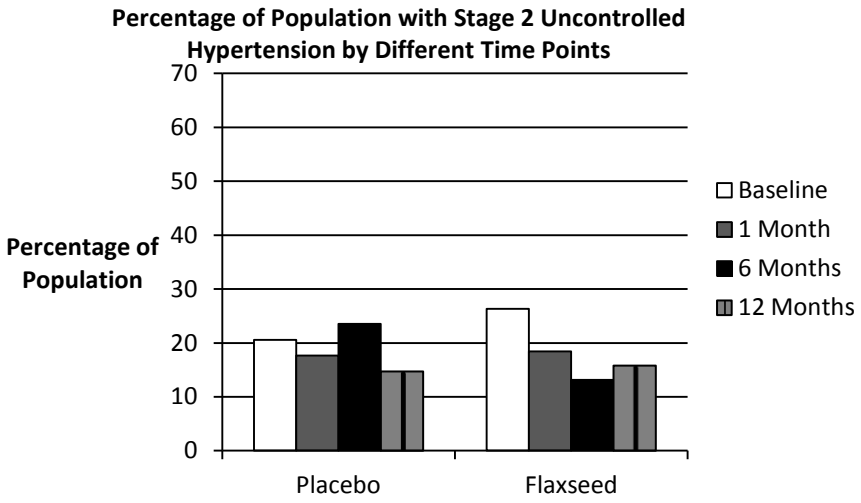


Figure 2: Distribution of Hypertension Categories (Controlled, Stage 1 Uncontrolled, Stage 2 Uncontrolled Blood Pressure) in the Placebo and Flax Group by Time Point. * - Chi-square test p<0.05. Data extracted from the FLAX-PAD Trial (23)

b) Flax oil and ALA

The anti-hypertensive effects of ALA were first observed in a small observational study where adipose ALA concentrations were inversely associated with SBP and DBP in men (26). Large epidemiological trials have concluded a similar relationship between ALA and cardiovascular disease. In a landmark trial examining a diet rich in ALA, the incidence of secondary myocardial infarctions and mortality was reduced in 605 patients post myocardial infarction compared to a typical diet (27). In another large epidemiological study of 4680 men and women (The INTERMAP trial), a significant inverse relationship between dietary ALA and SBP and DBP was observed (28).

Randomized controlled trials have concluded similar effects of ALA on BP. In a randomized, double blinded, controlled study, pre-hypertensive men and women (n=101), with an average SBP and DBP of 136 and 88 mm Hg, respectively, ingested 2.6 g of ALA/day for 12 weeks. After 12 weeks, the ALA group exhibited a significant reduction

in SBP and DBP of ~10 and 3 mm Hg, respectively. After a 4 week washout period, SBP and DBP increased by 4 and 3 mm Hg from the 12 week measurements (29). Therefore, individuals needed to continually consume a source of ALA in order to obtain the anti-hypertensive effect. In a trial of dyslipidemic patients, flaxseed oil containing 8 g of ALA was provided daily for 12 weeks. The flaxseed oil group exhibited a significant reduction in SBP, DBP and MAP of 10, 8, and 8 mm Hg, respectively, versus control (n6 oil) (30).

c) Flaxseed lignans

To ascertain whether lignans or ALA within flaxseed elicit anti-hypertensive action, three strains of flaxseed were compared in postmenopausal women with vascular disease using a randomized, Latin square, double-blind, three-way crossover design (31). Patients consumed 30 g/d of ground flaxseed either as high ALA, low lignan (Flanders), low ALA, high lignan (Linola 989) and moderate ALA and lignan (AC Linora). When each diet was compared to baseline or pre-treatment values, all three strains attenuated BP when individuals were given a stressful cognitive task. Peripheral resistance during stress was least affected when the low ALA, high lignan Linola 989 flaxseed strain was consumed (31).

In a randomized controlled trial involving adults >50 years of age participating in a walking program, normotensive subjects (14% had metabolic syndrome) were asked to consume 543 mg/d of SDG from flaxseed lignan for 6 months (32). When the data were stratified by gender, only males demonstrated a decrease in DBP when consuming a flax lignan diet compared to those in the control group. This significant group × sex × time interaction existed only for DBP and was not observed in females. When all individuals (no gender exclusions) with metabolic syndrome were sub-grouped, the flax lignan group

once again demonstrated a significant 7 mm Hg reduction in DBP over time compared to control.

Recently, a high SDG lignan supplement was assessed for its hypotensive effects in a healthy population >48 years of age (33). BeneFlax is a commercially available source of SDG lignan that provides 543 mg of SDG in a single tablet. Individuals consumed one BeneFlax tablet, daily for 6 months in this randomized, double blind, controlled trial. After study completion, there were no observable differences in either SBP or DBP between treatment groups. A limitation of this paper was that most of the participants were either healthy at baseline or had their BP controlled through medications (average baseline SBP was 127 ± 12 mm Hg and DBP was 81 ± 7 mm Hg).

d) Flaxseed fibre

Flaxseed is composed of 28% total fibre by weight. It is comprised of both soluble and insoluble fibre at a proportion of about 20:80 to 40:60 (34). The anti-hypertensive action of dietary fibre is well-established in several meta-analyses (35,36). In data from 24 trials published from 1966 – 2003, an average fibre dose of 11.5 g/d can decrease SBP by -1.13 mm Hg and DBP by -1.26 mm Hg (36). Based upon 8 clinical trials, 7.2 - 18.9 g/d of supplemented fibre, provides maximal reductions (-3.4 mm Hg in SBP and -1.97 mm Hg in DBP) (35). Fibre doses >19 g/d do not provide additional BP lowering. Soluble fibre produces most of the anti-hypertensive benefits, accounting for 85% of the fibre-associated decreases in SBP and 59% in DBP (36). In a randomized, controlled trial, hypertensive, type 2 diabetic patients consuming 5 g/d of flaxseed gum (soluble mucilage) for 12 weeks noted substantial, yet insignificant, decreases in SBP,

DBP and MAP (-16, -8 and -10 mm Hg) (37). Higher doses of soluble fibre may be required to elicit significant anti-hypertensive action.

Proposed Anti-Hypertensive Mechanism of Flaxseed

The underlying mechanisms for the anti-hypertensive properties of flaxseed are not entirely clear but the data above would suggest ALA may be responsible for most of the anti-hypertensive action of flaxseed. Circulating levels of ALA were significantly correlated with SBP and DBP (23). The mechanism for the anti-hypertensive action of ALA may involve its capacity to reduce the activity of soluble epoxide hydrolase (38). This enzyme currently is a target for anti-hypertensive treatment. Soluble epoxide hydrolase produces oxylipins that can cause a loss of vasodilation and promote inflammation. ALA reduced the activity of soluble epoxide hydrolase resulting in a reduction of the soluble epoxide hydrolase products, the dihydroxyoctadecenoic acids (DiHOMES) and dihydroxyeicostrienoic acids (DIHETREs). The DiHOMEs are associated with inflammation and cytotoxicity and the DiHETrEs are associated with a concomitant loss of vasodilation. As a result, a decrease in soluble epoxide hydrolase-derived oxylipins was associated with a significant reduction in SBP of about 8 mm Hg (38).

ALA may also exhibit its anti-hypertensive potential through an anti-inflammatory effect. In a randomized, controlled, crossover trial, 23 hypercholesterolemic patients were provided a high ALA (6.5% of energy), high linoleic acid (12.5% of energy), or a typical western diet for 6 week periods each. The high ALA diet significantly reduced peripheral blood mononuclear cell production of interleukin-6, interleukin-1, and tumor necrosis factor-alpha compared to the high linoleic acid diet (39). Ground flaxseed consumption also reduced pro-inflammatory oxylipins in the

plasma of older adults after 4 weeks (40). Essential hypertension has been hypothesized to be a result of inflammation and endothelial dysfunction which causes an imbalance between endothelial derived vasoconstrictive factors and vasodilative factors (41). If ALA has anti-inflammatory effects, it is possible that it may prevent the inflammation-induced imbalance of molecules that regulate vascular tone. ALA may also affect inflammation and BP through an alteration of the oxylipin profile, as discussed above.

Circulating levels of total enterolignans, and the enterolignan species END and ENL, following the ingestion of ground flaxseed, have been correlated with DBP (23). Enterodiol levels were inversely correlated with SBP (23). Although total enterolignan levels trended toward a significant inverse correlation with SBP, they were not statistically significant ($P = 0.06$) (23). The lignans within flaxseed may be anti-hypertensive through an anti-oxidative action. Reactive oxygen species have been suggested to play a role in hypertension (42). The antioxidant effects of lignans may not come from SDG itself but from its metabolites END and ENL. These behave as potent antioxidants in both *in vitro* (8,43,44) and *in vivo* (45) models. Furthermore, ENL can induce phase 2 protein activation which leads to decreased oxidative stress (46).

Flaxseed peptides may also induce an anti-hypertensive effect through their rich arginine content (22). Arginine is converted in the vascular endothelium to nitric oxide and citrulline. Nitric oxide produces vasodilation when released in the endothelium and thus represents a strategy for reducing hypertension (47). Another possible mechanism through which the cationic peptides may proceed is through the inhibition of angiotensin-converting enzyme and renin (22). The mechanism of action of dietary protein hydrolysates may be through inhibition of the renin-angiotensin system as has been

suggested in studies using *Lactobacillus helveticus* fermented milk (48). The mechanism may involve angiotensin-converting enzyme inhibition or angiotensin receptor blockage, thus preventing the production of the vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin.

Each of the components within flaxseed: ALA, lignans, fibre and peptides, all contribute towards BP reduction. The cumulative effects on BP may be a reflection of the contributions of each of these components. However, further work in tightly controlled trials over at least 6 months is necessary using appropriate concentrations of the bioactive isolates in hypertensive patients before firm conclusions can be reached. It is also relevant to note from animal work that dietary flaxseed can induce anti-arrhythmic (49), anti-atherosclerotic (50,51), anti-inflammatory (51), and anti-diabetic effects (52), plaque regression (53), and has cholesterol (54,55) and trans fat lowering capacity (50). Ultimately, any one of these actions may also contribute to the BP lowering effects of dietary flaxseed.

Implications for Hypertension Management

Dietary interventions have been recommended to be used in concert with current pharmacological treatments to reduce the burden of high BP (4-6). Dietary flaxseed may provide a suitable treatment option for patients. The strengths of flaxseed as an anti-hypertensive therapy include its ability to complement medication, accessibility, desirability, high nutritional content, capacity to simultaneously address other disease biomarkers and symptoms (i.e. hypercholesterolemia), its relative lack of side effects and its low cost.

Flaxseed as a treatment for hypertension may reduce the increasing financial burden that prescription medications put on governments and individuals. Nearly 80

million prescriptions for anti-hypertensive medications were given to patients in Canada alone (56). This cost over $3 billion in 2006 in Canada (56). This is more than double the expenditures on hypertension medications 10 years earlier (56). A greater emphasis needs to be placed on cost-effective strategies (56). The price of ground flaxseed for 1 month at 30 g/day would be ~$15 maximum based on current consumer prices in North America and Europe. By contrast, the cost of one prescription for anti-hypertensive medication averages $42-45 in Canada and the United States per month (56,57). Most patients must take more than one drug to control their BP which increases the expense further. For patients with resistant hypertension, flaxseed offers a potential solution when all other strategies have been inadequate (23).

Not only is flaxseed affordable, it is also highly accessible worldwide. It is grown in North America, Asia, Europe, Africa, Australia, Central America, and South America (Figure 3). Many of the countries that grow flaxseed are low to middle income (58). This is a critical point. In the *Global Brief on Hypertension* (2013, page 13), the World Health Organization stated, "*Nearly 80% of deaths due to cardiovascular disease occur in low and middle income countries. They are the countries that can least afford the social and economic consequences of ill health."* Many populations or communities in developing countries have limited financial resources or physical access to anti-hypertensive medications. These developing countries have the greatest need for hypertension management (1). As of 2011, flaxseed is grown in 54 countries (58) and according to the World Bank income classification (59), 50% of the flax growing countries shown in Figure 3 are low or middle income regions. Flaxseed, therefore, may prove to be a highly accessible and affordable hypertension treatment for many people worldwide.

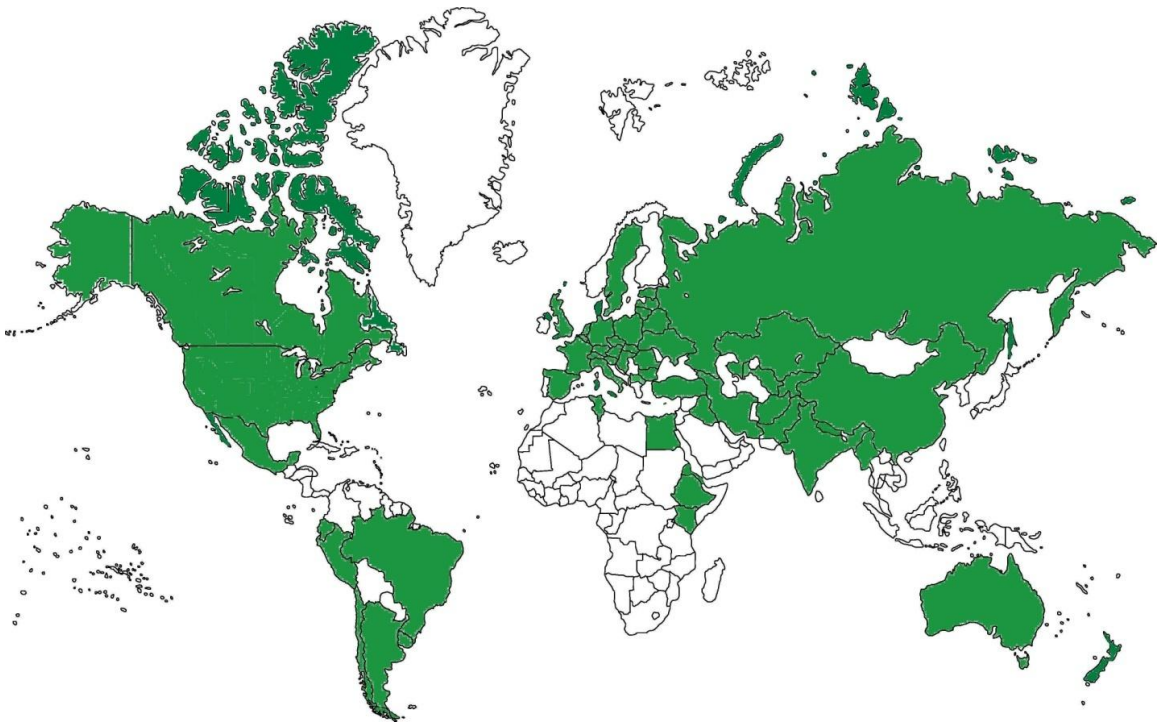


Figure 3: World Atlas of Countries Growing Flaxseed.

* Based on data from the Food and Agriculture Organization of United Nations Database (2011) (58)

The use of a dietary strategy like flaxseed to treat hypertension provides patients the ability to take their health into their own hands. This is desirable by the public. Most Americans (72%) believe that nutrition plays a very important role in overall health and 85% are interested in learning about foods with added health benefits (60). Public interest in functional foods such as flaxseed may motivate self-management of chronic disease. Self-management of chronic disease results in decreased hospitalizations, fewer nights in the hospital, and improved indications of health including: self-rated health, indicators of disability, energy, health distress, and social role/activity limitation (61).

Flaxseed is recognized as safe to ingest and has additional health-related benefits beyond its anti-hypertensive actions. Flaxseed has been granted GRAS (Generally Recognized As Safe) status by the Food and Drug Administration (62) and in Canada given a health claim for its cholesterol lowering capabilities (63). In addition, flaxseed has many nutrients including fibre, ALA, and antioxidants which most people do not consume in adequate quantities. For example, the daily mean intake of dietary fibre was estimated at 15.9 g/day in 2008 in the United States (64). This is well below the recommendation of the Institute of Medicine which is 25 g/day for women and 38 g/day for men (65). Thirty grams of flaxseed can provide up to 8 g of dietary fibre and can assist individuals in obtaining a healthy diet. In addition, the generation of oxygen derived free radicals and inflammation are becoming increasingly recognized as important factors in a variety of chronic diseases (66). The capacity of lignans to act as powerful antioxidants (43) and ALA to reduce inflammation (39,67) may be useful therapeutic mechanisms in other diseases or conditions besides hypertension.

Some patients may have difficulty with the side effects of medications. Flaxseed also has side-effects that can deter its ingestion for some people. Clinical trials of 30-45 g of flaxseed per day can result in mild gastrointestinal discomfort, flatulence, and bloating that disappears over time as the individual became accustomed to the high fibre load (68) (23). Patients can be gradually introduced to flaxseed in order to lessen the likelihood of these side effects (23). Approximately 20% of patients dropped out of the FLAX-PAD Trial when asked to ingest flaxseed daily for one year. The majority of those dropped out of the study within 6 months. However, the control group had a similar dropout rate indicating compliance was an issue primarily due to taste, limited food choice variety and maintaining dietary habits. This is an important obstacle to consider when flaxseed is being introduced into the diet. Flaxseed supplementation into a variety of foods available to the public will provide more dietary choices and improve adherence.

Conclusion

Due to the alarming situation of hypertension globally and the need for more desirable and effective strategies, research on new and inventive therapies is of the utmost importance. If flaxseed is proven to be effective, it has many advantages as an anti-hypertensive treatment option for patients. It provides an alternative or complementary strategy for patients who cannot control their BP with medication, for patients who cannot afford or do not have access to medication, or for patients who prefer a dietary approach. It has been observed that dietary flaxseed may be an excellent complementary strategy. In patients already taking anti-hypertensive medication, flaxseed provided additional BP lowering capabilities and decreased the percentage of patients with uncontrolled hypertension by 17% (23). The ability of flaxseed to independently replace medication will be explored in a trial currently underway (25). Even if some

individuals do not exhibit a decrease in BP with flaxseed, the addition to the diet is positive as it contains many nutrients that most individuals do not consume in adequate quantities. Flaxseed may help individuals gain control of their BP and, therefore, may decrease the risk of hypertension associated morbidity and mortality. Further research on specific parameters of this intriguing new approach is clearly required but current evidence is strongly in support of its role in controlling hypertension.

Acknowledgements

The work was supported through grants to GNP from the Canadian Institutes for Health Research (CIHR), the Agri-food Research and Development Initiative and SaskFlax. Indirect research support was obtained from St Boniface Hospital Foundation. SPBC was a recipient of the Patient Oriented Research Doctoral Award from CIHR. ALE was a recipient of a Doctoral Research Scholarship from the Heart and Stroke Foundation of Canada.

Chapter I Section V References

1. World Health Organization. A global brief on Hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization; 2013.
2. Mazzaglia G, Ambrosioni E, Alacqua M, Filippi A, Sessa E, Immordino V, et al. Adherence to antihypertensive medications and cardiovascular morbidity among newly diagnosed hypertensive patients. Circulation 2009 Oct 20;120(16):1598-1605.
3. Wolf-Maier K, Cooper RS, Kramer H, Banegas JR, Giampaoli S, Joffres MR, et al. Hypertension treatment and control in five European countries, Canada, and the United States. Hypertension 2004 Jan;43(1):10-17.
4. Hypertension Canada. The 2013 CHEP Recommendations: 2013. http://www.hypertension.ca/chep-recommendations Accessed May 1 2014.
5. 2013 ESH/ESC Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). 2013. http://www.esh2013.org/wordpress/wp-content/uploads/2013/06/ESC-ESH-Guidelines-2013.pdf Accessed May 1 2014
6. James PA, Oparil S, Carter BL, Cushman WC, Dennison-Himmelfarb C, Handler J, et al. 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). JAMA 2014 Feb 5;311(5):507-520.

7. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. N-3 Fatty Acid Dietary Recommendations and Food Sources to Achieve Essentiality and Cardiovascular Benefits. Am J Clin Nutr 2006 Jun;83(6 Suppl):1526S-1535S.
8. Prasad K. Antioxidant Activity of Secoisolariciresinol Diglucoside-derived Metabolites, Secoisolariciresinol, Enterodiol, and Enterolactone. Int J Angiol 2000 Oct;9(4):220-225.
9. Prasad K. Flax lignan complex slows down the progression of atherosclerosis in hyperlipidemic rabbits. J Cardiovasc Pharmacol Ther 2009 Mar;14(1):38-48.
10. Sicilia T, Niemeyer HB, Honig DM, Metzler M. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. J Agric Food Chem 2003 Feb 26;51(5):1181-8.
11. Carreau C, Flouriot G, Bennetau-Pelissero C, Potier M. Enterodiol and enterolactone, two major diet-derived polyphenol metabolites have different impact on ERalpha transcriptional activation in human breast cancer cells. J steroid biochem molec biol 2008 May;110(1-2):176-85.
12. Bassett CM, Rodriguez-Leyva D, Pierce GN. Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. Appl Physiol Nutr Metab 2009 Oct;34(5):965-974.
13. Austria JA, Richard MN, Chahine MN, Edel AL, Malcolmson LJ, Dupasquier CM, et al. Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. J Am Coll Nutr 2008 Apr;27(2):214-221.

14. Kuijsten A, Arts IC, van't Veer P, Hollman PC. The relative bioavailability of enterolignans in humans is enhanced by milling and crushing of flaxseed. J Nutr 2005 Dec;135(12):2812-6.
15. Talom RT, Judd SA, McIntosh DD, McNeill JR. High flaxseed (linseed) diet restores endothelial function in the mesenteric arterial bed of spontaneously hypertensive rats. Life Sci 1999;64(16):1415-1425.
16. Sekine S, Sasanuki S, Aoyama T, Takeuchi H. Lowering Systolic Blood Pressure and Increases in Vasodilator Levels in SHR with Oral alpha-Linolenic Acid Administration. J of Oleo Sci 2007;56(7):341-345.
17. Park JB, Velasquez MT. Potential effects of lignan-enriched flaxseed powder on bodyweight, visceral fat, lipid profile, and blood pressure in rats. Fitoterapia 2012 Jul;83(5):941-946.
18. Prasad K. Secoisolariciresinol Diglucoside (SDG) Isolated from Flaxseed, an Alternative to ACE Inhibitors in the Treatment of Hypertension. Int J Angiol 2013 Dec;22(4):235-8.
19. Prasad K. Antihypertensive activity of secoisolariciresinol diglucoside (SDG) isolated from flaxseed: role of guanylate cyclase. Int J Angiol 2004;13:7-14.
20. Setchell KDR, Brown NM, Zimmer-Nechemias L, Wolfe B, Jha P, Heubi JE. Metabolism of secoisolariciresinol-diglycoside the dietary precursor to the intestinally derived lignan enterolactone in humans. Food & Function 2014;5(3):491-501.
21. Doyen A, Udenigwe CC, Mitchell PL, Marette A, Aluko RE, Bazinet L. Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate

fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. Food Chem 2014 145:66-76.

22. Udenigwe CC, Adebiyi AP, Doyen A, Li H, Bazinet L, Aluko RE. Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. Food Chem 2012 132:468-475.
23. Rodriguez-Leyva D, Weighell W, Edel AL, Lavallee R, Dibrov E, Pinneker R, et al. Potent Antihypertensive Action of Dietary Flaxseed in Hypertensive Patients. Hypertension 2013 Oct 14;62:1081-1089.
24. Law MR, Wald NJ, Morris JK, Jordan RE. Value of low dose combination treatment with blood pressure lowering drugs: analysis of 354 randomised trials. BMJ 2003 Jun 28;326(7404):1427.
25. Caligiuri SP, Penner B, Pierce GN. The HYPERFlax trial for determining the anti-HYPERtensive effects of dietary flaxseed in newly diagnosed stage 1 hypertensive patients: study protocol for a randomized, double-blinded, controlled clinical trial. Trials 2014 Jun 18;15(1):232-6215-15-232.
26. Berry EM, Hirsch J. Does dietary linolenic acid influence blood pressure? Am J Clin Nutr 1986 Sep;44(3):336-340.
27. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet 1994 Jun 11;343(8911):1454-1459.

28. Ueshima H, Stamler J, Elliott P, Chan Q, Brown IJ, Carnethon MR, et al. Food omega-3 fatty acid intake of individuals (total, linolenic acid, long-chain) and their blood pressure: INTERMAP study. Hypertension 2007 Aug;50(2):313-319.
29. Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, et al. Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. J Oleo Sci 2007;56(7):347-360.
30. Paschos GK, Magkos F, Panagiotakos DB, Votteas V, Zampelas A. Dietary supplementation with flaxseed oil lowers blood pressure in dyslipidaemic patients. Eur J Clin Nutr 2007 Oct;61(10):1201-1206.
31. Spence JD, Thornton T, Muir AD, Westcott ND. The Effect of Flax Seed Cultivars with Differing Content of α-Linolenic Acid and Lignans on Responses to Mental Stress. J Amer Coll of Nutr December 1 2003;22(6):494-501.
32. Cornish SM, Chilibeck PD, Paus-Jennsen L, Biem HJ, Khozani T, Senanayake V, et al. A randomized controlled trial of the effects of flaxseed lignan complex on metabolic syndrome composite score and bone mineral in older adults. Appl Physiol Nutr Metab 2009 Apr;34(2):89-98.
33. Billinsky J, Glew RA, Cornish SM, Whiting SJ, Thorpe LU, Alcorn J, et al. No evidence of hypoglycemia or hypotension in older adults during 6 months of flax lignan supplementation in a randomized controlled trial: A safety evaluation. Pharmaceutical Biology 2013;51(6):778-782.

34. Mazza G, Oomah B. Flaxseed, dietary fiber and cyanogens. In: Cunnane S, Thompson L, editors. Flaxseed in human nutrition. Champaign, IL: AOCS Press; 1995. p. 56-81.

35. Whelton SP, Hyre AD, Pedersen B, Yi Y, Whelton PK, He J. Effect of dietary fiber intake on blood pressure: a meta-analysis of randomized, controlled clinical trials. J Hypertens 2005 Mar;23(3):475-481.

36. Streppel MT, Arends LR, van 't Veer P, Grobbee DE, Geleijnse JM. Dietary fiber and blood pressure: A meta-analysis of randomized placebo-controlled trials. Arch Intern Med 2005;165(2):150-156.

37. Thakur G, Mitra A, Pal K, Rousseau D. Effect of flaxseed gum on reduction of blood glucose and cholesterol in type 2 diabetic patients. Inter j food sci and nutr 2009;60 Suppl 6:126-36.

38. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, Pierce GN. Flaxseed Consumption Reduces Blood Pressure in Patients With Hypertension by Altering Circulating Oxylipins via an alpha-Linolenic Acid-Induced Inhibition of Soluble Epoxide Hydrolase. Hypertension 2014;64(1):53-59.

39. Zhao G, Etherton TD, Martin KR, Gillies PJ, West SG, Kris-Etherton PM. Dietary alpha-linolenic acid inhibits proinflammatory cytokine production by peripheral blood mononuclear cells in hypercholesterolemic subjects. Am J Clin Nutr 2007 Feb;85(2):385-391.

40. Caligiuri SP, Aukema HM, Ravandi A, Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. Exp Gerontol 2014 Apr 18.

41. Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. Acta Cardiol 2000 Aug;55(4):221-232.

42. Montezano AC, Touyz RM. Molecular Mechanisms of Hypertension—Reactive Oxygen Species and Antioxidants: A Basic Science Update for the Clinician. Can J Cardiol 2012;28(3):288-295.

43. Hu C, Yuan YV, Kitts DD. Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro. Food Chem Toxicol 2007 Nov;45(11):2219-2227.

44. Kitts DD, Yuan YV, Wijewickreme AN, Thompson LU. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Mol Cell Biochem 1999 Dec;202(1-2):91-100.

45. Vanharanta M, Voutilainen S, Nurmi T, Kaikkonen J, Roberts LJ, Morrow JD, et al. Association between low serum enterolactone and increased plasma F2-isoprostanes, a measure of lipid peroxidation. Atherosclerosis 2002 Feb;160(2):465-9.

46. Wang W, Liu LQ, Higuchi CM, Chen H. Induction of NADPH:Quinone Reductase by Dietary Phytoestrogens in Colonic Colo205 Cells. Biochem Pharmacol 1998;56(2):189-195.

47. Marin E, Sessa WC. Role of endothelial-derived nitric oxide in hypertension and renal disease. Curr opin in nephrol and hypertension 2007 Mar;16(2):105-10.

48. Seppo L, Jauhiainen T, Poussa T, Korpela R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. AJCN 2003 Feb;77(2):326-30.
49. Ander BP, Weber AR, Rampersad PP, Gilchrist JS, Pierce GN, Lukas A. Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic Rabbits. J Nutr 2004 Dec;134(12):3250-3256.
50. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. Am J Physiol Heart Circ Physiol 2011 Dec;301(6):H2220-6.
51. Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, et al. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am J Physiol Heart Circ Physiol 2007 Oct;293(4):H2394-402.
52. Abuelgassim A. Effect of flax seeds and date palm leaves extracts on serum concentrations of glucose and lipids in alloxan diabetic rats. Pak J Biol Sci 2010;13(23):1141-45.
53. Francis AA, Deniset JF, Austria JA M, Lavallee RK, Maddaford GG, Hedley TE, et al. The Effects of Dietary Flaxseed on Atherosclerotic Plaque Regression. Am J Physiol Heart Circ Physiol 2013;304(12):H1743-51.
54. Prim CR, Baroncini LA, Precoma LB, Caron PH, Winter G, Poletti MO, et al. Effects of linseed consumption for a short period of time on lipid profile and

atherosclerotic lesions in rabbits fed a hypercholesterolaemic diet. Brit J Nutr 2012 Mar;107(5):660-4.

55. Haliga R, Mocanu V, Oboroceanu T, Stitt PA, Luca VC. The effects of dietary flaxseed supplementation on lipid metabolism in streptozotocin-induced diabetic hamsters. Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi 2007 Apr-Jun;111(2):472-6.
56. Jackevicius CA, Cox JL, Carreon D, Tu JV, Rinfret S, So D, et al. Long-term trends in use of and expenditures for cardiovascular medications in Canada. CMAJ 2009 Jul 7;181(1-2):E19-28.
57. United States Food and Drug Administration. Drugs: Savings From Generic Drugs Purchased at Retail Pharmacies. 2009. http://www.fda.gov/drugs/resourcesforyou/ucm134205.htm Accessed July 22 2014
58. Food and Agriculture Organization of the United Nations. Linseed. 2014. http://faostat3.fao.org/faostat-gateway/go/to/search/flaxseed/E Accessed June 1 2014.
59. The World Bank Group. The World Bank: Income Levels. 2013. https://wdronline.worldbank.org/worldbank/a/incomelevel Accessed June 4 2014
60. International Food Information Council. Functional Foods Consumer Survey. 2013. http://www.foodinsight.org/2013_Functional_Foods_Consumer_Survey Accessed June 10 2014.
61. Lorig KR, Sobel DS, Stewart AL, Brown BW,Jr, Bandura A, Ritter P, et al. Evidence suggesting that a chronic disease self-management program can

improve health status while reducing hospitalization: a randomized trial. Med Care 1999 Jan;37(1):5-14.

62. Food and Drug Act. GRAS Notice 000280: Whole and Milled Flaxseed. 2009; Available at: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000280.pdf.
63. Health Canada. Summary of Health Canada's Assessment of a Health Claim about Ground Whole Flaxseed and Blood Cholesterol Lowering. 2014. http://www.hc-sc.gc.ca.proxy2.lib.umanitoba.ca/fn-an/label-etiquet/claims-reclam/assess-evalu/flaxseed-graines-de-lin-eng.php Accessed May 30 2014.
64. King DE, Mainous AG,3rd, Lambourne CA. Trends in dietary fiber intake in the United States, 1999-2008. J Acad Nutr Diet 2012 May;112(5):642-648.
65. Institute of Medicine. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. 2002. http://www.iom.edu/Reports/2002/Dietary-Reference-Intakes-for-Energy-Carbohydrate-Fiber-Fat-Fatty-Acids-Cholesterol-Protein-and-Amino-Acids.aspx Accessed May 12 2014.
66. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39(1):44-84.
67. Rallidis LS, Paschos G, Liakos GK, Velissaridou AH, Anastasiadis G, Zampelas A. Dietary alpha-linolenic acid decreases C-reactive protein, serum amyloid A and interleukin-6 in dyslipidaemic patients. Atherosclerosis 2003 Apr;167(2):237-242.

68. Bloedon LT, Balikai S, Chittams J, Cunnane SC, Berlin JA, Rader DJ, et al. Flaxseed and cardiovascular risk factors: results from a double blind, randomized, controlled clinical trial. J Am Coll Nutr 2008 Feb;27(1):65-74.

vi. Oxylipins

Oxylipins as Therapeutic Targets in Cardiovascular Disease

Stephanie P.B. Caligiuri

Abstract

Polyunsaturated fatty acids through oxygenation give rise to messenger molecules called the oxylipins. Oxylipins have the ability to regulate important physiological and pathophysiological processes such as immunity, vascular tone, inflammation, and coagulation. These processes are all essential in cardiovascular disease progression and onset. As a result, the relationship of these molecules to cardiovascular disease is likely to provide insight into potential therapeutic targets. In addition, concentrations of endogenous oxylipins are influenced by many factors including disease state and diet. Therefore, potential for diet to influence the concentration of oxylipins in different disease states may exist. This review highlights different roles of oxylipins in cardiovascular disease and several factors that influence endogenous oxylipin concentrations.

Introduction

Oxylipins are an intriguing area of research for hypertension and cardiovascular disease due to their role in innate immunity, inflammation, and vascular tone regulation. Oxylipins are signaling molecules that are endogenously produced from polyunsaturated fatty acids. As a result, interventions involving polyunsaturated fatty acids (ie: flaxseed) may prove to be an effective strategy in altering concentrations of deleterious or beneficial oxylipins. In addition, the majority of oxylipins are enzymatically produced. As a result, this gives rise to many targets for therapeutic development. Oxylipins can be beneficial, detrimental, or play both roles depending upon which receptor they bind. This chapter will highlight the physiological and pathophysiological role of oxylipins in cardiovascular disease as well as how concentrations of oxylipins can be altered. Characteristics of select oxylipins are indicated in Table 6.

Table 6: Select Oxylipins and Select Physiological/Pathophysiological Effects

Oxylipin	Oxylipin full name	Physiological/Pathophysiological Effect
Linoleic Acid		
9-HODE 13-HODE	9-hydroxyoctadecadienoic acid 13-hydroxyoctadecadienoic acid	Produced via the lipoxygenase pathway. HODEs are present in human monocyte-oxidized LDL (1) HODEs activate peroxisome proliferator –activated receptor γ (2) HODEs are transient receptor potential vanilloid-1 (TRPV1) channel agonists that can result in induction of pain and nociception during heat exposure in the skin of rodents (3) HODEs are associated with oxidative stress (4)
9,10-DiHOME 12,13-DiHOME	9,10-dihydroxydecenoic acid 12,13-dihydroxydecenoic acid	The DiHOMEs (leukotoxin diols) are produced by the metabolism of the EpOMEs (leukotoxins) by soluble epoxide hydrolase. The DiHOMEs are associated with cytotoxicty and oxidative stress (5, 6) DiHOMEs have induced cytotoxicity in proximal renal tubular cells (7), induced mitochondrial dysfunction (8), acute respiratory distress (9), and have been associated with inflammation in inflammatory bowel disease (10).
13-oxoODE	13-oxooctadecadienoic acid	Product of 13-HODE (11), weakly associated with hepatic fibrosis (12), is a TRPV1 agonist that can result in induction of pain and nociception during heat exposure in the skin of rodents(3)
9,10,13-TriHOME 9,12,13-TriHOME	9,10,13-trihydroxyoctadecenoic acid 9,12,13-trihydroxyoctadecenoic acid	Elevated concentrations of the TriHOMEs are present in the bronchoalveolar lumen in patients with asthma compared to normal healthy individuals and concentrations further increase after birch pollen provocation(13)

Dihomo Gamma Linolenic Acid		
15-HETrE	15-hydroxyeicosatrienoic acid	Produced via 15-lipoxygenase and can inhibit 5-lipoxgenase in human polymorphonuclear leukocytes (14)
Alpha-Linolenic Acid		
9-HOTrE 13-HOTrE	9-hydroxyoctadecatrienoic acid 13-hydroxyoctadecatrienoic acid	Produced via the lipoxygenase pathway. Concentration of renal HOTrEs increased with dietary alpha-linolenic acid and were inversely associated with mean glomerular volume in a model of diet-induced obesity (15)
Arachidonic Acid		
5,6-DiHETrE 8,9-DiHETrE 11,12-DiHETrE 14,15-DiHETrE	5,6-dihydroxyeicosatrienoic acid 8,9-dihydroxyeicosatrienoic acid 11,12-dihydroxyeicosatrienoic acid 14,15-dihydroxyeicosatrienoic acid	The DiHETrEs are produced by soluble epoxide hydrolase from the epoxyeicosatrienoic acids (EETs). The conversion of the EETs to the DiHETrEs causes a concomitant loss of vasodilation (16, 17)
5-HETE 8-HETE 11-HETE 12-HETE 15-HETE 16-HETE	5-hydroxyeicosatetraenoic acid 8-hydroxyeicosatetraenoic acid 11-hydroxyeicosatetraenoic acid 12-hydroxyeicosatetraenoic acid 15-hydroxyeicosatetraenoic acid 16-hydroxyeicosatetraenoic acid	HETEs are produced via the lipoxygenase and epoxygenase pathways. HETEs are chemotactic for neutrophils(18, 19). 16-HETE is produced by polymorphonuclear leukocytes *in vitro* (20) and is released upon angiotensin II stimulation. 16-HETE when administered intravenously lowered intracranial pressure following a thromboemoblic stroke in New Zealand white rabbits(21).

18-HETE	18-hydroxyeicosatetraenoic acid	
PGD_2	Prostaglandin D_2	PGD_2 is produced via the cyclooxygenase pathway and can inhibit platelet aggregation; however at higher concentrations can be pro-aggregatory. PGD_2 can increase cyclic adenosine monophosphate levels. Prostaglandin D_2 either has no effect on vascular tone or has can constrict and dilate mesenteric arteries in dogs (22). PGD_2 is implicated in the innate immune response (23). However, an analog of PGD_2 inhibited polymorphonuclear neutrophil recruitment *in vitro* (24).
PGE_2	Prostaglandin E_2	PGE_2 is produced via the cyclooxygenase pathway and can induce renal vascular resistance in rats (25). PGE_2 can either be pro-inflammatory and vasoconstrictive or anti-inflammatory and vasodilatative depending upon which receptor it is bound, EP_{1-4} (26).
$6ketoPGF_{1\alpha}$	6keto prostaglandin $F_{1\alpha}$	6ketoPGF1α is produced via the cyclooxygenase pathway and is the stable product and therefore marker of prostacyclin. Prostacyclin is an endothelial-derived vasodilatory and is anti-aggregatory (27, 28). Can reduce pulmonary vascular resistance in humans (29).
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$ is produced via the cyclooxygenase pathway and induces vasoconstriction in bovine, canine, and human coronary arteries (30)
TXB_2	Thromboxane B_2	TXB_2 is produced via the cyclooxygenase pathway and is the stable product of of the short-lived TXA_2. Therefore, TXB_2 is considered the marker for TXA_2. TXA_2 is an endothelial derived eicosanoid that potently induces vasoconstriction and aggregation of platelets (27).
Eicosapentanoic Acid		
5-HEPE	5-hydroxyeicosapentanoic acid	The HEPEs are produced via the lipoxygenase and cyclooxygenase pathways. The HEPEs can be produced by neutrophils and have 10% the potency on

12-HEPE 18-HEPE	12-hydroxyeicosapentanoic acid 18-hydroxyeicosapentanoic acid	neutrophil recruitment as 5-HETE (31)18-HEPE is the precursor to the E-series resolvins which can reduce neutrophil migration and inflammatory responses (32).
Docosahexanoic Acid		
4-HDOHE 7-HDOHE 10-HDOHE 11-HDOHE 13-HDOHE 14-HDOHE 16-HDOHE 17-HDOHE 20-HDOHE	4-hydroxydocosahexanoic acid 7-hydroxydocosahexanoic acid 10-hydroxydocosahexanoic acid 11-hydroxydocosahexanoic acid 13-hydroxydocosahexanoic acid 14-hydroxydocosahexanoic acid 16-hydroxydocosahexanoic acid 17-hydroxydocosahexanoic acid 20-hydroxydocosahexanoic acid	Docosahexanoic acid, 14-HDOHE, and 17-HDOHE are precursors to the maresins which are inflammation resolving mediators. The HDOHEs can also be produced via auto-oxidation *in vitro* and therefore have been implicated as potential markers of oxidative stress (33).
19,20-DiHDPA	19,20-dihydroxydocosapentanoic acid	19,20-DiDHPA is produced from docosahexanoic acid likely via the epoxygenase pathway. 19,20-DiHDPA has been detected in seminal vesicles of monkeys (34) and liver microsomes from rats (35).

Nomenclature

The naming of oxylipins is complex but provides information on the structure of the molecule. Information regarding the fatty acid substrate, number of double bonds, and location of the hydroxyl groups are identified within the name. For example, 9-hydroxyoctadecadienoic acid (HODE) indicates that 9-HODE has a hydroxyl group present at the 9^{th} carbon from the carboxylic acid end, 9-HODE originated from an 18 carbon fatty acid (octadeca), and it has two double bonds (dienoic). As another example, 5,6-dihydroxyeicosatrienoic acid (DiHETrE) indicates the presence of two hydroxyl groups, one at the 5^{th} and one at the 6^{th} carbon. DiHETrE indicates it is derived from a 20 carbon fatty acid (eicosa) and has 3 double bonds (trienoic). The chemical structure is also determined by the enzyme through which it was produced.

Enzymes in the Production of Oxylipins

Cyclooxygenase (COX) is one of the three primary enzymes involved in the production of oxylipins from polyunsaturated fatty acids. COX is a dioxygenase enzyme, thus donating two oxygen molecules to its substrate. The substrates for COX are primarily arachidonic acid and eicosapentanoic acid. COX largely produces a family of oxylipins called the prostaglandins (36). Prostaglandins are signaling molecules produced from arachidonic acid. Prostaglandins are produced via two different isomers of COX, COX-1 and COX-2. COX-1 is constitutively expressed. COX-2 is induced by inflammation in order to produce oxylipins that can propagate the inflammatory response (36). Inhibitors of COX such as acetaminophen, ibuprofen, and acetylsalicylic acid reduce the concentration of COX products such as prostaglandins. COX inhibitors are used to reduce fever, pain, or coagulation (36).

Lipoxygenase (LOX) is similar to COX as it donates two oxygen molecules to its substrate in order to convert polyunsaturated fatty acids into oxylipins. There are three known human isomers of lipoxygenase: 5-LOX, 12-LOX, and 15-LOX. Lipoxygenase primarily produces leukotrienes and hydroxyeicosatetraenoic acids (HETEs) from arachidonic acid. Leukotrienes can cause bronchoconstriction, increase blood vessel permeability and infiltration of neutrophils (36, 37). Inhibitors of 5-lipoxgenase have been developed in order to reduce inflammatory conditions such as asthma. Zileuton is an example of a 5-LOX inhibitor (38).

Cytochrome P450 (CYP450)/Epoxygenase donates oxygen to polyunsaturated fatty acids to create the epoxyeicosatrienoic acids (EETs) and the HETEs. There are many isoforms of CYP450, including CYP1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 20, 21, 24, and 26 (39). CYP450 not only produces oxylipins but is also a key enzyme in drug metabolism. Because many drugs interact with CYP450, many compounds are known to either increase or decrease the activity of this enzyme (39). For example, St John's Wort is a potent inducer of CYP3A thereby increasing its activity and resulting in an increased breakdown of drugs such as midazolem (40). In contrast, grapefruit juice can inhibit the activity of CYP3A thereby decreasing the breakdown of drugs (41). The impact of CYP inhibitors or activators on plasma oxylipins is not well characterized.

In contrast, a well characterized enzyme and pharmacological target is soluble epoxide hydrolase (sEH). SEH is responsible for metabolizing the epoxygenase products EETs to the DiHETrEs. When sEH adds a H_2O molecule to an EET to create the diol DiHETrE, a significant loss of vasodilation occurs. The half-life of EETs is in the milliseconds, so they are quickly metabolized to the DiHETrEs by sEH (42, 43). Many of

the enzymes responsible for oxylipin metabolism are present in the endoplasmic reticulum. However, soluble epoxide hydrolase is present in the cytoplasm of cells. sEH is ubiquitously expressed, and in particularly high concentrations in the liver, kidney and blood vessels (44, 45). The latest pharmaceutical inhibitors of sEH are derived from urea and amides that are active site transition state mimetics. Inhibition of sEH is done with the purpose of increasing the EETs and decreasing their de-activation into the DiHETrEs (42, 43).

Many other intermediate enzymes are present in oxylipin metabolism. For example, thromboxane synthase is responsible for converting prostaglandin H_2 to thromboxane A_2. Ozagrel is a pharmaceutical thromboxane synthase inhibitor that improves neurological function after an acute ischemic stroke in humans (46). However, the use of ozagrel is not supported by the Food and Drug Act as its clinical safety and efficacy is still being investigated (47). Another enzyme of interest in oxylipin metabolism is prostacyclin synthase. Prostacyclin synthase converts PGH_2 to prostaglandin I_2 which is further metabolized to prostacyclin, a vasodilator (36). An experimental agonist of a prostacyclin agonist and a thromboxane synthase inhibitor (ONO-1301) was tested in an animal model of hypertension and it resulted in a decrease in blood pressure (48).

Many enzymes involved in oxylipin metabolism have been targeted in order to reduce inflammation, platelet aggregation and vasoconstriction. The potential to investigate the pathophysiological role of these highly bioactive signaling molecules holds a great amount of potential in order to discover novel therapeutic targets and to maintain homeostasis and cardiovascular health.

Relationship of Oxylipins to Hypertension and Cardiovascular Disease

Oxylipins, and more specifically the eicosanoids produced from arachidonic acid, are implicated in atherosclerosis, platelet aggregation and vascular constriction. Because of this, the enzymes mentioned above have been targeted in order to reduce the risk of cardiovascular disease. Therefore, eicosanoids have been and continue to be targets for the treatment and prevention of heart disease.

A well researched inhibitor of eicosanoids is the cyclooxygenase inhibitor, acetylsalicylic acid (ie: aspirin). The association of cyclooxygenase products with cardiovascular disease has been well documented. For example, patients with stable angina pectoris exhibit significantly higher concentrations of circulating TXB_2 versus normal subjects. Those with variant angina exhibit markedly elevated levels of TXB_2 during an episode of angina (49). In addition, patients with genetic variations for oxylipin producing enzymes such as thromboxane synthase are at a higher risk for developing a myocardial infarction or ischemic stroke (50). Aspirin has been implicated in lower cardiovascular disease risk because it can decrease the concentration of pro-aggregatory and vasoconstrictive oxylipins including TXA_2 and $PGF_{2\alpha}$ (51-53).

In two landmark trials, aspirin significantly reduced the incidence of myocardial infarction and cardiovascular events (54). The Physician's Health Study and the British Doctor's Trial both observed an average 32% reduction in the incidence of a first myocardial infarction and a 15% reduction in vascular events with aspirin (54). However, in another landmark trial including nearly 40,000 women, low dose aspirin (100 mg), provided every second day for 10 years did not significantly lower the risk of

cardiovascular events in women aged > 45 years. However, it did significantly reduce the risk of cardiovascular events in women aged >65 years (55). It is important to note that selective COX-2 inhibitors such as rofecoxib have resulted in a greater risk of developing cardiovascular events compared to naproxen. This is likely due to COX-2 inhibition reducing the production of the protective anti-aggregatory prostacyclin (56). These trials provide strong support for the concept of an important involvement of oxylipins in cardiovascular disease.

Another group of eicosanoids that have been highly investigated for their impact on cardiovascular disease are the leukotrienes. Leukotrienes have been implicated in the progression of atherosclerosis due to their role in inflammation. The leukotrienes are produced via lipoxygenase from arachidonic acid. Leukotrienes are thought to have a role in endothelial dysfunction, intimal hyperplasia and cytokine release (57). Inhibition of leukotrienes by competitive exclusion to their respective receptors reduced infarct size, intimal hyperplasia and atherosclerosis in experimental models (57).

Like the leukotrienes, the hydroxyeicosatetraenoic acids (HETEs) have been implicated in oxidative stress and vasoconstriction. For example, the production of 20-HETE is stimulated by angiotensin II and endothelin-1 in vascular smooth muscle cells (58). Inhibiting the formation of 20-HETE reduces the impact of angiotensin II and endothelin-1 on the vasculature (58). In rats, 20-HETE increases tubuloglomerular feedback, vasoconstriction of preglomerular vasculature, and can increase total peripheral resistance in experimental models (58).

Although oxylipins are largely implicated in cardiovascular disease progression, they may also have a protective role against inflammation and cardiovascular disease.

Oxylipins produced from the long chain n3 fatty acids compete for production with oxylipins derived from arachidonic acid. Oxylipins produced from n3 fatty acids tend to be less inflammatory. The maresins and resolvins in particular possess inflammatory resolving characteristics by preventing neutrophil transmigration in murine models of inflammation (23, 32, 59). In relation to cardiovascular disease, the resolvins produced from eicosapentanoic acid and docosahexanoic acid can increase the production of nitric oxide and prostacyclin, and can decrease the production of adhesion molecules and reactive oxygen species in endothelial cells (60).

The CYP450 derived epoxyeicosatrienoic acids (EETs) are a rapidly developing area of research due to their impact on vasodilation. EETs are characterized as endothelial derived hyperpolarizing factors. EETs increase the open-state probability of Ca^{2+}-activated K^{+} channels in coronary smooth muscle cells (61). EETs accomplish this by activating the TRPV4 channel to import calcium which activates the ryanodine receptor to induce a calcium spark. The calcium sparks activate the calcium-activated potassium channel to induce hyperpolarization and relaxation of the vasculature (36). The EETs are quickly metabolized by soluble epoxide hydrolase to the DiHETrEs in which a concomitant loss of vasodilation occurs. Maintaining EET concentrations by reducing the production of DiHETrEs through pharmacological sEH inhibition (62) induces a reduction in blood pressure in models of hypertension (62-64), reduces vascular smooth muscle cell proliferation (65), and inhibits inflammatory pain processes (66).

Because oxylipins possess many beneficial or deleterious effects, the ability to alter their concentration may result in significant physiological and pathophysiological effects. As a result, any factors that modulate oxylipin concentrations are important to

consider. These factors can provide information on patients at high risk for cardiovascular disease or means by which we can alter oxylipins in a beneficial manner.

Many limitations still exist within the breadth of knowledge of oxylipins and cardiovascular disease. The novel octadecanoids and docosanoids produced from 18 and 22 carbon fatty acids, respectively, have little known about them. The impact of these oxylipins on risk for cardiovascular disease is unknown.

Factors that Modulate Oxylipin Concentrations

Many lifestyle factors including smoking, diet, body weight, and the presence of a disease can influence the concentration of oxylipins. The inflammatory response associated with smoking, for example, may be mediated in part by oxylipins. Concentrations of the proinflammatory and vasconstrictive oxylipins $PGF_{2\alpha}$ and thromboxane B_2 were in significantly higher concentrations in the bronchoalveolar lavage of smokers vs. non-smokers (67).

Patients with coronary artery disease exhibited significantly higher concentrations of the vasodilatitive EETs but had no observed differences in 20-HETE versus healthy subjects (68). Within this study, significantly lower plasma EET levels and higher plasma DiHETrE levels were observed in obese versus normal weight subjects. Age, diabetes, or cigarette smoking did not significantly impact plasma oxylipin concentrations in this study (68).

Individuals with inflammatory conditions such as rheumatoid arthritis and asthma tend to have much higher concentrations of plasma pro-inflammatory oxylipins such as 5-HETE and the TriHOMEs which are elevated up to 5,000 fold (69). However, in contrast to most documented findings, a recent analysis observed that of 60 oxylipins quantified,

the concentration of oxylipins were not different between patients having low or high inflammatory statuses (as indicated by C-reactive protein). Within the study, plasma oxylipins were assessed before and after weight reduction. There was no impact of weight reduction of plasma oxylipins (70).

In a trial comparing those with diabetes versus those without, significantly higher concentrations of total oxylipins and specifically higher concentrations of 13-HODE, the EETs, and the DiHETrEs, were observed. Dyslipidemia does not appear to have a significant impact on circulating oxylipin concentrations. However, it was noted that erthyrocyte polyunsaturated fatty acid concentrations were strongly correlated to the concentration of its oxylipin product (71).

Because polyunsaturated fatty acids are the precursors to oxylipins, dietary intake of polyunsaturated fatty acids may influence endogenous oxylipin concentrations. Intake of fatty acids can have an acute effect on circulating oxylipin levels as demonstrated by 2 and 4 hour blood collection following consumption of a high fat milkshake in healthy adults. Oxylipins derived from n3 fatty acids increased in the circulation 2 and 4 hour post consumption of an n3 fatty acid enriched milkshake (72). Dietary intake of differing concentrations of n3 and n6 fatty acids can influence renal oxylipin concentrations in an animal model of obesity (73). Therefore, there is great potential through dietary intervention to alter the endogenous lipidomics profile and through this mechanism reduce the risk of pro-inflammatory conditions such as hypertension and cardiovascular disease.

The next chapter includes findings about i) the impact of age on plasma oxylipins and the influence of dietary flaxseed on plasma oxylipins in healthy adults, ii) the

mechanism of the anti-hypertensive effects of flaxseed in patients with hypertension and peripheral artery disease iii) the impact of dietary flaxseed on central blood pressure, cardiac function, and arterial stiffness in patients with hypertension and peripheral artery disease, iv) the relationship among plasma oxylipins and cardiovascular events, v) a study protocol to assesss the influence of dietary flaxseed on patients with newly diagnosed hypertension, and lastly vi) the findings and implications of the hypertension awareness campaign.

Chapter I Section vi References

1. Folcik VA, Cathcart MK. Predominance of esterified hydroperoxy-linoleic acid in human monocyte-oxidized LDL. J Lipid Res. 1994;35:1570-82.

2. Marx N, Bourcier T, Sukhova GK, Libby P, Plutzky J. PPARgamma activation in human endothelial cells increases plasminogen activator inhibitor type-1 expression: PPARgamma as a potential mediator in vascular disease. Arterioscler Thromb Vasc Biol. 1999;19:546-51.

3. Patwardhan AM, Akopian AN, Ruparel NB, *et al*. Heat generates oxidized linoleic acid metabolites that activate TRPV1 and produce pain in rodents. J Clin Invest. 2010;120:1617-26.

4. Horie M, Fukui H, Endoh S, *et al*. Comparison of acute oxidative stress on rat lung induced by nano and fine-scale, soluble and insoluble metal oxide particles: NiO and TiO2. Inhal Toxicol. 2012;24:391-400.

5. Moran JH, Weise R, Schnellmann RG, Freeman JP, Grant DF. Cytotoxicity of linoleic acid diols to renal proximal tubular cells. Toxicol Appl Pharmacol. 1997;146:53-9.

6. Viswanathan S, Hammock BD, Newman JW, Meerarani P, Toborek M, Hennig B. Involvement of CYP 2C9 in mediating the proinflammatory effects of linoleic acid in vascular endothelial cells. J Am Coll Nutr. 2003;22:502-10.

7. Moran JH, Weise R, Schnellmann RG, Freeman JP, Grant DF. Cytotoxicity of linoleic acid diols to renal proximal tubular cells. Toxicol Appl Pharmacol. 1997;146:53-9.

8. Sisemore MF, Zheng J, Yang JC, *et al*. Cellular characterization of leukotoxin diol-induced mitochondrial dysfunction. Arch Biochem Biophys. 2001;392:32-7.

9. Zheng J, Plopper CG, Lakritz J, Storms DH, Hammock BD. Leukotoxin-diol: A putative toxic mediator involved in acute respiratory distress syndrome. Am J Respir Cell Mol Biol. 2001;25:434-8.

10. Zhang W, Yang AL, Liao J, *et al*. Soluble epoxide hydrolase gene deficiency or inhibition attenuates chronic active inflammatory bowel disease in IL-10(-/-) mice. Dig Dis Sci. 2012;57:2580-91.

11. Earles SM, Bronstein JC, Winner DL, Bull AW. Metabolism of oxidized linoleic acid: Characterization of 13-hydroxyoctadecadienoic acid dehydrogenase activity from rat colonic tissue. Biochim Biophys Acta. 1991;1081:174-80.

12. Zein CO, Lopez R, Fu X, *et al*. Pentoxifylline decreases oxidized lipid products in nonalcoholic steatohepatitis: New evidence on the potential therapeutic mechanism. Hepatology. 2012;56:1291-9.

13. Lundstrom SL, Yang J, Kallberg HJ, *et al*. Allergic asthmatics show divergent lipid mediator profiles from healthy controls both at baseline and following birch pollen provocation. PLoS One. 2012;7:e33780.

14. Petrich K, Ludwig P, Kuhn H, Schewe T. The suppression of 5-lipoxygenation of arachidonic acid in human polymorphonuclear leucocytes by the 15-lipoxygenase product (15S)-hydroxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid: Structure-activity relationship and mechanism of action. Biochem J. 1996;314 (Pt 3):911-6.

15. Caligiuri SP, Love K, Winter T, *et al*. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr. 2013;143:1421-31.

16. Hercule HC, Schunck WH, Gross V, *et al*. Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. Arterioscler Thromb Vasc Biol. 2009;29:54-60.

17. Kopkan L, Huskova Z, Sporkova A, *et al*. Soluble epoxide hydrolase inhibition exhibits antihypertensive actions independently of nitric oxide in mice with renovascular hypertension. Kidney Blood Press Res. 2012;35:595-607.

18. Goetzl EJ, Pickett WC. The human PMN leukocyte chemotactic activity of complex hydroxy-eicosatetraenoic acids (HETEs). J Immunol. 1980;125:1789-91.

19. Goetzl EJ. A role for endogenous mono-hydroxy-eicosatetraenoic acids (HETEs) in the regulation of human neutrophil migration. Immunology. 1980;40:709-19.

20. Bednar MM, Gross CE, Balazy MK, *et al*. 16(R)-hydroxy-5,8,11,14-eicosatetraenoic acid, a new arachidonate metabolite in human polymorphonuclear leukocytes. Biochem Pharmacol. 2000;60:447-55.

21. Bednar MM, Gross CE, Russell SR, *et al*. 16(R)-hydroxyeicosatetraenoic acid, a novel cytochrome P450 product of arachidonic acid, suppresses activation of human polymorphonuclear leukocyte and reduces intracranial pressure in a rabbit model of thromboembolic stroke. Neurosurgery. 2000;47:1410,8; discussion 1418-9.

22. Giles H, Leff P. The biology and pharmacology of PGD2. Prostaglandins. 1988;35:277-300.

23. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev. 2011;111:5922-43.

24. Darius H, Michael-Hepp J, Thierauch KH, Fisch A. Inhibition of human platelets and polymorphonuclear neutrophils by the potent and metabolically stable prostaglandin D2 analog ZK 118.182. Eur J Pharmacol. 1994;258:207-13.

25. Baer PG, McGiff JC. Comparison of effects of prostaglandins E2 and I2 on rat renal vascular resistance. Eur J Pharmacol. 1979;54:359-63.

26. Coleman RA, Smith WL, Narumiya S. International union of pharmacology classification of prostanoid receptors: Properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev. 1994;46:205-29.

27. Cheng Y, Austin SC, Rocca B, *et al*. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science. 2002;296:539-41.

28. Dusting GJ, Moncada S, Vane JR. Prostacyclin (PGX) is the endogenous metabolite responsible for relaxation of coronary arteries induced by arachindonic acid. Prostaglandins. 1977;13:3-15.

29. McLaughlin VV, Genthner DE, Panella MM, Rich S. Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. N Engl J Med. 1998;338:273-7.

30. Kulkarni PS, Roberts R, Needleman P. Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate. Prostaglandins. 1976;12:337-53.

31. Powell WS, Gravel S, Gravelle F. Formation of a 5-oxo metabolite of 5,8,11,14,17-eicosapentaenoic acid and its effects on human neutrophils and eosinophils. J Lipid Res. 1995;36:2590-8.

32. Oh SF, Pillai PS, Recchiuti A, Yang R, Serhan CN. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. J Clin Invest. 2011;121:569-81.

33. VanRollins M, Murphy RC. Autooxidation of docosahexaenoic acid: Analysis of ten isomers of hydroxydocosahexaenoate. J Lipid Res. 1984;25:507-17.

34. Oliw EH, Sprecher HW. Metabolism of polyunsaturated (n-3) fatty acids by monkey seminal vesicles: Isolation and biosynthesis of omega-3 epoxides. Biochim Biophys Acta. 1991;1086:287-94.

35. VanRollins M, Baker RC, Sprecher HW, Murphy RC. Oxidation of docosahexaenoic acid by rat liver microsomes. J Biol Chem. 1984;259:5776-83.

36. Koeppen B, Stanton B. Cellular physiology. In: Koeppen B, Stanton B, eds. Berne & Levy Physiology. Sixth ed. Mosby Elsevier, 2010. 1-51.

37. Samuelsson B. Leukotrienes: A new class of mediators of immediate hypersensitivity reactions and inflammation. Adv Prostaglandin Thromboxane Leukot Res. 1983;11:1-13.

38. Silva BC, de Miranda AS, Rodrigues FG, *et al*. The 5-lipoxygenase (5-LOX) inhibitor zileuton reduces inflammation and infarct size with improvement in neurological outcome following cerebral ischemia. Curr Neurovasc Res. 2015;12:398-403.

39. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics. 2004;14:1-18.

40. Wang Z, Gorski JC, Hamman MA, Huang SM, Lesko LJ, Hall SD. The effects of st john's wort (hypericum perforatum) on human cytochrome P450 activity. Clin Pharmacol Ther. 2001;70:317-26.

41. Dahan A, Altman H. Food-drug interaction: Grapefruit juice augments drug bioavailability--mechanism, extent and relevance. Eur J Clin Nutr. 2004;58:1-9.

42. Kato Y, Fuchi N, Saburi H, *et al*. Discovery of 2,8-diazaspiro[4.5]decane-based trisubstituted urea derivatives as highly potent soluble epoxide hydrolase inhibitors and orally active drug candidates for treating hypertension. Bioorg Med Chem Lett. 2013;23:5975-9.

43. Imig JD, Walsh KA, Hye Khan MA, *et al*. Soluble epoxide hydrolase inhibition and peroxisome proliferator activated receptor gamma agonist improve vascular function and decrease renal injury in hypertensive obese rats. Exp Biol Med (Maywood). 2012;237:1402-12.

44. Spector AA, Fang X, Snyder GD, Weintraub NL. Epoxyeicosatrienoic acids (EETs): Metabolism and biochemical function. Prog Lipid Res. 2004;43:55-90.

45. Enayetallah AE, French RA, Barber M, Grant DF. Cell-specific subcellular localization of soluble epoxide hydrolase in human tissues. J Histochem Cytochem. 2006;54:329-35.

46. Zhang J, Yang J, Chang X, Zhang C, Zhou H, Liu M. Ozagrel for acute ischemic stroke: A meta-analysis of data from randomized controlled trials. Neurol Res. 2012;34:346-53.

47. Government of Canada. Food and drugs act. 2016. http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ retrieved March 11 2016.

48. Nakamura A, Nagaya N, Obata H, *et al*. Oral administration of a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity for pulmonary arterial hypertension. Circ J. 2013;77:2127-33.

49. Tada M, Kuzuya T, Inoue M, *et al*. Elevation of thromboxane B2 levels in patients with classic and variant angina pectoris. Circulation. 1981;64:1107-15.

50. Lemaitre RN, Rice K, Marciante K, *et al*. Variation in eicosanoid genes, non-fatal myocardial infarction and ischemic stroke. Atherosclerosis. 2009;204:e58-63.

51. Tohgi H, Konno S, Tamura K, Kimura B, Kawano K. Effects of low-to-high doses of aspirin on platelet aggregability and metabolites of thromboxane A2 and prostacyclin. Stroke. 1992;23:1400-3.

52. Findings from the aspirin component of the ongoing physicians' health study. N Engl J Med. 1988;318:262-4.

53. van Diemen JJ, Fuijkschot WW, Wessels TJ, Veen G, Smulders YM, Thijs A. Evening intake of aspirin is associated with a more stable 24-h platelet inhibition compared to morning intake: A study in chronic aspirin users. Platelets. 2015:1-6.

54. Eidelman RS, Hebert PR, Weisman SM, Hennekens CH. An update on aspirin in the primary prevention of cardiovascular disease. Arch Intern Med. 2003;163:2006-10.

55. Ridker PM, Cook NR, Lee IM, *et al*. A randomized trial of low-dose aspirin in the primary prevention of cardiovascular disease in women. N Engl J Med. 2005;352:1293-304.

56. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA. 2001;286:954-9.

57. Back M. Leukotriene signaling in atherosclerosis and ischemia. Cardiovasc Drugs Ther. 2009;23:41-8.

58. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev. 2002;82:131-85.

59. Serhan CN, Krishnamoorthy S, Recchiuti A, Chiang N. Novel anti-inflammatory--pro-resolving mediators and their receptors. Curr Top Med Chem. 2011;11:629-47.

60. Spite M, Serhan CN. Novel lipid mediators promote resolution of acute inflammation: Impact of aspirin and statins. Circ Res. 2010;107:1170-84.

61. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ Res. 1996;78:415-23.

62. Inceoglu B, Schmelzer KR, Morisseau C, Jinks SL, Hammock BD. Soluble epoxide hydrolase inhibition reveals novel biological functions of epoxyeicosatrienoic acids (EETs). Prostaglandins Other Lipid Mediat. 2007;82:42-9.

63. Neckar J, Kopkan L, Huskova Z, *et al*. Inhibition of soluble epoxide hydrolase by cis-4-[4-(3-adamantan-1-ylureido)cyclohexyl-oxy]benzoic acid exhibits antihypertensive and cardioprotective actions in transgenic rats with angiotensin II-dependent hypertension. Clin Sci (Lond). 2012;122:513-25.

64. Imig JD, Zhao X, Zaharis CZ, *et al*. An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. Hypertension. 2005;46:975-81.

65. Davis BB, Thompson DA, Howard LL, Morisseau C, Hammock BD, Weiss RH. Inhibitors of soluble epoxide hydrolase attenuate vascular smooth muscle cell proliferation. Proc Natl Acad Sci U S A. 2002;99:2222-7.

66. Node K, Huo Y, Ruan X, *et al*. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science. 1999;285:1276-9.

67. Zijlstra FJ, Vincent JE, Mol WM, Hoogsteden HC, Van Hal PT, Jongejan RC. Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and non-smokers. Eur J Clin Invest. 1992;22:301-6.

68. Theken KN, Schuck RN, Edin ML, *et al*. Evaluation of cytochrome P450-derived eicosanoids in humans with stable atherosclerotic cardiovascular disease. Atherosclerosis. 2012;222:530-6.

69. The Human Serum Metabalome. The human serum metabalome. 2016.

70. Moller K, Ostermann AI, Rund K, *et al*. Influence of weight reduction on blood levels of C-reactive protein, tumor necrosis factor-alpha, interleukin-6, and oxylipins in obese subjects. Prostaglandins Leukot Essent Fatty Acids. 2016;106:39-49.

71. Schuchardt JP, Schmidt S, Kressel G, *et al*. Comparison of free serum oxylipin concentrations in hyper- vs. normolipidemic men. Prostaglandins Leukot Essent Fatty Acids. 2013;89:19-29.

72. Strassburg K, Esser D, Vreeken RJ, *et al*. Postprandial fatty acid specific changes in circulating oxylipins in lean and obese men after high-fat challenge tests. Mol Nutr Food Res. 2014;58:591-600.

73. Caligiuri SP, Love K, Winter T, *et al*. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr. 2013;143:1421-31.

Chapter II - Methods and Results

i. Oxylipins in Aging

It is unknown if many of the newly discovered plasma oxylipins are influenced by age. This is important to identify because oxylipins may be implicated in age associated inflammation and the increased risk of developing pro-inflammatory disorders. Identifying any age-associated oxylipins may provide direction on potential therapeutic targets. In addition, it is unknown if a functional food containing such as flaxseed that provides a source of the polyunsaturated fatty acid, alpha-linolenic acid and antioxidant lignans can alter the plasma oxylipin profile. Therefore, the current objectives of the following study include:

1) To determine if age influences the plasma oxylipins profile;

2) To observe if dietary flaxseed alters the plasma oxylipin profile;

3) To determine if age influences the flaxseed induced changes in the plasma oxylipin profile.

The hypothesis of the current study is: older individuals will exhibit significantly higher concentrations of many pro-inflammatory oxylipins and flaxseed will reduce the concentration of these pro-inflammatory oxylipins. In order to test the above hypothesis, the following study was carried out.

Elevated Levels of Pro-Inflammatory Oxylipins in Older Subjects are Normalized by Flaxseed Consumption

Published in: Exp Gerontol. 2014 Nov;59:51-7.

Stephanie P.B. Caligiuri[1,2,3], Harold M. Aukema[1,4], Amir Ravandi[2,5] and Grant N. Pierce[1,2,3]

[1]Canadian Centre for Agri-food Research in Health and Medicine (CCARM) and [2]the Institute of Cardiovascular Sciences St. Boniface Hospital Research Centre, and the [3]Departments of Physiology, [4]Human Nutritional Sciences, and [5]Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Abbreviations: ALA - alpha-linolenic acid, ARA - arachidonic acid, DGLA - Dihomo-γ-linolenic acid, DiHDPA - dihydroxydocosapentanoic acid, DHET - dihydroxyeicosatrienoic acid, DiHOME - dihydroxyoctadecenoic acid, DHA - docosahexanoic acid, EPA - eicosapentanoic acid, EpOME - epoxyoctadecenoic acid, HDOHE - hydroxydocosahexanoic acid, HEPE - hydroxyeicosapentanoic acid, HETE - hydroxyeicosatetraenoic acid, HETrE - hydroxyeicosatrienoic acid, HHTrE - hydroxyheptadecatrienoic acid, HODE - hydroxyoctadecadienoic acid, HOTrE - hydroxyoctadecatrienoic acid, LA - linoleic acid, oxoODE - oxooctadecadienoic acid, oxoOTrE - oxooctadecatrienoic acid, TriHOME - trihydroxyoctadecenoic acid

Abstract

Background and Aims: Oxylipins, including the eicosanoids, are highly bioactive molecules endogenously produced from polyunsaturated fatty acids. Oxylipins play a key role in chronic disease progression. It is possible, but unknown, if oxylipin concentrations change with the consumption of functional foods or differ with subject age.

Methods: Therefore, in a parallel comparator trial, 20 healthy individuals were recruited into a younger (19-28 years) or older (45-64 years) age group (n=10/group). Participants ingested one muffin/day containing 30 g of milled flaxseed (6 g alpha-linolenic acid) for 4 weeks. Plasma oxylipins were isolated through solid phase extraction, analyzed with HPLC-MS/MS targeted lipidomics, and quantified with the stable isotope dilution method.

Results: At baseline, the older group exhibited 13 oxylipins ≥2-fold the concentration of the younger group. Specifically, pro-inflammatory oxylipins 5-hydroxyeicosatetraenoic acid, 9,10,13-trihydroxyoctadecenoic acid, and 9,12,13-trihydroxyoctadecenoic acid were significantly greater in the older (1.1±0.23 nM, 5.6±0.84 nM, and 4.5±0.58 nM, respectively) versus the younger group (0.34±0.12 nM, 3.5±0.33 nM, and 3.0±0.24 nM, respectively) ($p<0.05$). After 4 weeks of flaxseed consumption the number of oxylipins that were ≥2-fold higher in the older versus the younger group was reduced to 3. 5-hydroxyeicosatetraenoic acid, 9,10,13-trihydroxyoctadecenoic acid, and 9,12,13-trihydroxyoctadecenoic acid decreased in the older group to concentrations equivalent to the younger group after flaxseed consumption.

Conclusion: These data suggest an important role for oxylipins in the aging process and how nutritional interventions like flaxseed can beneficially disrupt these biological changes associated with inflammation and aging.

Key Words: Eicosanoids, Alpha-Linolenic Acid, Aging, Lipidomics

Introduction

Oxylipins, including octadecanoids, eicosanoids and docosanoids, play an essential role in both progressing and ameliorating chronic disease. Those derived from the long chain n3 fatty acids such as resolvins, protectins and maresins can resolve inflammatory processes by preventing polymorphonuclear neutrophil recruitment (1-6). In contrast, some of the oxylipins produced from arachidonic acid (ARA) or linoleic acid (LA) have been attributed to inflammation (7-9), tissue damage (10, 11), vasoconstriction (12, 13), oxidative stress (14), and chronic disease (7, 9-11, 15-17). However, some arachidonic acid-derived eicosanoids have also exhibited protective effects. For example, epoxyeicosatrienoic acids have potent vasodilatory properties (15, 18) and 15-hydroxyeicosatrienoic acid (15-HETE) possesses anti-inflammatory properties by preventing chemotaxis in rat neutrophils (19). It is likely the balance of pro- and anti-inflammatory oxylipins that determines the overall effects of these compounds.

Despite the importance of oxylipins in health and disease, we know relatively little about the factors that influence their concentrations in the blood. For example, although metabolism changes dramatically with age (20, 21), there are no reports comparing the profiles of oxylipins in younger and older individuals. As well, since fatty acid concentrations differ between younger and older individuals (22), it is possible that oxylipin profiles may differ as well. Therefore, the first objective of the study was to compare the oxylipin profiles of healthy younger and older individuals.

In previous trials, dietary supplementation with the long chain n-3 fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) increased the concentration of plasma oxylipins derived from EPA and DHA in humans (23, 24) and diets high in alpha-linolenic acid (ALA) increased renal ALA-derived oxylipins in rats (25). Flaxseed,

which is rich in the n3 fatty acid, ALA, is easily incorporated into food products (26-28). Ingestion of flaxseed will increase plasma (29) and tissue ALA fatty acid concentrations, (30) increase plasma enterolignans, (31) reduce atherogenicity (32, 33), plasma cholesterol (34), plasma glucose (34), plasma trans fats (35) and blood pressure (36). It is possible that these health benefits of flaxseed may be the result of altering the generation of highly bioactive oxylipins. However, this has yet to be determined. Therefore, the second objective of the study was to determine if oxylipin profiles could be affected when individuals consumed a food enriched in flaxseed, and if the age of the subject would influence any differences in the response of the oxylipin profile to flaxseed consumption.

Materials and Methods

Study Participants

Twenty apparently healthy participants were recruited and grouped into either a younger (19-28 years) or older (45-64 years) age group (n=10/group). The study was a parallel single-blinded design where the individuals involved in the data analysis were unaware of age group. The participants exhibited no clinical evidence of disease and were not using any medication including cholesterol-lowering, anti-hypertensive, anti-histamine, or hormone therapy medications. Study participants were not allowed to consume supplements, oils, salad dressings with oils or more than 2 fish servings/week for 1 month prior to and during the study. The younger group had an average age of 22 years with an average body mass index of 25.5 ± 1.3 and contained 6 males and 4 females. The average age of the older group was 53 years with an average body mass index of 24.3 ± 0.7 with 5 males and 5 females. The study design was approved by and

performed in accordance with the University of Manitoba Research Ethics Board and St. Boniface Hospital Research Review Committee.

Dietary Intervention

Participants were provided two flavours of muffins and instructed to ingest one muffin per day for four weeks. Each muffin contained 30 g of milled flaxseed which provided 6 g of ALA. The change in plasma ALA, nutrient composition and flavour acceptability of the muffins has been previously published (27, 29, 37)

Oxylipin Analysis

Blood was collected at baseline and after 4 weeks of dietary intervention. The blood was centrifuged at 4°C to isolate the plasma and stored at -80°C for up to 8 years before analysis of the oxylipin profile. Previous publications have confirmed the stability of the serum fatty acid profile when stored at -80°C for up to ten years (38).

To isolate the oxylipins, 200 µL of plasma underwent solid phase extraction and were analyzed by HPLC-MS/MS (AB SCIEX 4000 QTRAP) and multiple-reaction monitoring as previously described (25, 39). Details of the deuterated internal standards, collision-induced dissociation mass transitions, retention times, and detector response factors for analytes can be found in Table 7. The quantification limit was set at 3 levels above the background. Quantification of oxylipins was determined using the stable isotope dilution method (40) and expressed as nM.

Table 7: Collision Induced Dissociation Mass Transitions, Analyte Retention Time, Internal Standard and Detector Response Factors for All Oxylipins Quantified

Oxylipin	CID Mass Transition	Analyte Retention Time	Internal Standard	Detector Response Factor
	Da	*min*		
LA Derived Oxylipins				
9-HODE	295.1>170.6	15.2	9-HODE-d_4	1.54
13-HODE	295.1>194.9	15.1	13-HODE-d_4	1.44
9,10-DIHOME	313.0>200.8	13.4	9,10-DiHOME-d_4	2.78
12,13-DIHOME	313.0>183.0	13.1	12,13-DiHOME-d_4	2.36
9-oxoODE	293.0>184.8	15.3	5-OxoETE-d_7	1.27
13-oxoODE	293.0>112.9	15.0	5-OXoETE-d_7	3.86
9,10,13-TriHOME	329.0>171.0	7.3	9,10-DiHOME-d_4	1.85
9,12,13-TriHOME	329.0>211.0	7.2	12,13-DiHOME- d_4	0.74
DGLA Derived Oxylipins				
15-HETr E	321.0>221.0	15.8	15-HETE-d_8	2.30
ALA Derived Oxylipins				
9-HOTrE	293.0>170.8	14.0	9-HODE-d_4	1.34
ARA Derived Oxylipins				
6-keto-$PGF_{1\alpha}$	369.0>162.9	4.8	6-keto-$PGF_{1\alpha}$-d_4	1.85
12-HHTre	279.1>178.9	13.4	15-HETE-d_8	0.42
5,6-DiHETrE	337.0>144.8	14.9	11,12-DHET-d_{11}	0.69
8,9-DiHETrE	337.0>185.0	14.3	8,9-DHET-d_{11}	0.86
11,12 DiHETrE	337.0>167.0	13.9	11,12-DHET-d_{11}	1.37

14,15-DiHETrE	337.0>206.8	13.5	14,15-DHET-d_{11}	1.18
5-HETE	319.0>115.0	16.2	5-HETE-d_8	1.15
8-HETE	319.0>155.0	15.8	5-HETE-d_8	0.76
9-HETE	319.0>151.0	15.9	5-HETE-d_8	0.36
11-HETE	319.0>167.0	15.5	5-HETE-d_8	4.12
12-HETE	319.0>179.0	15.8	5-HETE-d_8	1.73
15-HETE	319.0>219.0	15.2	15-HETE-d_8	1.57
16-HETE	319.0> 232.8	14.7	15-HETE-d_8	2.04
18-HETE	319.0>261.0	14.5	15-HETE-d_8	1.41
20-HETE	319.0>280.0	14.4	20-HETE-d_6	1.30
EPA Derived Oxylipins				
5-HEPE	317.0>115.0	14.9	5-HETE-d_8	0.83
9-HEPE	317.0>123.0	14.6	5-HETE-d_8	0.38
12-HEPE	317.0>179.0	14.5	5-HETE-d_8	1.49
DHA Derived Oxylipins				
4-HDoHE	343.0>101.1	16.2	5-HETE-d_8	1.40
7-HDOHE	343.0>140.7	15.6	5-HETE-d_8	1.03
10-HDOHE	343.0>152.7	15.2	5-HETE-d_8	2.41
11-HDOHE	343.0>149.1	15.5	5-HETE-d_8	1.78
13-HDOHE	343.0>192.9	15.1	15-HETE-d_8	3.10
14-HDOHE	343.0>161.0	15.3	15-HETE-d_8	1.06
16-HDOHE	319.0>219.0	15.0	15-HETE-d_8	3.13
17-HDOHE	343.0>245.2	15.3	15-HETE-d_8	0.32
20-HDOHE	343.0>240.9	14.8	15-HETE-d_8	1.12
19,20-DiHDPA	361.0>229.0	13.3	14,15-DHET-d_{11}	0.21

[1]Abbreviations: alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-γ-linolenic acid, DGLA; dihydroxydocosapentaenoic acid, DiHDPA; dihydroxyeicosatrienoic acid, DHET; dihydroxyoctadecenoic acid, DiHOME; docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA; hydroxydocosahexaenoic acid, HDOHE; hydroxyeicosapentaenoic acid, HEPE; hydroxyeicosatetraenoic acid, HETE; hydroxyeicosatrienoic acid, HETrE; hydroxyheptadecatrienoic acid, HHTrE; hydroxyoctadecadienoic acid, HODE; hydroxyoctadecatrienoic acid, HOTrE; linoleic acid, LA; oxooctadecadienoic acid, oxoODE; oxooctadecatrienoic acid, OXoOTrE trihydroxyoctadecenoic acid, TriHOME.

Statistical Analyses

Data were analyzed using SAS version 9.2 (SAS Institute Inc, Cary, NC) and tested for normality and homogeneity of variance using Shapiro Wilk's and Levene's test, respectively. In Figure 4, the first two columns were created by pairing an individual from the older and from the younger group based on body mass index and gender. The fold differences were calculated for each pair and averaged. The last two columns of Figure 4 were created by calculating the fold changes for each individual from week 0 to week 4 and averaged. Data for Figures 4-6 were analyzed using a two-way general linear model for age and time interaction. Data are represented as mean ± SEM. The level of significance was set at $p<0.05$.

Results

3.1 Plasma Oxylipin Concentrations in Healthy Individuals

Of the 81 plasma oxylipins scanned, 38 were quantified according to the quantification limit. **Table 8** provides the plasma concentrations (nM) of the oxylipins. Of the oxylipins quantified, LA-derived oxylipins were of the highest concentration. Total LA-derived oxylipins averaged 38.5 nM in both the younger and older groups. By comparison, the one ALA derived oxylipin detected averaged 0.99 nM. The DGLA derived oxylipin averaged a plasma concentration of 0.29 nM. The ARA-derived oxylipins averaged 6.9 nM. Long chain n3 derived oxylipins averaged a plasma concentration of 1.1 nM and 4.3 nM for EPA and DHA derived oxylipins, respectively.

Table 8: Plasma oxylipin concentrations by age group, before and after 4 weeks of dietary intervention with milled flaxseed

	Younger Week 0	Younger Week 4	Older Week 0	Older Week 4
		Oxylipin (nM)		
LA Derived Oxylipins				
9-HODE	11.6 ± 1.6	9.6 ± 1.1	9.4 ± 1.8	6.4 ± 0.62
13-HODE	9.4 ± 0.73	7.9 ± 0.82	10 ± 1.3	7.6 ± 0.76
9,10-DiHOME	4.5 ± 1.1	2.7 ± 0.37	2.4 ± 0.34	2.2 ± 0.28
12,13-DiHOME	2.7 ± 0.25	3.4 ± 0.51	3.0 ± 0.44	2.6 ± 0.29
9-OXOoDE	2.9 ± 1.6	2.1 ± 1.1	4.6 ± 1.7	1.6 ± 1.1
13-OXOoDE	5.7 ± 2.2	4.0 ± 1.0	4.8 ± 1.1	4.4 ± 1.0
9,10,13-TriHOME	3.5 ± 0.33	2.9 ± 0.25	5.6 ± 0.84	3.2 ± 1.4
9,12,13-TriHOME	3.0 ± 0.24	3.0 ± 0.69	4.5 ± 0.58	2.7 ± 0.31
DGLA Derived Oxylipins				
15-HETrE	0.31 ± 0.10	0.31 ± 0.10	0.38 ± 0.14	0.17 ± 0.04
ALA Derived Oxylipins				

9-HOTrE	1.2 ± 0.31	1.2 ± 0.13	0.87 ± 0.27	0.69 ± 0.34
ARA Derived Oxylipins				
$6kPGF_{1\alpha}$	1.1 ± 0.10	0.85 ± 0.13	1.0 ± 0.092	1.1 ± 0.071
12-HHTrE	0.59 ± 0.28	0.0 ± 0.0	0.30 ± 0.14	0.05 ± 0.05
5,6-DHET	0.14 ± 0.032	0.20 ± 0.031	0.19 ± 0.025	0.17 ± 0.030
8,9-DHET	0.24 ± 0.052	0.20 ± 0.067	0.26 ± 0.036	0.24 ± 0.049
11,12-DHET	0.43 ± 0.045	0.48 ± 0.067	0.52 ± 0.054	0.42 ± 0.044
14,15-DHET	0.51 ± 0.042	0.52 ± 0.058	0.54 ± 0.05	0.42 ± 0.036
5-HETE	0.34 ± 0.12	0.37 ± 0.18	1.1 ± 0.23	0.39 ± 0.17
8-HETE	0.18 ± 0.10	0.26 ± 0.19	0.38 ± 0.12	0.32 ± 0.11
9-HETE	0.27 ± 0.27	0.086 ± 0.086	0.78 ± 0.30	0.19 ± 0.10
11-HETE	0.69 ± 0.40	0.85 ± 0.48	0.95 ± 0.19	0.65 ± 0.06
12-HETE	0.77 ± 0.34	0.45 ± 0.12	1.0 ± 0.20	0.56 ± 0.12
15-HETE	0.68 ± 0.12	0.75 ± 0.14	0.91 ± 0.22	0.57 ± 0.15
16-HETE	0.18 ± 0.10	0.18 ± 0.09	0.20 ± 0.065	0.19 ± 0.081

18-HETE	0.048 ± 0.037	0.026 ± 0.026	0.46 ± 0.18	0.13 ± 0.059
20-HETE	0.12 ± 0.082	0.59 ± 0.27	0.70 ± 0.29	0.89 ± 0.41
EPA Derived Oxylipins				
5-HEPE	0.24 ± 0.14	0.39 ± 0.14	1.0 ± 0.34	0.56 ± 0.18
9-HEPE	0.047 ± 0.047	0.0 ± 0.0	0.82 ± 0.31	0.49 ± 0.29
12-HEPE	0.056 ± 0.056	0.16 ± 0.08	0.32 ± 0.12	0.12 ± 0.062
DHA Derived Oxylipins				
4-HDOHE	0.29 ± 0.079	0.25 ± 0.076	0.56 ± 0.18	0.20 ± 0.07
7-HDOHE	0.051 ± 0.027	0.057 ± 0.029	0.40 ± 0.18	0.10 ± 0.069
10-HDOHE	0.28 ± 0.10	0.14 ± 0.06	0.53 ± 0.24	0.21 ± 0.09
11-HDOHE	0.039 ± 0.026	0.086 ± 0.044	0.57 ± 0.22	0.09 ± 0.06
13-HDOHE	0.15 ± 0.051	0.18 ± 0.046	0.49 ± 0.14	0.11 ± 0.063
14-HDOHE	0.039 ± 0.17	0.18 ± 0.065	0.57 ± 0.13	0.10 ± 0.056
16-HDOHE	0.21 ± 0.05	0.31 ± 0.08	0.44 ± 0.13	0.23 ± 0.050
17-HDOHE	0.91 ± 0.31	0.41 ± 0.22	0.96 ± 0.57	0.26 ± 0.19

20-HDOHE	0.22 ± 0.17	1.6 ± 0.66	1.0 ± 0.48	0.86 ± 0.38
19,20-DiHDPA	0.99 ± 0.12	1.3 ± 0.16	1.2 ± 0.14	1.0 ± 0.07

Data are represented as mean ± SEM.

Abbreviations: 6-keto prostaglandin $F_{1\alpha}$, $6kPGF_{1\alpha}$; alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-γ-linolenic acid, DGLA; dihydroxydocosapentanoic acid, DiHDPA; dihydroxyeicosatrienoic acid, DHET; dihydroxyoctadecenoic acid, DiHOME; docosahexanoic acid, DHA; eicosapentanoic acid, EPA; epoxyoctadecenoic acid, EpOME; hydroxydocosahexanoic acid, HDOHE; hydroxyeicosapentanoic acid, HEPE; hydroxyeicosatetraenoic acid, HETE; hydroxyeicosatrienoic acid, HETrE; hydroxyheptadecatrienoic acid, HHTrE; hydroxyoctadecadienoic acid, HODE; hydroxyoctadecatrienoic acid, HOTrE; linoleic acid, LA; oxooctadecadienoic acid, oxoODE; oxooctadecatrienoic acid, OXoOTrE; trihydroxyoctadecenoic acid, TriHOME.

3.2 Oxylipin Profiles Differ As a Function of Age

Figure 4 illustrates the average of the individual fold differences between each pair or fold differences for each individual over time. At baseline, the most prominent finding was the higher concentrations of ARA-, EPA-, and DHA-derived oxylipins in the older group versus their younger counterparts in column 1 of Figure 4. The older group had a total of 13 oxylipins ≥2-fold the concentration observed in the younger group. This included 5-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE, 9-HETE, 18-HETE, 20-HETE, 5-hydroxyeicosapentaenoic acid (5-HEPE), 9-HEPE, 12-HEPE, 7-hydroxydocosahexaenoic acid (7-HDOHE), 11-HDOHE, 13-HDOHE, 16-HDOHE, and 20-HDOHE. In contrast, only two n6 derived oxylipins, 9,10-dihydroxyoctadecenoic acid (DiHOME) and 12-hydroxyheptadecatrienoic acid (HHTrE) were ≥2-fold lower than the concentrations observed in the younger group (Figure 4).

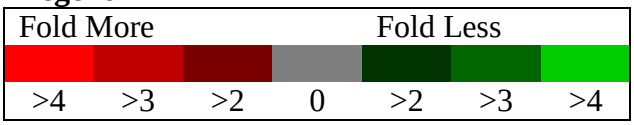
3.3 Response to Flaxseed Consumption Differs Between Age Groups

The response to the dietary intervention differed between the younger and older groups. The older group exhibited a ≥2-fold decrease in 17 oxylipins, mostly those derived from EPA and DHA (Figure 4). In contrast, the younger group exhibited a ≥2-fold decrease in 7 oxylipins. In the older group, there were no oxylipins that exhibited a ≥2-fold increase after flaxseed consumption, whereas, the younger group exhibited a ≥2-fold increase in 3 oxylipins, of which 2 were derived from n3 long chain fatty acids (Column 3 and 4 of Figure 4).

Substrate	Oxylipin	Baseline Older/ Younger	4 weeks Older/ Younger	Younger 4/0 weeks	Older 4/0 weeks
LA[a]	9-HODE				
	13-HODE				
	9,10-DiHOME				
	12,13-DiHOME				
	9-OXOoDE				
	13-OXOoDE				
	9,10,13-TriHOME				
	9,12,13-TriHOME				
DGLA	15-HETrE				
ALA	9-HOTrE				
ARA	6-k-$PGF_{1\alpha}$				
	12-HHTrE				
	5-HETE				
	8-HETE				
	9-HETE				
	11-HETE				
	12-HETE				
	15-HETE				
	16-HETE				
	18-HETE				
	20-HETE				
	5,6-DiHETrE				
	8,9-DiHETrE				
	11,12-DiHETrE				
	14,15-DiHETrE				
EPA	5-HEPE				
	9-HEPE				
	12-HEPE				
DHA	4-HDOHE				
	7-HDOHE				
	10-HDOHE				
	11-HDOHE				
	13-HDOHE				
	14-HDOHE				
	16-HDOHE				
	17-HDOHE				
	20-HDOHE				
	19,20-DiHDPA				

Figure 4: Heat Map of Average Individual Fold Differences Between Age Groups and Fold Changes Between Time Points in the Plasma Oxylipin Profile

Legend



[a]Abbreviations: alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-γ-linolenic acid, DGLA; dihydroxydocosapentanoic acid, DiHDPA; dihydroxyeicosatrienoic acid, DHET; dihydroxyoctadecenoic acid, DiHOME; docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA; epoxyoctadecenoic acid, EpOME; hydroxydocosahexaenoic acid, HDOHE; hydroxyeicosapentaenoic acid, HEPE; hydroxyeicosatetraenoic acid, HETE; hydroxyeicosatrienoic acid, HETrE; hydroxyheptadecatrienoic acid, HHTrE; hydroxyoctadecadienoic acid, HODE; hydroxyoctadecatrienoic acid, HOTrE; linoleic acid, LA; oxooctadecadienoic acid, oxoODE; oxooctadecatrienoic acid, OXoOTrE; trihydroxyoctadecenoic acid, TriHOME.

3.4 Flaxseed Consumption Produces Similar Oxylipin Profiles between Age Groups after Four Weeks

The majority of the differences between the two age groups that were previously observed at baseline were no longer evident at 4 weeks (Column 2 of Figure 4). After 4 weeks of dietary supplementation with flaxseed, the older age group now had only 3 oxylipins ≥2-fold greater and 8 oxylipins ≥2-fold less than the younger group.

After 4 weeks of consuming functional foods containing flaxseed, the older group exhibited changes in their oxylipin profiles which resulted in similar profiles to their younger counterparts. More specifically, using a 2-way general linear model, at baseline the older group exhibited significantly higher concentrations of pro-inflammatory 5-HETE compared to the younger group. The average plasma concentration in the older group at baseline was 1.12 ± 0.23 nM. After consuming muffins containing 30 g of milled flaxseed for 4 weeks, the older group exhibited a significant decrease to 0.39 ± 0.17 nM which was equivalent to the concentrations observed in the younger group (0.37 ± 0.18 nM) **(Figure 5).** At baseline, the older group had significantly higher concentrations of the pro-inflammatory 9,10,13-TriHOME and 9,12,13-TriHOME versus the younger group **(Figure 6).** After consuming the functional foods with flaxseed for 4 weeks, the older group exhibited a significant decrease (-2.4 nM and -1.87 nM, respectively) to concentrations equivalent to that of the younger group. Plasma 11-HDOHE concentrations at baseline were significantly higher than the younger group (0.57 ± 0.22 nM and 0.04 ± 0.03 nM, respectively) **(Figure 7).** However, at 4 weeks, the older group exhibited a significant decrease to concentrations similar to those observed in the younger group (0.09 ± 0.06 nM). A similar pattern was observed for the metabolite of EPA, 5-HEPE. At baseline, the older group had an average plasma concentration of 1.0 ± 0.34 nM. At 4 weeks, the older group exhibited a decrease to 0.56 ±

0.18 nM which was similar to the concentrations in the younger group (0.39 ± 0.14 nM) (Figure 7).

The younger group also exhibited changes in the plasma oxylipin profile to concentrations equivalent to the older group as a result of the flaxseed intervention. A cytotoxic metabolite of linoleic acid, 9,10-DiHOME, was significantly higher in the younger group at baseline (4.5 ± 1.1 nM). After 4 weeks of flaxseed ingestion, the younger group demonstrated a significant decrease to concentrations similar to the older group (2.7 ± 0.35 nM) **(**Figure 6**).**

A metabolite of ALA did not change significantly after 4 weeks of consuming muffins containing milled flaxseed. However, after 4 weeks of flaxseed ingestion, the younger group exhibited significantly higher concentrations of the ALA metabolite, 9-hydroxyoctadecatrieonic acid (HOTrE), versus the older group (1.2 ± 0.13 nM versus 0.69 ± 0.11 nM, respectively) **(**Figure 7**).**

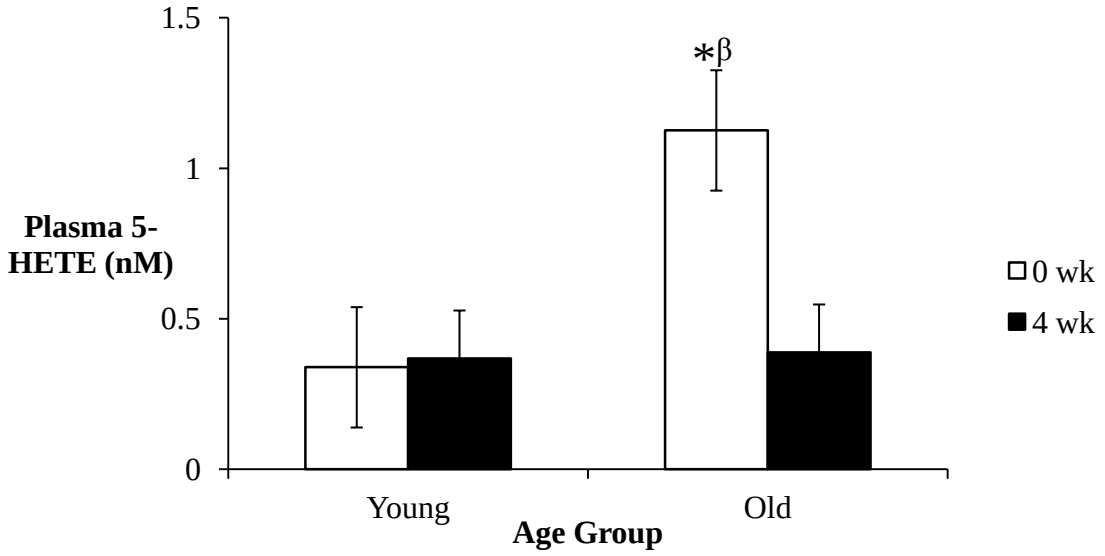
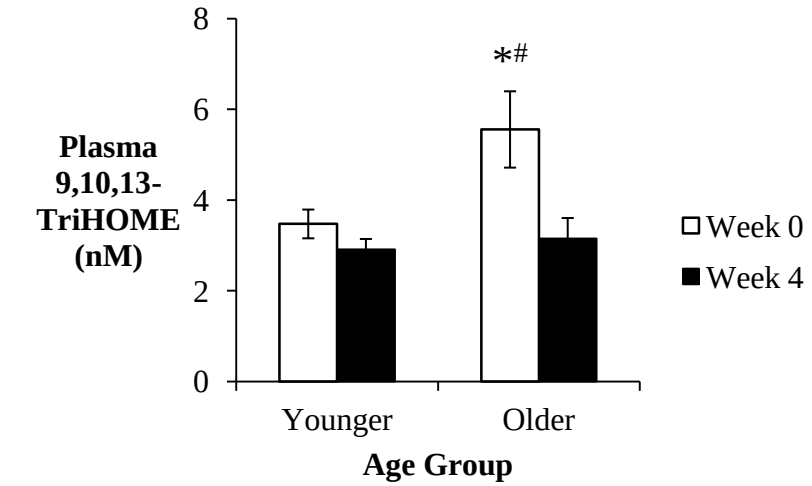
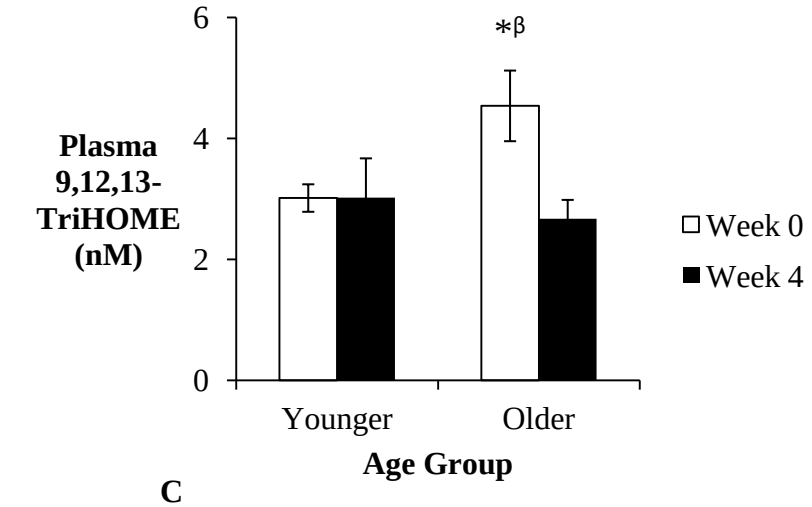


Figure 5: Plasma levels of the arachidonic acid-derived 5-HETE (5-hydroxyeicosatetraenoic acid) as a function of age and flaxseed consumption.
Values represent mean ± SEM. *Significant difference between age groups at same time point. $^{\beta}$Significant age*time interaction. The data were analyzed using a 2-way general linear model.



B



C

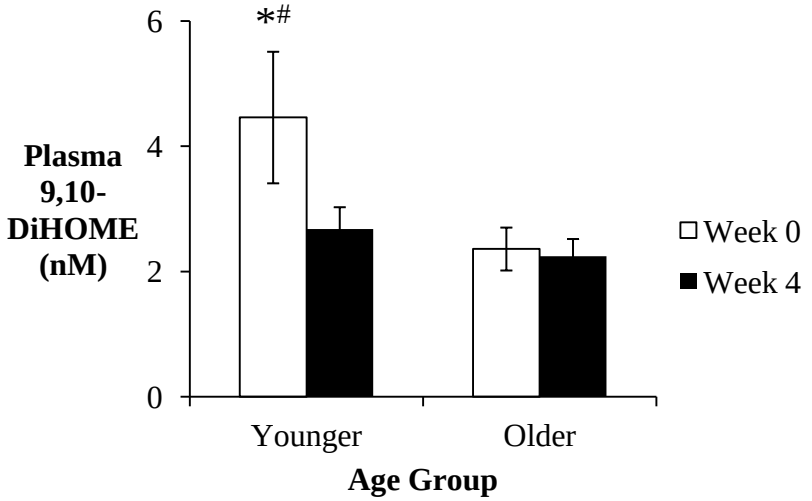


Figure 6: Linoleic acid-derived oxylipins in plasma from healthy subjects as a function of their age and the consumption of flaxseed.

Values represent the mean ± SEM. A) 9,10,13-TriHOME (9,10,13-trihydroxyoctadecenoic acid). B) 9,12,13-TriHOME (9,12,13-trihydroxyoctadecenoic acid). C) 9,10-DiHOME (9,10-dihydroxyoctadecenoic acid).*Significant difference between age groups at same time point. $^{\#}$ Significant difference between time points for the same age group. $^{\beta}$Significant age*time interaction ($p<0.05$). Data were analyzed using a 2-way general linear model.

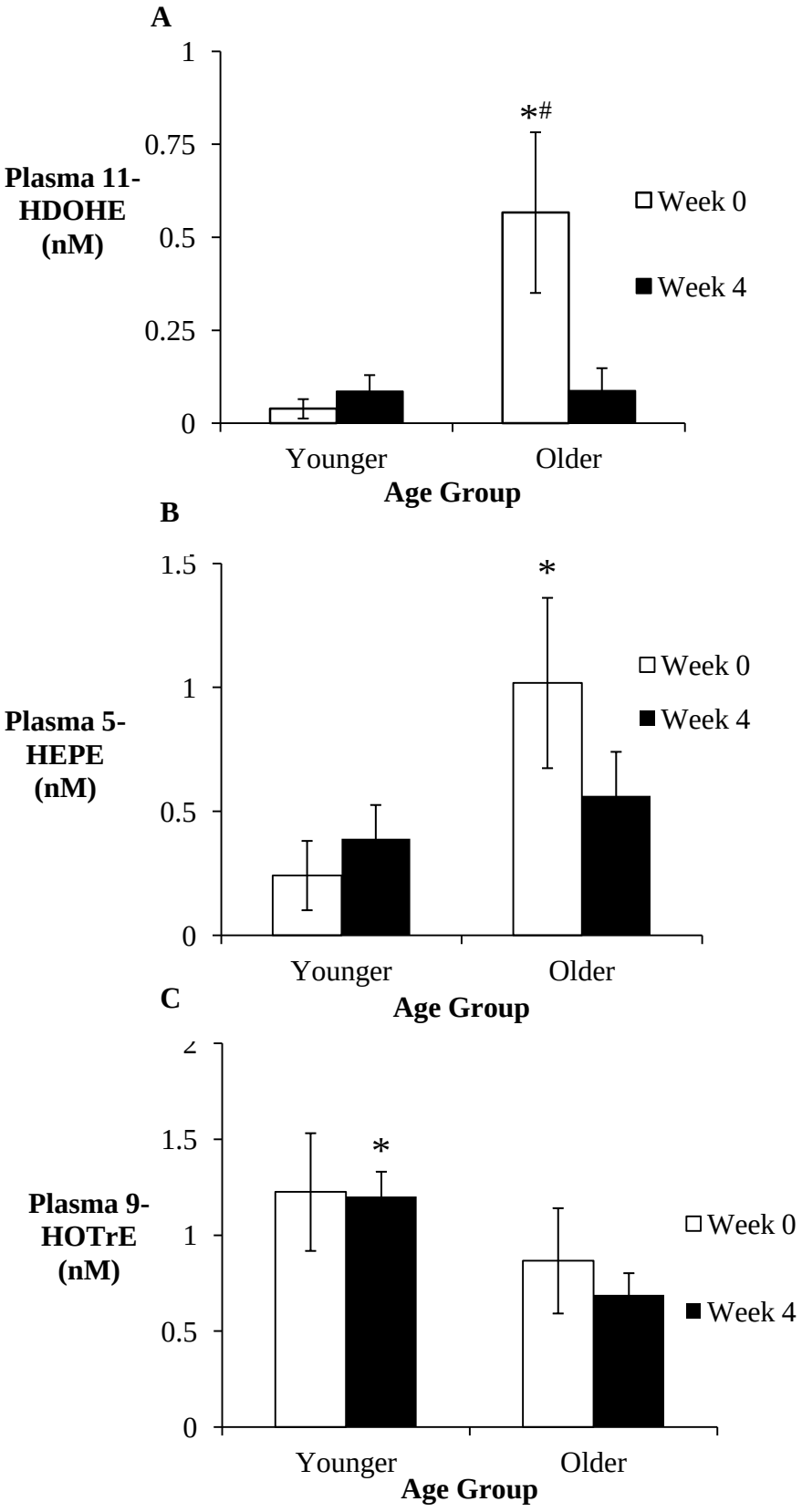

A
Plasma 11-HDOHE (nM)
1
0.75
0.5
0.25
0
*#
Week 0
Week 4
Younger
Older
Age Group
B
Plasma 5-HEPE (nM)
1.5
1
0.5
0
*
Week 0
Week 4
Younger
Older
Age Group
C
Plasma 9-HOTrE (nM)
2
1.5
1
0.5
0
*
Week 0
Week 4
Younger
Older
Age Group

Figure 7: Polyunsaturated n-3 fatty acid derived oxylipins in plasma from healthy subjects as a function of their age and the consumption of flaxseed.
Values represent the mean ± SEM. A) 11-HDOHE (11-hydroxydocosahexanoic acid). B) 5-HEPE (5-hydroxyeicosapentanoic acid). C) 9-HOTrE (9-hydroxyoctadecatrienoic acid). *Significant difference between age groups at same time point. # Significant difference between time points for the same age group ($p<0.05$). Data were analyzed using a 2-way general linear model.

3.5 Gender Differences in Plasma Oxylipin Concentrations and Response to Flaxseed Consumption

Comparisons between genders were also examined utilizing the Student's T-test or Wilcoxin Rank Sum test for Gaussian and non-Gaussian data, respectively. Of the 38 oxylipins quantified, only 14,15-dihydroxyeicosatrienoic acid (DHET) differed significantly between males and females at baseline. Females had significantly higher concentrations (0.61 ± 0.027 nM) than males (0.47 ± 0.047 nM). In response to flaxseed consumption, the change in three oxylipins differed between genders. Females exhibited a decrease in 11,12-DHET (-0.14 ± 0.055 nM), 14,15-DHET (-0.15 ± 0.024 nM), and 12-HEPE (-0.39 ± 0.22), whereas men showed a slight increase in the same oxylipins (0.085 ± 0.063, 0.052 ± 0.060, and 0.070 ± 0.088, respectively).

Discussion

The results of the present study demonstrate that there are significant differences in plasma oxylipin profiles in older and younger individuals. This study also demonstrates that consuming functional foods with flaxseed can alter the oxylipin profile and these changes can differ dependent upon age and, to a lesser extent, gender.

The older age group had significantly higher concentrations of several pro-inflammatory oxylipins including 5-HETE, 9,10,13-TriHOME, and 9,12-13-TriHOME compared to the younger group. 5-HETE is the most chemotactic HETE for neutrophil recruitment and progression of inflammation (8, 41). Serum and plasma concentrations of 5-HETE up to 1000 × higher have been observed in individuals with chronic inflammatory conditions such as asthma and rheumatoid arthritis (42). TriHOMEs are involved in the propagation of inflammation. Elevated concentrations have been measured in the fluid of the bronchoalveolar lumen in patients with asthma compared to normal healthy individuals and

concentrations further increase after birch pollen provocation (9). These elevated concentrations of pro-inflammatory oxylipins in the older age group reported here, therefore, may provide mechanistic information to explain the higher levels of inflammation in older versus younger individuals (43). Our findings are also consistent with the increased prevalence of inflammatory related conditions such as arthritis, diabetes, hypertension, and obesity with increased age (44). The elevation of pro-inflammatory oxylipins shown here may pre-dispose individuals to chronic disease conditions (45-47).

The differences in oxylipin profiles between age groups were not due to confounding factors such as the presence of chronic disease conditions, medications, differences in body mass index or differences in PUFA intake. The inclusion and exclusion criteria removed the former three factors and, although diet was not strictly controlled, plasma ALA, EPA, and DHA levels did not differ significantly at baseline or at 4 weeks between the two age groups (37). In addition, the plasma n6:n3 ratios for the younger and older groups at 4 weeks were also similar (10.8 ± 1.4 and 9.25 ± 1.1, respectively) (37). We can conclude, therefore, that age may represent an independent risk factor for the generation of pro-inflammatory oxylipins.

The present data demonstrate that a potential therapeutic strategy to correct the deleterious pro-inflammatory plasma oxylipin profile is via a dietary supplementation with flaxseed. After 4 weeks of consuming food products containing milled flaxseed, the plasma 5-HETE, 9,10,13-TriHOME and 9,12,13-TriHOME profiles of the older individuals became similar to that of the younger individuals and potentially less inflammatory. However, the benefits of dietary flaxseed on the oxylipin profile were not only observed in the older group but the younger group as well. The leukotoxin diol, 9,10-DiHOME, induces neutrophil chemotaxis (48) and is cytotoxic to renal proximal tubular cells (49). At baseline, the younger

group had significantly higher concentrations than the older age group, and after 4 weeks of flaxseed consumption, the younger group exhibited a significant decline from 4.5 ± 1.1 nM to 2.7 ± 0.37 nM.

The data above demonstrate a generalized capacity of dietary flaxseed and/or its bioactive components to reduce inflammation. Flaxseed supplementation in animal models of acute lung disease have exhibited reductions in neutrophil concentrations in bronchoalveolar lavage (50). Flaxseed supplementation has inhibited atherogenesis in animals through a reduction in the expression of inflammatory mediators like interleukin-6, mac-3 and vascular cell adhesion molecule-1 (32). Flaxseed lignans in food products reduced C-reactive protein in post-menopausal women (51) and dietary ALA reduced C-reactive protein, fibronectin, serum amyloid A, and white blood cell count in morbidly obese patients (52). These reports of a reduction in inflammation may be in part explained by the capacity of flaxseed to reduce the levels of the neutrophil aggregating oxylipins demonstrated in the present investigation. This is the first report of a human mechanistic study to potentially explain the anti-inflammatory effects of flaxseed.

There were two unexpected findings associated with the results from this study. First, although consuming flaxseed for 4 weeks resulted in an increase in plasma ALA in these subjects (37), there were no observed changes in ALA-derived oxylipins. Secondly, although there were no changes in plasma EPA and DHA, there were significant changes in oxylipins derived from these long chain n-3 PUFAs. It is possible that the high concentrations of ALA in flaxseed may have reduced the metabolism of long chain n-3 fatty acids to oxylipins. A recent study which provided 7.9 g of ALA to participants resulted in a reduced conversion of ALA to EPA and docosapentanoic acid compared to diets with lower ALA quantities (0.51-0.71 g) (53). It is possible, therefore, that fatty acid profiles are not always indicative of

oxylipin profiles. This is in agreement with a previous report on renal oxylipin profiles in diet-induced obese rats (25). In contrast, clinical trials providing participants with long chain n-3 ethyl ester supplementation resulted in increased plasma n-3 fatty acids and n-3 derived oxylipins after 4 weeks (23, 24). It is possible that functional foods take longer than ethyl ester fatty acid supplements to alter tissue fatty acid levels. In support of this hypothesis, ALA is more bioavailable when presented to the body as an oil in comparison to milled flaxseed (29). As oxylipins are produced from fatty acids bound to membrane phospholipids (54, 55), a dietary intervention period longer than 4 weeks may be required in order to observe increases in ALA-derived oxylipins in the plasma.

In addition to the influence of age on the oxylipin profile, the effect of gender was assessed. In the current study, only 14,15-DHET was significantly higher in the females at baseline. However, the women exhibited a decrease in 11,12-DHET, 14,15-DHET, and 12-HEPE, after 4 weeks of dietary intervention, whereas the men showed a slight increase. This supports the contention that not only do gender-based differences exist in fatty acid metabolism but that the two genders respond quite differently to dietary interventions that alter oxylipin metabolism and, ultimately, physiological functions. Gender based differences in fatty acid metabolism are frequently attributed to estrogenic effects (56-58). However, this will require further study to prove an association.

In conclusion, this is the first study to report differences in plasma oxylipin profiles based upon age and gender. The consumption of milled flaxseed resulted in potentially beneficial alterations in the plasma oxylipin profiles of both the younger and older individuals by reducing oxylipins that induce neutrophil chemotaxis and inflammation. The older groups' plasma oxylipin profile became similar to that of their younger counterparts as a function of the dietary flaxseed. The findings of this study, therefore, have implications for understanding

the biochemical changes associated with aging, the potential mechanisms of action responsible for the beneficial physiological effects of dietary flaxseed, and therapeutic strategies for disturbing inflammatory-related conditions and diseases.

Chapter II Section i References

1. Calder PC. Omega-3 fatty acids and inflammatory processes. Nutrients. 2010;2:355-74.

2. Oh SF, Pillai PS, Recchiuti A, Yang R, Serhan CN. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. J Clin Invest. 2011;121:569-81.

3. Serhan CN, Krishnamoorthy S, Recchiuti A, Chiang N. Novel anti-inflammatory--pro-resolving mediators and their receptors. Curr Top Med Chem. 2011;11:629-47.

4. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev. 2011;111:5922-43.

5. Spite M, Norling LV, Summers L, *et al*. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. Nature. 2009;461:1287-91.

6. Petasis NA, Akritopoulou-Zanze I, Fokin VV, *et al*. Design, synthesis and bioactions of novel stable mimetics of lipoxins and aspirin-triggered lipoxins. Prostaglandins Leukot Essent Fatty Acids. 2005;73:301-21.

7. Shearer GC, Newman JW. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. Curr Atheroscler Rep. 2009;11:403-10.

8. O'Flaherty JT, Thomas MJ, Lees CJ, McCall CE. Neutrophil-aggregating activity of monohydroxyeicosatetraenoic acids. Am J Pathol. 1981;104:55-62.

9. Lundstrom SL, Yang J, Kallberg HJ, *et al*. Allergic asthmatics show divergent lipid mediator profiles from healthy controls both at baseline and following birch pollen provocation. PLoS One. 2012;7:e33780.

10. Wakefield AP, Ogborn MR, Ibrahim N, Aukema HM. A dietary conjugated linoleic acid treatment that slows renal disease progression alters renal cyclooxygenase-2-derived prostanoids in the han: SPRD-cy rat. J Nutr Biochem. 2011.

11. Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in han:SPRD-cy rats with inherited kidney disease. Am J Physiol Renal Physiol. 2007;293:F821-30.

12. Ogletree ML. Overview of physiological and pathophysiological effects of thromboxane A2. Fed Proc. 1987;46:133-8.

13. Kulkarni PS, Roberts R, Needleman P. Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate. Prostaglandins. 1976;12:337-53.

14. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev. 2011;111:5922-43.

15. Zhao X, Yamamoto T, Newman JW, *et al*. Soluble epoxide hydrolase inhibition protects the kidney from hypertension-induced damage. J Am Soc Nephrol. 2004;15:1244-53.

16. Warford-Woolgar L, Peng CY, Shuhyta J, *et al*. Selectivity of cyclooxygenase isoform activity and prostanoid production in normal and diseased han:SPRD-cy rat kidneys. Am J Physiol Renal Physiol. 2006;290:F897-904.

17. Ogborn MR, Nitschmann E, Bankovic-Calic N, Weiler HA, Fitzpatrick-Wong S, Aukema HM. Dietary conjugated linoleic acid reduces PGE2 release and interstitial injury in rat polycystic kidney disease. Kidney Int. 2003;64:1214-21.

18. Oltman CL, Weintraub NL, VanRollins M, Dellsperger KC. Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. Circ Res. 1998;83:932-9.

19. Fischer DB, Christman JW, Badr KF. Fifteen-S-hydroxyeicosatetraenoic acid (15-S-HETE) specifically antagonizes the chemotactic action and glomerular synthesis of leukotriene B4 in the rat. Kidney Int. 1992;41:1155-60.

20. Lonnqvist F, Nyberg B, Wahrenberg H, Arner P. Catecholamine-induced lipolysis in adipose tissue of the elderly. J Clin Invest. 1990;85:1614-21.

21. Petersen KF, Befroy D, Dufour S, *et al*. Mitochondrial dysfunction in the elderly: Possible role in insulin resistance. Science. 2003;300:1140-2.

22. Carver JD, Benford VJ, Han B, Cantor AB. The relationship between age and the fatty acid composition of cerebral cortex and erythrocytes in human subjects. Brain Res Bull. 2001;56:79-85.

23. Keenan AH, Pedersen TL, Fillaus K, Larson MK, Shearer GC, Newman JW. Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers. J Lipid Res. 2012.

24. Shearer GC, Harris WS, Pedersen TL, Newman JW. Detection of omega-3 oxylipins in human plasma and response to treatment with omega-3 acid ethyl esters. J Lipid Res. 2010;51:2074-81.

25. Caligiuri SP, Love K, Winter T, *et al*. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr. 2013;143:1421-31.

26. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile and acceptability of bagels. J Food Sci. 2012;77:S62-70.

27. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile of muffins and snack bars. Food Res Int. 2011;44:2489.

28. Bassett CM, Rodriguez-Leyva D, Pierce GN. Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. Appl Physiol Nutr Metab. 2009;34:965-74.

29. Austria JA, Richard MN, Chahine MN, *et al*. Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. J Am Coll Nutr. 2008;27:214-21.

30. Ander BP, Edel AL, McCullough R, *et al*. Distribution of omega-3 fatty acids in tissues of rabbits fed a flaxseed-supplemented diet. Metabolism. 2010;59:620-7.

31. Edel AL, Aliani M, Pierce GN. Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults. J Chromatogr B Analyt Technol Biomed Life Sci. 2013;912:24-32.

32. Dupasquier CM, Dibrov E, Kneesh AL, *et al*. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am J Physiol Heart Circ Physiol. 2007;293:H2394-402.

33. Francis AA, Deniset JF, Austria JA M, *et al*. The effects of dietary flaxseed on atherosclerotic plaque regression. Am J Physiol Heart Circ Physiol. 2013.

34. Tomaz Pacheco J, Beltrame Daleprame J, Teles Boaventura G. Impact of dietary flaxseed (linum usitatissimum) supplementation on biochemical profile in healthy rats. Nutr Hosp. 2011;26:798-802.

35. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. Am J Physiol Heart Circ Physiol. 2011;301:H2220-6.

36. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013.

37. Patenaude A, Rodriguez-Leyva D, Edel AL, *et al*. Bioavailability of alpha-linolenic acid from flaxseed diets as a function of the age of the subject. Eur J Clin Nutr. 2009;63:1123-9.

38. Matthan NR, Ip B, Resteghini N, Ausman LM, Lichtenstein AH. Long-term fatty acid stability in human serum cholesteryl ester, triglyceride, and phospholipid fractions. J Lipid Res. 2010;51:2826-32.

39. Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. Methods Enzymol. 2007;432:59-82.

40. Hall LM, Murphy RC. Electrospray mass spectrometric analysis of 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids generated by lipid peroxidation of red blood cell ghost phospholipids. J Am Soc Mass Spectrom. 1998;9:527-32.

41. Goetzl EJ, Brash AR, Tauber AI, Oates JA, Hubbard WC. Modulation of human neutrophil function by monohydroxy-eicosatetraenoic acids. Immunology. 1980;39:491-501.

42. Chavis C, Fraissinet L, Chanez P, Thomas E, Bousquet J. A method for the measurement of plasma hydroxyeicosatetraenoic acid levels. Anal Biochem. 1999;271:105-8.

43. Franceschi C. Inflammaging as a major characteristic of old people: Can it be prevented or cured? Nutr Rev. 2007;65:S173-6.

44. Statistics Canada. 2013. http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/health51a-eng.htm Retrieved Feb 2014.

45. De Martinis M, Franceschi C, Monti D, Ginaldi L. Inflammation markers predicting frailty and mortality in the elderly. Exp Mol Pathol. 2006;80:219-27.

46. Vasan RS, Sullivan LM, Roubenoff R, *et al*. Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: The framingham heart study. Circulation. 2003;107:1486-91.

47. Gan WQ, Man SF, Senthilselvan A, Sin DD. Association between chronic obstructive pulmonary disease and systemic inflammation: A systematic review and a meta-analysis. Thorax. 2004;59:574-80.

48. Totani Y, Saito Y, Ishizaki T, Sasaki F, Ameshima S, Miyamori I. Leukotoxin and its diol induce neutrophil chemotaxis through signal transduction different from that of fMLP. Eur Respir J. 2000;15:75-9.

49. Moran JH, Weise R, Schnellmann RG, Freeman JP, Grant DF. Cytotoxicity of linoleic acid diols to renal proximal tubular cells. Toxicol Appl Pharmacol. 1997;146:53-9.

50. Kinniry P, Amrani Y, Vachani A, *et al*. Dietary flaxseed supplementation ameliorates inflammation and oxidative tissue damage in experimental models of acute lung injury in mice. J Nutr. 2006;136:1545-51.

51. Hallund J, Tetens I, Bugel S, Tholstrup T, Bruun JM. The effect of a lignan complex isolated from flaxseed on inflammation markers in healthy postmenopausal women. Nutr Metab Cardiovasc Dis. 2008;18:497-502.

52. Faintuch J, Horie LM, Barbeiro HV, *et al*. Systemic inflammation in morbidly obese subjects: Response to oral supplementation with alpha-linolenic acid. Obes Surg. 2007;17:341-7.

53. Gillingham LG, Harding SV, Rideout TC, *et al*. Dietary oils and FADS1-FADS2 genetic variants modulate [13C]alpha-linolenic acid metabolism and plasma fatty acid composition. Am J Clin Nutr. 2013;97:195-207.

54. Hammond VJ, O'Donnell VB. Esterified eicosanoids: Generation, characterization and function. Biochim Biophys Acta. 2012;1818:2403-12.

55. Buczynski MW, Dumlao DS, Dennis EA. Proteomics. an integrated omics analysis of eicosanoid biology. J Lipid Res. 2009;50:1015-38.

56. Giltay EJ, Gooren LJ, Toorians AW, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. Am J Clin Nutr. 2004;80:1167-74.

57. Romanski SA, Nelson RM, Jensen MD. Meal fatty acid uptake in adipose tissue: Gender effects in nonobese humans. Am J Physiol Endocrinol Metab. 2000;279:E455-62.

58. Jensen MD. Gender differences in regional fatty acid metabolism before and after meal ingestion. J Clin Invest. 1995;96:2297-303.

Acknowledgements

We gratefully acknowledge the technical support of Joy Gauthier and Tanja Winter.

Statement of Authorship: SPBC carried out the sample analyses, data analyses, statistical analyses, and helped draft the manuscript. HMA and AR provided intellectual insight with methodology and data analyses and helped to draft the manuscript. GP designed, conceived, and carried out the study, helped draft the manuscript and had final approval on all content. All authors read and approved the final manuscript.

Sources of Support: The work was supported through grants from Flax2015 and the Canadian Institutes for Health Research. The flaxseed was provided by the Flax Council of Canada. The Canadian International Grain Institute, Winnipeg, Manitoba, Canada created and produced the muffins for this study. Indirect research support was obtained from St Boniface Hospital Foundation. SPBC was a recipient of the Patient-Oriented Research Doctoral Award from the Canadian Institutes of Health Research. None of the sources of support influenced or had say in the study design, collection, analysis or interpretation of data, manuscript preparation or in the decision to submit for publication.

Conflict of Interest Statement: The authors have no conflicts of interest or disclosure

ii. Dietary Flaxseed and Blood Pressure in Patients with Peripheral Artery Disease

Due to the effects observed with dietary flaxseed on pro-inflammatory oxylipins in the age dependency trial, it was hypothesized that dietary flaxseed can lower blood pressure in the FlaxPAD trial by decreasing the concentration of pro-inflammatory and vasoconstrictive oxylipins. To test the current hypothesis, the following objectives were investigated:

1) Quantify the plasma oxylipin concentration in patients with hypertension and peripheral artery disease;
2) Identify any significant differences in the control and flaxseed groups after 6 months of the intervention; and
3) Identify any relationships between plasma oxylipins and blood pressure in the population.

In order to assess the current objectives, the following study was carried out.

Flaxseed Consumption Reduces Blood Pressure in Patients with Hypertension by Altering Circulating Oxylipins via an Alpha-Linolenic Acid Induced Inhibition of Soluble Epoxide Hydrolase

Published in: Hypertension. 2014 Jul;64(1):53-9.

Stephanie P.B. Caligiuri M.Sc,[1,2,4] Harold M. Aukema Ph.D,[1,5] Amir Ravandi M.D.,Ph.D., [2,4,6] Randy Guzman M.D.,[3] Elena Dibrov Ph.D,[1,2] Grant N. Pierce Ph.D[1,2,4]

[1]Canadian Centre for Agri-food Research in Health and Medicine (CCARM) and [2]the Institute of Cardiovascular Sciences, [3]Asper Clinical Research Institute, St. Boniface Hospital Research Centre, and [4]the Departments of Physiology, [5]Human Nutritional Sciences, and [6]Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Abstract

In a randomized, double-blinded, controlled clinical trial, participants with peripheral arterial disease (75% hypertensive) consumed 30 g of milled flaxseed/day for 6 months. The flaxseed group exhibited significant reductions in systolic (-10 mmHg) and diastolic (-7 mmHg) blood pressure. Flaxseed contains the n3 fatty acid alpha-linolenic acid. Plasma alpha-linolenic acid increased with ingestion of flaxseed and was inversely associated with blood pressure. However, the anti-hypertensive mechanism was unclear. Oxylipins derived from polyunsaturated fatty acids regulate vascular tone. Therefore, the objective was to examine if flaxseed consumption altered plasma oxylipins in a manner that influenced blood pressure. Plasma of FlaxPAD participants underwent solid phase extraction and HPLC-MS/MS analysis. The flaxseed group exhibited significant decreases in 8 plasma oxylipins versus control. Six of these (5,6-, 8,9-, 11,12-, 14,15-dihydroxyeicosatrienoic acid, 9,10- and 12,13-dihydroxyoctadecenoic acid) were products of soluble epoxide hydrolase, a pharmacological target for anti-hypertensive treatment. Patients exhibiting a decrease in total plasma soluble epoxide hydrolase-derived oxylipins, exhibited a significant decrease in systolic blood pressure (mean (95% confidence interval)) (-7.97 mmHg (-14.4, -1.50)) versus those who exhibited increased plasma soluble epoxide hydrolase-derived oxylipins (+3.17 mmHg (-4.78, 11.13)). These data suggest that a flaxseed bioactive may have decreased blood pressure via soluble epoxide hydrolase inhibition. Using a soluble epoxide hydrolase inhibitor screening assay, increasing concentrations of alpha-linolenic acid decreased soluble epoxide hydrolase activity ($p=0.0048$, $\rho=-0.94$). In conclusion, alpha-linolenic acid in flaxseed may have inhibited soluble epoxide hydrolase which altered oxylipin concentrations that contributed to the anti-hypertensive effects in patients with peripheral arterial disease.

Key Words: Lipids, eicosanoids, nutrition, peripheral vascular disease, oxylipin

Introduction

The World Health Organization identified high blood pressure as a global public crisis as it is the leading risk factor attributed to death worldwide.[1] Globally, 40% of individuals have hypertension and a significant proportion of individuals are undiagnosed, untreated, or have uncontrolled high blood pressure (1). Therefore, it is important to identify effective and desirable treatment strategies in order to reduce the prevalence and incidence of hypertension globally.

A potential therapeutic strategy for high blood pressure may be through dietary intervention. Recently, patients with peripheral arterial disease (PAD) (75% with hypertension) who consumed 30 g of milled flaxseed daily for 6 months exhibited significant reductions in systolic (-10 mmHg) and diastolic (-7 mmHg) blood pressure (the FlaxPAD Trial) (2). Flaxseed contains the n3 fatty acid alpha-linolenic acid (ALA), lignans and fibre. The participants consuming flaxseed exhibited significant increases in plasma ALA and enterolignans which were inversely associated with blood pressure (2). However, the biological mechanism of action responsible for the blood pressure lowering effects was unclear.

Oxylipins are a class of highly bioactive molecules that include the octadecanoids, eicosanoids and docosanoids. Oxylipins are derived endogenously from polyunsaturated fatty acids. As plasma concentrations of the polyunsaturated fatty acids, ALA and eicosapentanoic acid, increased in the flax group (2), we hypothesized that concentrations of oxylipins also changed. Oxylipins have a fundamental role in regulating vascular tone and inflammation. For example, some oxylipins produced from arachidonic acid or linoleic acid have been associated with inflammation (3-5), tissue damage (6-7), vasoconstriction (8-9), and oxidative stress (10). In contrast, the epoxyeicosatrienoic acids (EETs) produced from arachidonic acid are endothelial derived hyperpolarizing factors that are associated with vasodilation (11-13),

natriuresis (14-15), and may indirectly propagate vasodilation by activating endothelial nitric oxide synthase (16,17). EETs can be rapidly metabolized to dihydroxyeicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase (sEH) resulting in a concomitant loss of vasodilation (18,19). SEH also converts the protoxins, epoxyoctadecenoic acids (EpOMEs), to the dihydroxyoctadecenoic acids (DiHOMEs) which are potent cytotoxic and pro-inflammatory metabolites (21-23). Because products of sEH propagate loss of vascular relaxation and promote inflammation, targeting sEH inhibition pharmacologically has been used successfully to reduce blood pressure (15,24,25), hypertension associated renal damage (24), and infarction size (25) in animal models. The proposed relationship between epoxygenase-derived oxylipins and hypertension is depicted in **Figure 8.**

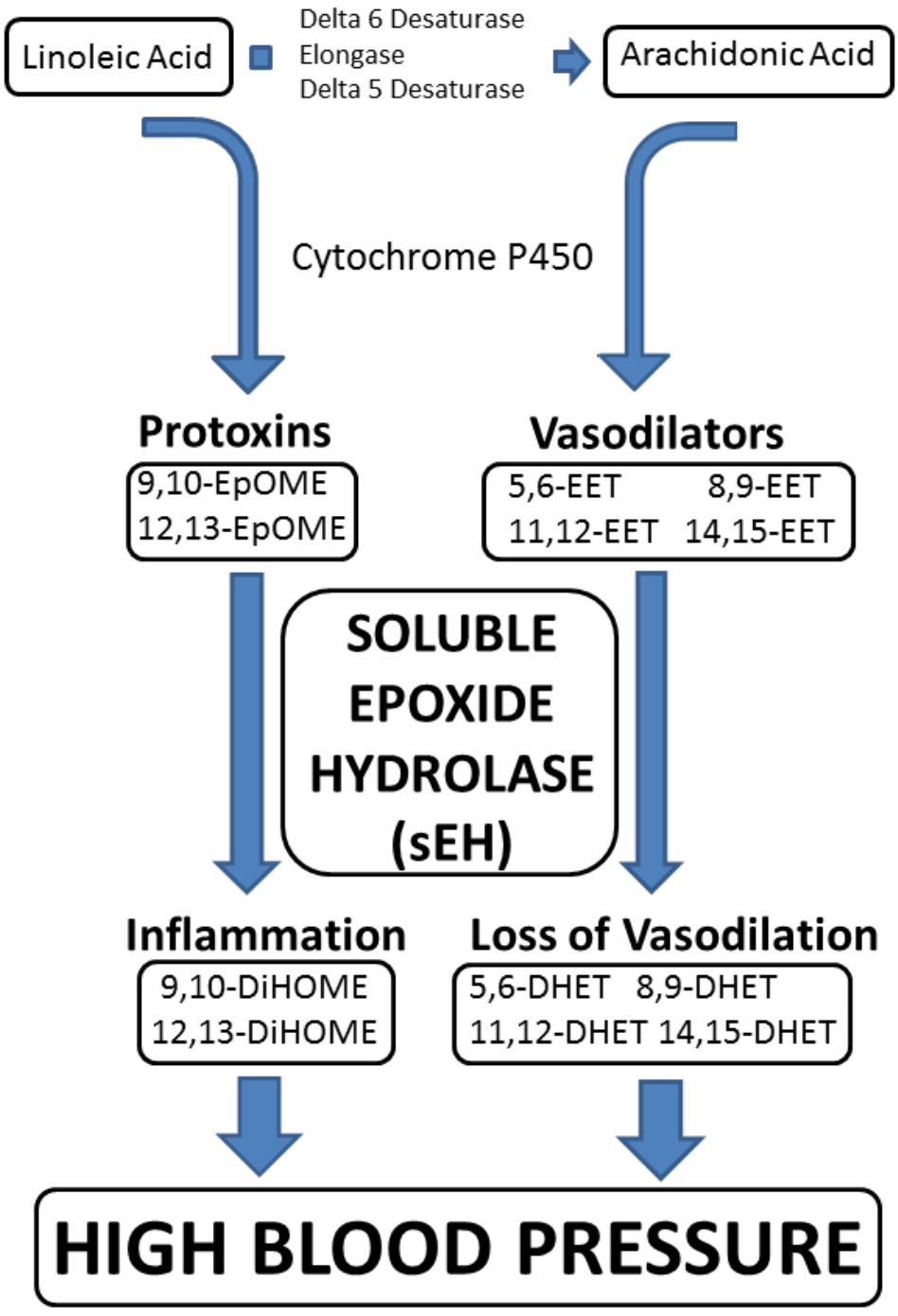


Figure 8: Schematic of Dihydroxyoctadecenoic Acid and Dihydroxyeicosatrienoic acid Production by Soluble Epoxide Hydrolase.

In order to test the hypothesis that flaxseed decreased the levels of oxylipins associated with inflammation and vasoconstriction, the study objectives were three-fold: (1) To characterize and quantify the plasma oxylipin profile of participants with peripheral arterial disease, (2) to compare changes in plasma oxylipin concentrations in the control and flaxseed groups, and (3) to determine the mechanism whereby any changes in oxylipins may have influenced blood pressure in the FlaxPAD Trial.

Methods

Participants and Food Products

A randomized, double-blinded, parallel, controlled clinical trial was established to determine the cardiovascular effects of dietary flaxseed in patients with peripheral arterial disease (26). Seventy-five percent of the participants were diagnosed as hypertensive at baseline (blood pressure ≥140/90 mmHg) (2,26). Participants (n=110) consumed food products containing either 30 g of milled flaxseed (treatment) or a combination of mixed dietary oils, milled wheat and bran (control) for 6 months. Further details of the food product composition (27,28), participant enrollment, intervention allocation, clinical characteristics, and blood pressure outcomes have been previously published (2,26).

The trial is registered on clinicaltrials.gov with the identifier: NCT00781950 and available at http://www.clinicaltrials.gov/ct2/show/NCT00781950?term=grant+pierce+flax&rank=1
All participants provided informed consent and all procedures were performed according to institutional guidelines. The trial was approved by Health Canada and its Natural Health Product Directorate, the University of Manitoba Research Ethics Board, and the St. Boniface Hospital Research Review Committee.

Oxylipin Analysis

Blood from baseline and 6 months was collected into ethylenediaminetetraacetic acid tubes and centrifuged at 4°C to isolate the plasma fraction and stored at -80°C. A total of 98 participants at baseline and 76 participants at 6 months had adequate plasma remaining for oxylipin analysis (200 µL). The oxylipin extraction and analysis methodology have been previously published (29,30) and are included in **Table 9.** Quantification of oxylipins was determined using the stable isotope dilution method (31) and expressed as nM.

Table 9: Collision Induced Dissociation Mass Transitions, Analyte Retention Time, Internal Standard and Detector Response Factors for All Oxylipins Quantified

Oxylipin	CID Mass Transition	Analyte Retention Time	Internal Standard	Detector Response Factor
	Da	*min*		
LA Derived Oxylipins				
9-HODE	295.1>170.6	15.2	9-HODE-d_4	1.54
13-HODE	295.1>194.9	15.1	13-HODE-d_4	1.44
9,10-DIHOME	313.0>200.8	13.4	9,10-DiHOME-d_4	2.78
12,13-DIHOME	313.0>183.0	13.1	12,13-DiHOME-d_4	2.36
13-oxoODE	293.0>112.9	15.0	5-OXoETE-d_7	3.86
9,10,13-TriHOME	329.0>171.0	7.3	9,10-DiHOME-d_4	1.85
9,12,13-TriHOME	329.0>211.0	7.2	12,13-DiHOME- d_4	0.740
12,13-EpOME	295.4>195.0	15.0	12,13-DiHOME- d_4	0.160
DGLA Derived Oxylipins				
15-HETrE	321.0>221.0	15.8	15-HETE-d_8	2.30
ALA Derived Oxylipins				
9-HOTrE	293.0>170.8	14.0	9-HODE-d_4	1.34
ARA Derived Oxylipins				
5,6-DHET	337.0>144.8	14.9	11,12-DHET-d_{11}	0.690
8,9-DHET	337.0>185.0	14.3	8,9-DHET-d_{11}	0.860
11,12 DHET	337.0>167.0	13.9	11,12-DHET-d_{11}	1.37
14,15-DHET	337.0>206.8	13.5	14,15-DHET-d_{11}	1.18
5-HETE	319.0>115.0	16.2	5-HETE-d_8	1.15
8-HETE	319.0>155.0	15.8	5-HETE-d_8	0.760

11-HETE	319.0>167.0	15.5	5-HETE-d_8	4.12
12-HETE	319.0>179.0	15.8	5-HETE-d_8	1.73
15-HETE	319.0>219.0	15.2	15-HETE-d_8	1.57
16-HETE	319.0> 232.8	14.7	15-HETE-d_8	2.04
18-HETE	319.0>261.0	14.5	15-HETE-d_8	1.41
PGD2	351>188.9	8.10	PGD_2-d_4	2.52
PGE2	351.2>271.0	7.67	PGE_2-d_4	1.61
6-keto-$PGF_{1\alpha}$	369.0>162.9	4.8	6-keto-$PGF_{1\alpha}$-d_4	1.85
$PGF_{2\alpha}$	352.9>192.8	7.35	$PGF_{2\alpha}$-d_4	2.22
TXB_2	369.1>168.9	6.57	TXB_2-d_4	1.52
EPA Derived Oxylipins				
5-HEPE	317.0>115.0	14.9	5-HETE-d_8	0.830
12-HEPE	317.0>179.0	14.5	5-HETE-d_8	1.49
18-HEPE	317.0>215.0	13.8	15-HETE-d_8	0.662
DHA Derived Oxylipins				
4-HDoHE	343.0>101.1	16.2	5-HETE-d_8	1.40
7-HDOHE	343.0>140.7	15.6	5-HETE-d_8	1.03
10-HDOHE	343.0>152.7	15.2	5-HETE-d_8	2.41
11-HDOHE	343.0>149.1	15.5	5-HETE-d_8	1.78
13-HDOHE	343.0>192.9	15.1	15-HETE-d_8	3.10
14-HDOHE	343.0>161.0	15.3	15-HETE-d_8	1.06
16-HDOHE	319.0>219.0	15.0	15-HETE-d_8	3.13
17-HDOHE	343.0>245.2	15.3	15-HETE-d_8	0.320
20-HDOHE	343.0>240.9	14.8	15-HETE-d_8	1.12
19,20-DiHDPA	361.0>229.0	13.3	14,15-DHET-d_{11}	0.210
Maresin	358.9>172.8	11.6	LTB_4-d_4	0.0499

[1]Abbreviations: alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-γ-linolenic acid, DGLA; dihydroxydocosapentaenoic acid, DiHDPA; dihydroxyeicosatrienoic acid, DHET; dihydroxyoctadecenoic acid, DiHOME; docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA; epoxyoctadecenoic acid, EpOME; hydroxydocosahexaenoic acid, HDOHE; hydroxyeicosapentaenoic acid, HEPE; hydroxyeicosatetraenoic acid, HETE; hydroxyeicosatrienoic acid, HETrE; hydroxyheptadecatrienoic acid, HHTrE; hydroxyoctadecadienoic acid, HODE; hydroxyoctadecatrienoic acid, HOTrE; linoleic acid, LA; oxooctadecadienoic acid, oxoODE; oxooctadecatrienoic acid, OXoOTrE; prostaglandin, PG; trihydroxyoctadecenoic acid, TriHOME ; thromboxane, TX.

sEH Percent Inhibition

The Soluble Epoxide Hydrolase Inhibitor Screening Assay Kit from Cayman Chemical (Item no. 10011671, Ann Arbor, Michigan, USA) was used to determine the influence of flaxseed bioactives or patient plasma lipid extract on sEH activity. Lipid extraction of patient plasma was performed according to the Folch method (32). ALA (Nu-Chek Prep Inc. MN, USA, item no. U-62-A), enterodiol (Sigma Aldrich Oakville, Ontario, Canada, item no. 45198) and enterolactone (Sigma Aldrich Oakville, Ontario, Canada, item no. 45199) were tested for their relationship to sEH activity. Linoleic acid (Nu-Chek Prep Inc. MN, USA, item no. U-59-A), was assessed as a potential negative control, as concentration did not change in either the flax or control group throughout the trial. Six physiological concentrations of each compound were utilized within the minimum and maximum plasma concentrations observed in the current population.

Inflammatory Markers

C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), and interleukin-10 (IL-10) were quantified in plasma using a Multiplex technology (Milliplex Cytokine/Chemokine magnetic bead panel, performed on Luminex MAGPIX, Millipore, Billerica, MA) and C-reactive protein (as part of WideScreen Human CVD Panel, EMD Millipore, Billerica, MA, performed on BioPlex 200, Bio-Rad Laboratories, Hercules, CA). Plasma samples were prepared according to the commercial protocols established for the Multiplex assay system.

Statistical Analysis

Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's Test, respectively. Due to the non-Gaussian distribution, data were analyzed with multiple 2-sided Mann-U-Whitney tests to assess differences between groups or time points.

Due to multiple testing, p-values were corrected with the false discovery rate correction. SEH inhibition data (Figure 3) were analyzed using Spearman's correlation. Sample sizes for each analysis are provided within tables and figures. Data are represented as a mean ± 95% confidence intervals.

Results

Absolute Plasma Oxylipin Concentrations

The plasma oxylipin concentrations of patients with peripheral arterial disease were quantified before and after dietary intervention **(Table 10).** Baseline plasma oxylipin profiles did not differ between the flaxseed and control group. After 6 months of dietary intervention, 6 oxylipins were significantly different between the control and flaxseed group ($p<0.05$). The ALA-derived 9-hydroxyoctadecatrieonic acid was significantly higher in the flax versus control group at 6 months. 11,12-DHET, 14,15-DHET, 4-hydroxydocosahexanoic acid (HDOHE), 20-HDOHE, and 19,20-dihydroxydocosapentanoic acid (DiHDPA) were significantly lower in the flax versus control group at 6 months. By comparison of time, the control group did not exhibit any significant differences in the absolute concentrations of plasma oxylipins from baseline to 6 months. The flax group exhibited a significantly lower concentration only in 14,15-DHET at 6 months versus baseline (Table 10).

Table 10: Plasma oxylipin concentration at baseline and 6 months in the control and flaxseed groups

Oxylipin	Control Baseline (n=45)	Control 6 months (n=38)	Flax Baseline (n=53)	Flax 6 months (n=38)
Linoleic Acid Derived				
9-HODE	8.49 (6.48, 10.5)	7.79 (6.48, 9.09)	8.90 (6.62, 11.2)	8.27 (6.70, 9.84)
13-HODE	9.25 (7.38, 11.1)	8.90 (7.72, 10.1)	10.6 (8.03, 13.2)	8.34 (7.30, 9.38)
9,10-DiHOME	2.97 (2.44, 3.51)	3.29 (2.47, 4.12)	4.08 (2.73, 5.42)	2.66 (2.32, 3.01)
12,13-DiHOME	2.86 (2.07, 3.65)	3.02 (2.50, 3.53)	3.55 (2.63, 4.46)	2.52 (2.22, 2.83)
13-OXoODE	6.89 (4.21, 9.57)	5.82 (4.61, 7.03)	10.1 (3.51, 16.7)	5.57 (4.25, 6.88)
9,10,13-TriHOME	3.39 (0.760, 6.03)	2.40 (1.94, 2.85)	6.28 (0.209, 12.3)	2.37 (1.88, 2.86)
9,12,13-TriHOME	2.04 (0.933, 3.15)	1.63 (1.35, 1.91)	3.70 (0.548, 6.86)	1.51 (1.17, 1.84)
Alpha-Linolenic Acid Derived				
9-HOTrE	0.701 (0.488, 0.914)	0.727 (0.541, 0.912)	0.862 (0.672, 1.05)	**1.05 (0.801, 1.30)**†
Dihomo Gamma Linolenic Acid Derived				
15-HETrE	0.461 (0.373, 1.06)	0.405 (0.324, 0.486)	0.720 (0.373, 1.06)	0.490 (0.207, 0.773)
Arachidonic Acid Derived				
5,6-DHET	0.253 (0.215, 0.291)	0.279 (0.226, 0.332)	0.289 (0.225, 0.353)	0.258 (0.170, 0.347)
8,9-DHET	0.161 (0.128, 0.194)	0.164 (0.129, 0.200)	0.221 (0.157, 0.284)	0.186 (0.128, 0.243)
11,12-DHET	0.657 (0.582, 0.732)	0.697 (0.587, 0.807)	0.678 (0.597, 0.759)	**0.587 (0.505, 0.668)**†
14,15-DHET	0.783 (0.719, 0.848)	0.867 (0.778, 0.956)	0.870 (0.785, 0.954)	**0.756 (0.670, 0.841)**†‡
5-HETE	1.27 (0.994, 1.55)	1.30 (1.03, 1.58)	1.58 (1.03, 2.13)	1.46 (0.850, 2.08)
8-HETE	0.609 (0.443, 0.775)	0.580 (0.401, 0.760)	0.878 (0.536, 1.22)	0.690 (0.337, 1.04)
11-HETE	0.742 (0.594, 0.891)	0.704 (0.546, 0.863)	0.897 (0.522, 0.127)	0.787 (0.242, 1.33)
12-HETE	3.63 (2.46, 4.79)	4.64 (2.55, 6.73)	4.07 (2.51, 5.62)	4.54 (2.88, 6.20)
15-HETE	1.13 (0.942, 1.32)	1.15 (0.915, 1.38)	1.22 (0.882, 1.57)	1.13 (0.751, 1.52)

16-HETE	0.116 (0.0570, 0.175)	0.185 (0.0638, 0.306)	0.153 (0.0805, 0.226)	0.111 (0.0426, 0.179)
18-HETE	0.257 (0.179, 0.335)	0.293 (0.214, 0.373)	0.379 (0.194, 0.564)	0.278 (0.0793, 0.477)
PGD2	5.32 (4.24, 6.41)	4.77 (3.46, 6.07)	8.00 (4.04, 12.0)	6.32 (4.49, 8.15)
PGE2	0.364 (0.312, 0.416)	0.340 (0.291, 0.390)	0.334 (0.290, 0.379)	0.357 (0.310, 0.403)
6k-PGF1alpha	0.714 (0.657, 0.772)	0.728 (0.650, 0.806)	0.719 (0.642, 0.795)	0.773 (0.666, 0.880)
PGF2a	0.288 (0.172, 0.404)	0.253 (0.112, 0.394)	0.279 (0.135, 0.424)	0.174 (0.0553, 0.292)
11dh-TXB2	124 (80.2, 168)	109 (73.3, 145)	97.5 (71.6, 124)	109 (79.2, 138)
TXB2	0.178 (0.0533, 0.302)	0.0888 (0.0262, 0.152)	0.0961 (0.0475, 0.145)	0.0813 (0.0329, 0.130)
Eicosapentanoic Acid Derived				
5-HEPE	0.553 (0.318, 0.788)	0.423 (0.263, 0.582)	0.787 (0.384, 1.19)	0.822 (0.299, 1.35)
12-HEPE	0.724 (0.435, 1.01)	1.08 (0.451, 1.71)	1.04 (0.590, 1.49)	1.29 (0.644, 1.94)
18-HEPE	0.0774 (0.00357, 0.158)	0.149 (0.0540, 0.245)	0.180 (0.0375, 0.322)	0.225 (0.00757, 0.442)
Docosahexanoic Acid Derived				
4-HDOHE	0.461 (0.263, 0.660)	0.460 (0.286, 0.634)	0.738 (0.379, 1.10)	**0.586 (0.0173, 1.19)†**
7-HDOHE	0.163 (0.0604, 0.265)	0.153 (0.0555, 0.251)	0.344 (0.0772, 0.611)	0.280 (0.0988, 0.659)
10-HDOHE	0.370 (0.227, 0.989)	0.340 (0.191, 0.490)	0.613 (0.236, 0.989)	0.526 (0.0417, 1.09)
11-HDOHE	0.701 (0.429, 0.974)	0.687 (0.463, 0.912)	1.05 (0.674, 1.43)	0.964 (0.394, 1.53)
13-HDOHE	0.213 (0.0560, 0.371)	0.175 (0.0791, 0.271)	0.350 (0.124, 0.575)	0.262 (0.0880, 0.613)
14-HDOHE	1.48 (0.929, 2.03)	2.07 (1.01, 3.14)	1.51 (0.900,2.12)	2.21 (0.979, 3.44)
16-HDOHE	0.275 (0.159, 0.392)	0.254 (0.156, 0.353)	0.417 (0.147, 0.687)	0.337 (0.00112, 0.672)
17-HDOHE	0.900 (0.578, 1.22)	0.814 (0.493, 1.14)	0.961 (0.381, 1.54)	0.816 (0.221, 1.41)
20-HDOHE	0.387 (0.254, 0.520)	0.366 (0.266, 0.466)	0.441 (0.191, 0.691)	**0.310 (0.0234, 0.643)†**
19,20-DiHDPA	1.50 (1.30, 1.69)	1.74 (1.53, 1.95)	1.54 (1.33,1.75)	**1.45 (1.19, 1.71)†**

Data is represented as mean (95% Confidence Interval).

† indicates at 6 months, the flax and control groups had significantly different concentrations of the respective oxylipin ($p<0.05$)

ǂ indicates the flax group had significantly lower levels of 14,15-DHET at 6 months versus baseline ($p<0.05$).

Change in Plasma Oxylipin Concentrations

Change in plasma oxylipin concentrations were calculated for those individuals who provided blood samples at baseline and 6 months with an adequate volume of plasma of at least 200 µL (n=76). Nine plasma oxylipins were significantly different between the control and flax group for change from baseline to 6 months ($p<0.05$). Participants in the flax group exhibited a decrease in docosahexanoic acid derived 19,20-DiHDPA (-0.248 nM (-0.73, 0.23)) and 20-HDOHE (-0.0239 nM (-0.41, 0.36)) which was significantly different from the control group that exhibited an increase (+0.19 nM (0.00267, 0.3728)) and (+0.023 nM (-0.13, 0.17)), respectively ($p<0.05$). By contrast, the flax group exhibited an increase in the docosahexanoic acid derived 4-HDOHE (+0.075 nM (-0.55, 0.71)) which was significantly greater than the control group (+0.047 nM (-0.24, 0.34)). The remaining six significant oxylipins were all derived from sEH ($p<0.05$) **(Figure 9).**

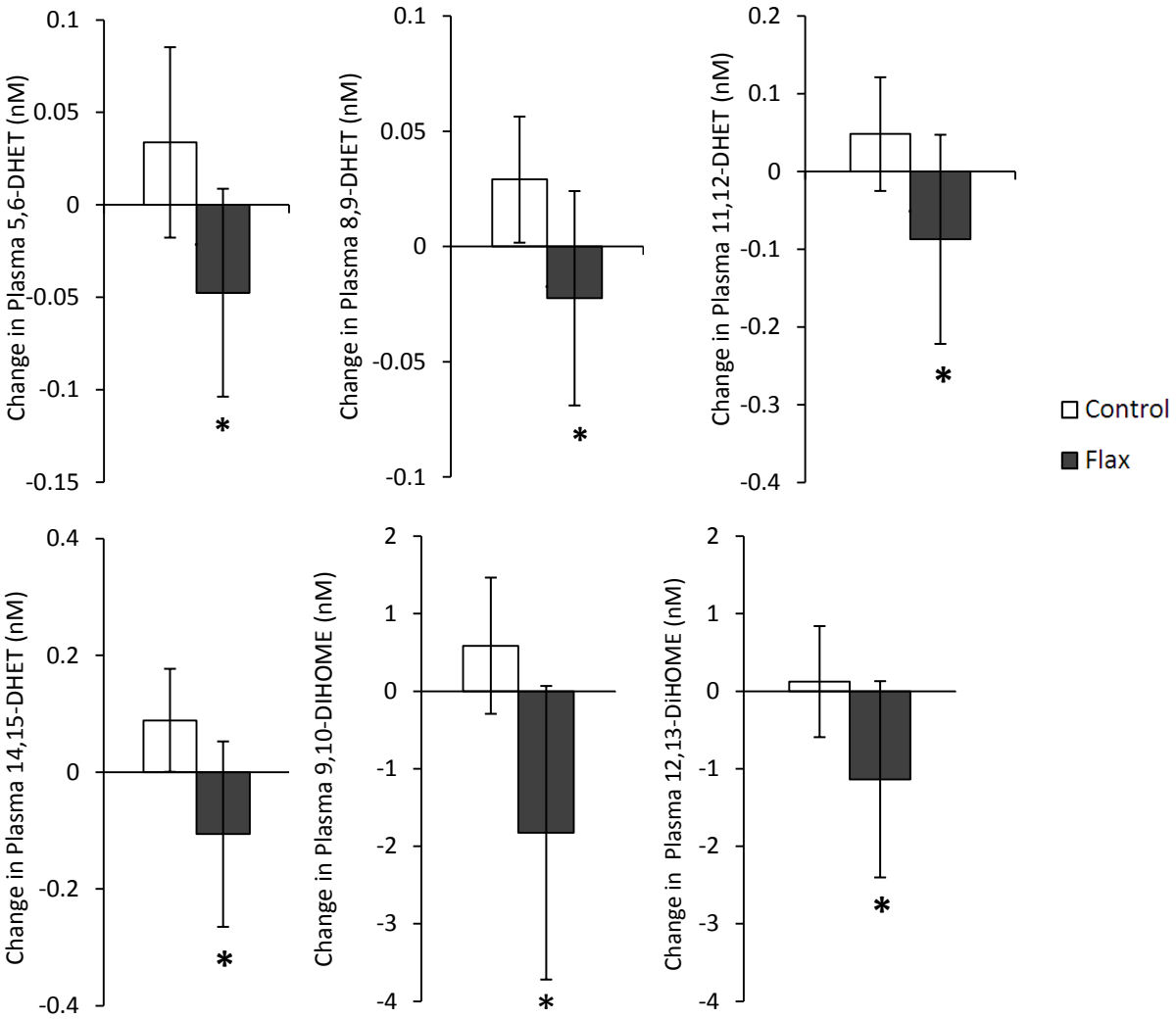


Figure 9: Change in Plasma Concentrations of sEH Derived Oxylipins in the Control and Flax Group from Baseline to 6 Months

Graphs represent mean ± 95% confidence intervals. * indicates statistical significance.

Association between Change in sEH Derived Oxylipins and Change in Blood Pressure

A reduction in total plasma sEH derived oxylipins was associated with a reduction in systolic blood pressure (-7.97 mmHg (-14.4, -1.50)). This was significantly different from those who exhibited an increase in total plasma sEH products who on average exhibited an increase in systolic blood pressure (+3.17 mmHg (-4.78, 11.1)) ($p = 0.048$). Change in diastolic blood pressure or mean arterial pressure were not significantly influenced by a change in total plasma sEH derived oxylipins ($p = 0.28$ and 0.11, respectively) **(Figure 10)**. When the change in sEH derived oxylipins was grouped by DHETs and DiHOMEs there was no significant association to change in blood pressure ($p > 0.05$). Upon analysis of individual sEH derived oxylipins, 9,10-DiHOME and 8,9-DHET were significantly associated with change in systolic blood pressure ($p = 0.048$ and 0.035, respectively) and mean arterial pressure ($p = 0.035$ and 0.035, respectively) (Figure 10).

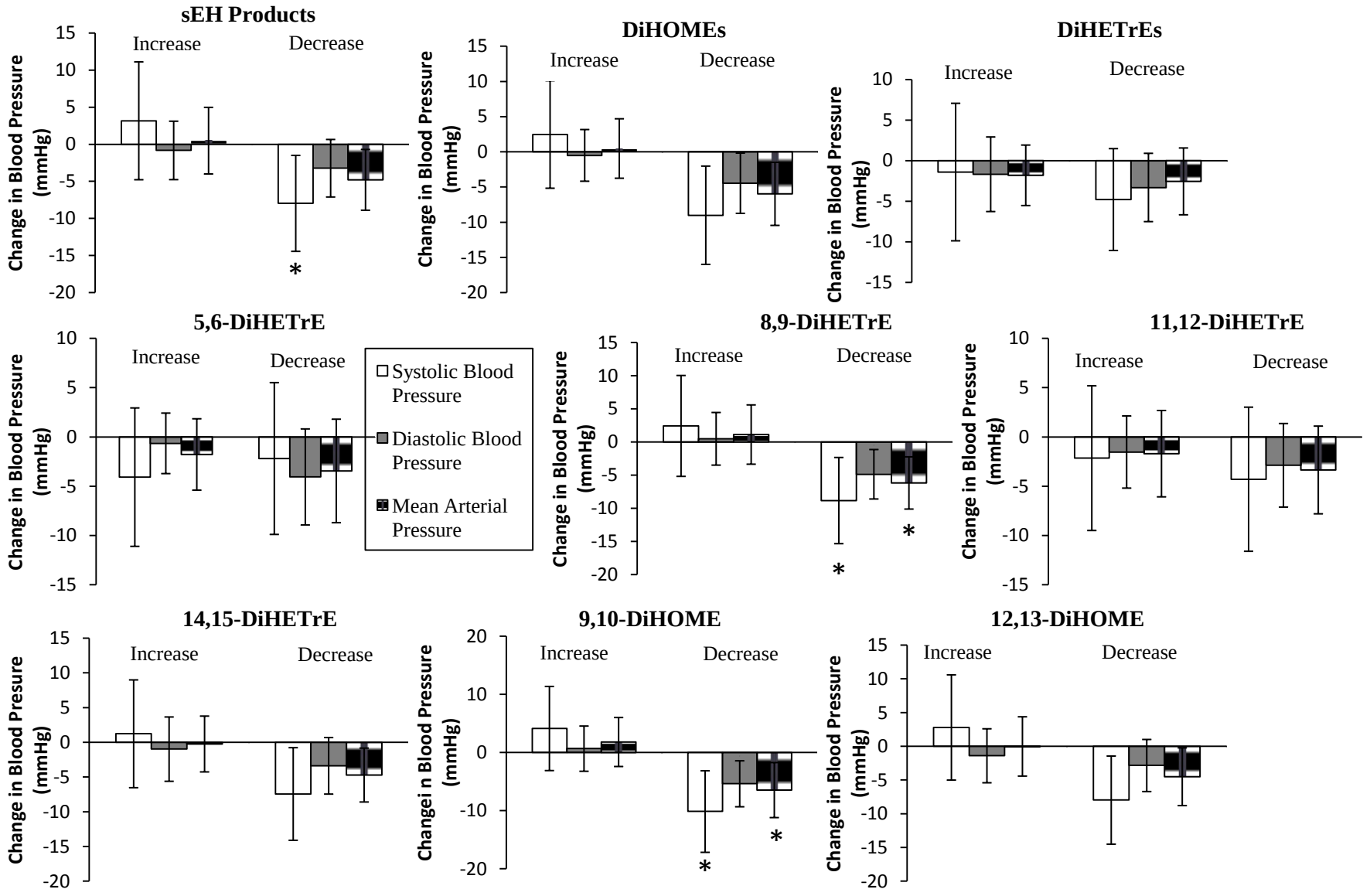


Figure 10: Change in Systolic, Diastolic, and Mean Arterial Pressure (mmHg) by Change in Plasma sEH Derived Oxylipins

Graphs represent mean ± 95% confidence intervals. * indicate statistical significance.

Inhibition of sEH

A strong inverse relationship was observed for ALA and % initial activity of sEH ($p=0.0048$, $\rho = -0.94$) **(Figure 11).** From the lowest ALA concentration (0.0075 mg/mL) to the highest (0.11 mg/mL), % initial activity of sEH declined from 43% to 12%. By contrast, enterodiol ($p=0.27$), enterolactone ($p=0.87$), and linoleic acid ($p=0.62$) were not significantly associated with sEH activity (Figure 4).

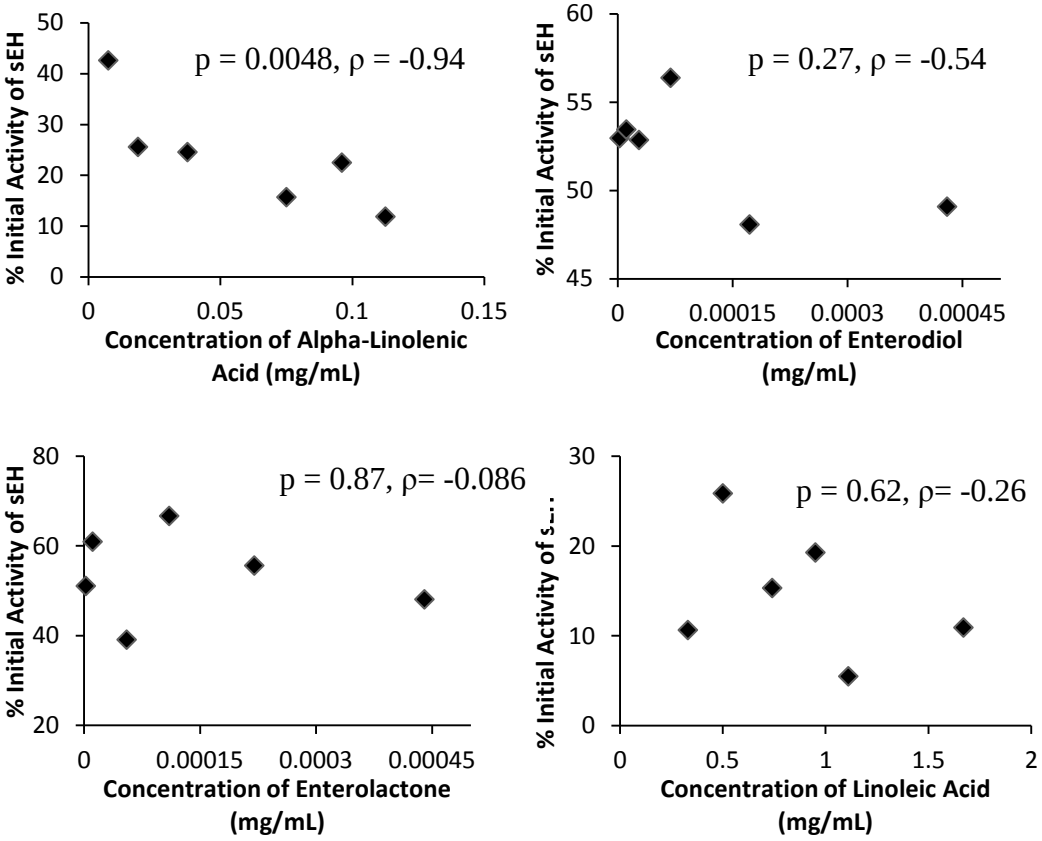


Figure 11: The Association Between Flaxseed Bioactives, Linoleic Acid, and sEH Inhibition.

Because increasing concentrations of ALA lowered sEH activity in the assay, it was hypothesized that plasma from patients in the flax group who had higher ALA concentrations by 6 months should lower sEH activity more than plasma from patients in the control group who had lower ALA concentrations. ALA concentrations in randomly selected plasma samples from patients (n=5/group) in the control group were 0.037 ± 0.008 mg/mL at baseline and 0.021 ± 0.007 mg/mL at 6 months. ALA concentrations in plasma samples from patients (n=5/group) in the flax group were 0.024 ± 0.006 mg/mL at baseline and 0.067 ± 0.0060 mg/mL at 6 months. Lipid extracts from these plasma samples were applied to the sEH assay to determine if the patient plasma could inhibit sEH activity. Percent initial activity of sEH fell by -10.2% (-30.7, 10.3) from baseline to 6 months in the flax group and by only -3.10% (-13.6, 7.44) from baseline to 6 months in the control group. Although this did not achieve statistical significance, the trend for the response was consistent with the hypothesis that ALA can inhibit sEH activity ($p = 0.13$). Interestingly, the change in plasma ALA concentration from 0.024 to 0.067 mg/mL in the flax group demonstrated the same 10% decrease in sEH activity as obtained in Figure 4 using 0.0375 to 0.075 mg/mL ALA. Similarly, the decrease in plasma ALA from 0.037 mg/mL to 0.021 mg/mL in the control group, resulted in a similar increase in sEH activity as obtained in Figure 4 using 0.0375 mg/mL to 0.01875 mg/mL, as both resulted in comparable 1-3% increases in sEH activity.

Inflammatory Markers

Plasma CRP, MCP-1, TNF-α, and IL-10 did not differ significantly by group or time point **(Table 11).** None of the inflammatory markers were significantly associated with the sEH derived oxylipins ($p>0.05$).

Table 11: Plasma Inflammatory Markers by Group and Time

Inflammatory Marker	Control Baseline (n=58)	Control 6 Months (n=41)	Flax Baseline (n=52)	Flax 6 Months (n=45)
CRP (ng/mL)	2782 (1929, 3636)	3178 (1315, 5041)	5186 (2522, 7852)	2563 (1859, 3257)
MCP-1 (pg/mL)	175.6 (163.3, 188.0)	200.9 (163.3, 238.6)	165.4 (154.5, 176.3)	179.4 (163.9, 194.9)
TNF-α (pg/mL)	7.378 (4.547, 10.21)	8.503 (5.405, 11.60)	8.305 (6.133, 10.48)	7.054 (5.193, 8.915)
IL-10 (pg/mL)	9.730 (3.639, 15.82)	8.901 (4.262, 13.54)	8.589 (4.792, 12.39)	6.688 (3.299, 10.08)

Data is presented as mean ± 95% Confidence Interval. Values were not significantly different from one another. C-reactive protein (CRP), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α), and interleukin-10 (IL-10).

Discussion

The ingestion of flaxseed in the FlaxPAD Trial resulted in a large decrease in systolic and diastolic blood pressure in patients with peripheral arterial disease and hypertension (2). Identifying the biological mechanism for this effect is critical in order to provide confidence in the antihypertensive action and insight into therapeutic targets. The present data provides evidence that consumption of flaxseed may reduce blood pressure by altering the plasma oxylipin profile via an inhibition of sEH. All six of the sEH derived oxylipins detected, significantly decreased in the flax group and increased in the control.

The relationship between sEH derived oxylipins and blood pressure was confirmed by the positive relationship between sEH derived oxylipins and systolic blood pressure. More specifically, the reductions in plasma 9,10-DiHOME and 8,9-DiHETrE were strongly associated with reductions in systolic and mean arterial blood pressure. A similar trend existed for a positive relationship between sEH products and diastolic blood pressure but this did not achieve statistical significance. A larger sample size may have been required. The relatively small reduction in diastolic blood pressure in comparison to systolic and mean arterial pressure may also have made it more difficult to detect a significant difference.

Since the flaxseed group exhibited significant reductions in all sEH derived oxylipins detected, it is reasonable to hypothesize that a bioactive component of flaxseed inhibited sEH. Of the flaxseed bioactives tested, ALA had the only significant relationship with sEH activity. This data is the first to demonstrate an n3 fatty acid inhibiting sEH. Utilizing lipid extracts from plasma obtained from the control and flax groups, the same trend was shown consistent with the hypothesis that plasma ALA had

the capacity to lower sEH activity. However, these results warrant further investigation in an *in vivo* animal model or in human tissue biopsy samples where sEH protein levels and sEH activity can be quantified. **Figure 12** represents the hypothesized ALA-induced alteration of the oxylipin profile and the influence on blood pressure.

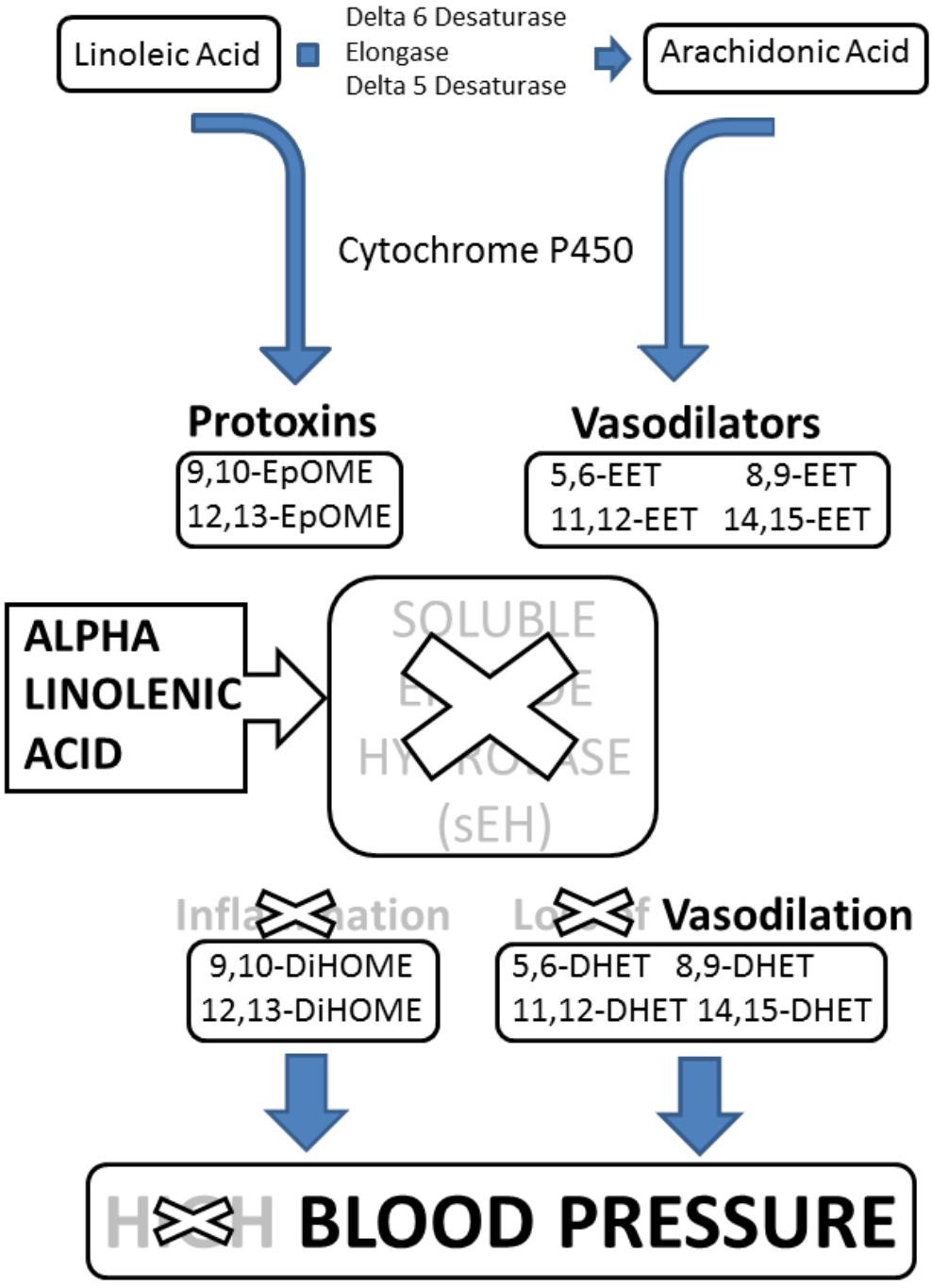


Figure 12: Schematic of Proposed ALA-Induced Alterations in the Oxylipin Profile

The association between sEH derived oxylipins and blood pressure in the current trial is consistent with previous animal studies. Pharmacological inhibition of sEH has resulted in a reduction of sEH-derived DIHETREs and blood pressure in animal studies (24, 25, 33, 34). The potential mechanisms behind the relationship between sEH derived oxylipins and blood pressure are numerous. The sEH substrates, EETs, have known vasodilatory actions (11-13, 15-19, 24, 25). However, due to the short half-life of EETs (35), these sEH substrates were below the level of detection and it was not possible to confirm if EETs increased in the patient population. Despite this, it is well established that the products of sEH have deleterious effects (18,19, 21-23), and a decrease in sEH products may provide protection against vasoconstriction and inflammation. Interestingly, CRP concentrations decreased to half the baseline concentration by 6 months in the flax group. However, none of the inflammatory markers achieved statistical significance. This could have been due to the large variation observed in this population. The above hypothesis for a mechanism of anti-hypertensive action does not discount the possibility that other oxylipins may have contributed as well. For example, reductions in docosahexanoic acid derived 19,20-DiHDPA and 20-HDOHE were observed in the flax group. Notably, although the biological actions of 19,20-DiHDPA are unknown, it is also thought to be an sEH product (36,37). By contrast, an increase in the docosahexanoic acid derived 4-HDOHE and 9-hydroxyoctadecatrienoic acid was observed in the flax group. However, the influence of these oxylipins on vascular tone or blood pressure has yet to be investigated.

Implications and Future Directions

The data in the present study provide evidence for the anti-hypertensive effects of

dietary flaxseed to be through an alteration in oxylipins that may be induced via an ALA-mediated inhibition of sEH. This knowledge lays the foundation to further investigate the influence of ALA on hypertension in both clinical settings and animal models. Most importantly, the data provide important mechanistic information to partially explain the significant anti-hypertensive action of dietary flaxseed (2) and ALA (38) which may be a desirable and effective treatment strategy for hypertensive patients.

Perspectives

The anti-hypertensive effects of dietary flaxseed are potent for hypertensive patients (2). The ALA content of flaxseed may provide this anti-hypertensive effect partly through an alteration of the oxylipin profile via an inhibition of soluble epoxide hydrolase. This enzyme has been identified in animal work as an anti-hypertensive target. Our work now supports this assertion and identifies the potential of ALA as a natural inhibitor of the enzyme. In view of the growing risk of hypertension as a burden of death today in most of the world (1), and the rising economic burden of the disease (2), having a nutritional strategy available as a complimentary approach is significant. Identifying the biological mechanism adds confidence to the anti-hypertensive actions of dietary flaxseed.

Acknowledgments

Flaxseed was provided by Flax Council of Canada. Food products were produced by Canada Bread, the Canadian International Grain Institute, and the Food Development Centre, Kelley Fitzpatrick is gratefully acknowledged for her support in the start up of the trial. We would like to thank Dr. Louise Simard and Ms. Valentina Ly in the Department of Biochemistry and Medical Genetics at the University of Manitoba for the use of their equipment to measure sEH activity. We are grateful to Renée LaVallée for her help with the plasma lipid extraction procedure. Lastly, we would like to acknowledge Joy Gauthier and Tanja Winter for establishing the HPLC-MS/MS methodology.

Disclosures

The authors have no disclosures or conflicts of interest.

Funding Sources

The work was supported through Flax2015, the Agri-food Research and Development Initiative, Canola Council of Canada, Canadian Institutes for Health Research (GNP), Natural Sciences and Engineering Research Council (to HMA), and the Heart and Stroke Foundation of Canada (AR). The support of St. Boniface Hospital Foundation is gratefully acknowledged. SPBC was a recipient of the Patient-Oriented Research Doctoral Award from the Canadian Institutes of Health Research.

Chapter II Section ii References

1. World Health Organization. A global brief on Hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization; 2013.

2. Rodriguez-Leyva D, Weighell W, Edel AL, Lavallee R, Dibrov E, Pinneker R, Maddaford TG, Ramjiawan B, Aliani M, Guzman R, Pierce GN. Potent antihypertensive action of dietary flaxseed in hypertensive patients. *Hypertension.* 2013; 62:1081-1089.

3. Shearer GC, Newman JW. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. *Curr Atheroscler Rep.* 2009; 11: 403-410.

4. O'Flaherty JT, Thomas MJ, Lees CJ, McCall CE. Neutrophil-aggregating activity of monohydroxyeicosatetraenoic acids. *Am J Pathol.* 1981; 104: 55-62.

5. Lundstrom SL, Yang J, Kallberg HJ, Thunberg S, Gafvelin G, Haeggstrom JZ, Gronneberg R, Grunewald J, van Hage M, Hammock BD, Eklund A, Wheelock AM, Wheelock CE. Allergic asthmatics show divergent lipid mediator profiles from healthy controls both at baseline and following birch pollen provocation. *PLoS One.* 2012; 7: e33780.

6. Wakefield AP, Ogborn MR, Ibrahim N, Aukema HM. A dietary conjugated linoleic acid treatment that slows renal disease progression alters renal cyclooxygenase-2-derived prostanoids in the Han: SPRD-cy rat. *J Nutr Biochem.* 2012; 23: 908-914.

7. Sankaran D, Bankovic-Calic N, Ogborn MR, Crow G, Aukema HM. Selective COX-2 inhibition markedly slows disease progression and attenuates altered prostanoid production in Han:SPRD-cy rats with inherited kidney disease. *Am J Physiol Renal Physiol.* 2007; 293: F821-30.

8. Ogletree ML. Overview of physiological and pathophysiological effects of thromboxane A2. *Fed Proc.* 1987; 46: 133-138.

9. Kulkarni PS, Roberts R, Needleman P. Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate. *Prostaglandins.* 1976; 12: 337-353.

10. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. *Chem Rev.* 2011; 111: 5922-5943.

11. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res.* 1996; 78: 415-423.

12. Oltman CL, Weintraub NL, VanRollins M, Dellsperger KC. Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. *Circ Res.* 1998; 83: 932-939.

13. Zhao X, Yamamoto T, Newman JW, Kim IH, Watanabe T, Hammock BD, Stewart J, Pollock JS, Pollock DM, Imig JD. Soluble epoxide hydrolase inhibition protects the kidney from hypertension-induced damage. *J Am Soc Nephrol.* 2004; 15: 1244-1253.

14. Romero MF, Madhun ZT, Hopfer U, Douglas JG. An epoxygenase metabolite of arachidonic acid 5,6 epoxy-eicosatrienoic acid mediates angiotensin-induced natriuresis in proximal tubular epithelium. *Adv Prostaglandin Thromboxane Leukot Res.* 1991; 21A: 205-208.

15. Kopkan L, Huskova Z, Sporkova A, Varcabova S, Honetschlagerova Z, Hwang SH, Tsai HJ, Hammock BD, Imig JD, Kramer HJ, Burgelova M, Vojtiskova A, Kujal P, Vernerova Z, Cervenka L. Soluble epoxide hydrolase inhibition exhibits antihypertensive

actions independently of nitric oxide in mice with renovascular hypertension. *Kidney Blood Press Res.* 2012; 35: 595-607.

16. Hercule HC, Schunck WH, Gross V, Seringer J, Leung FP, Weldon SM, da Costa Goncalves AC, Huang Y, Luft FC, Gollasch M. Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Arterioscler Thromb Vasc Biol.* 2009; 29: 54-60.

17. Honetschlagerova Z, Kitada K, Huskova Z, Sporkova A, Kopkan L, Burgelova M, Varcabova S, Nishiyama A, Hwang SH, Hammock BD, Imig JD, Kramer HJ, Kujal P, Vernerova Z, Cervenka L. Antihypertensive and renoprotective actions of soluble epoxide hydrolase inhibition in ANG II-dependent malignant hypertension are abolished by pretreatment with L-NAME. *J Hypertens.* 2013; 31: 321-332.

18. Sudhahar V, Shaw S, Imig JD. Epoxyeicosatrienoic acid analogs and vascular function. *Curr Med Chem.* 2010; 17: 1181-1190.

19. Falck JR, Krishna UM, Reddy YK, Kumar PS, Reddy KM, Hittner SB, Deeter C, Sharma KK, Gauthier KM, Campbell WB. Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation. *Am J Physiol Heart Circ Physiol.* 2003; 284: H337-49.

21. Moghaddam MF, Grant DF, Cheek JM, Greene JF, Williamson KC, Hammock BD. Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. *Nat Med.* 1997; 3: 562-566.

22. Moran JH, Weise R, Schnellmann RG, Freeman JP, Grant DF. Cytotoxicity of linoleic acid diols to renal proximal tubular cells. *Toxicol Appl Pharmacol.* 1997; 146: 53-59.

23. Zheng J, Plopper CG, Lakritz J, Storms DH, Hammock BD. Leukotoxin-diol: a putative toxic mediator involved in acute respiratory distress syndrome. *Am J Respir Cell Mol Biol.* 2001; 25: 434-438.

24. Honetschlagerova Z, Kitada K, Huskova Z, Sporkova A, Kopkan L, Burgelova M, Varcabova S, Nishiyama A, Hwang SH, Hammock BD, Imig JD, Kramer HJ, Kujal P, Vernerova Z, Cervenka L. Antihypertensive and renoprotective actions of soluble epoxide hydrolase inhibition in ANG II-dependent malignant hypertension are abolished by pretreatment with L-NAME. *J Hypertens.* 2013; 31: 321-332.

25. Neckar J, Kopkan L, Huskova Z, Kolar F, Papousek F, Kramer HJ, Hwang SH, Hammock BD, Imig JD, Maly J, Netuka I, Ostadal B, Cervenka L. Inhibition of soluble epoxide hydrolase by cis-4-(4-(3-adamantan-1-ylureido)cyclohexyl-oxy)benzoic acid exhibits antihypertensive and cardioprotective actions in transgenic rats with angiotensin II-dependent hypertension. *Clin Sci (Lond).* 2012; 122: 513-525.

26. Leyva DR, Zahradka P, Ramjiawan B, Guzman R, Aliani M, Pierce GN. The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with peripheral artery disease: rationale and design of the FLAX-PAD randomized controlled trial. *Contemp Clin Trials.* 2011; 32: 724-730.

27. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile and acceptability of bagels. *J Food Sci.* 2012; 77: S62-70.

28. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile of muffins and snack bars. *Food Res Int.* 2011; 44: 2489-2496.

29. Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and quantitation of eicosanoids via high performance liquid chromatography-electrospray ionization-mass spectrometry. *Methods Enzymol.* 2007; 432: 59-82.

30. Caligiuri SP, Love K, Winter T, Gauthier J, Taylor CG, Blydt-Hansen T, Zahradka P, Aukema HM. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. *J Nutr.* 2013; 143: 1421-1431.

31. Hall LM, Murphy RC. Electrospray mass spectrometric analysis of 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids generated by lipid peroxidation of red blood cell ghost phospholipids. *J Am Soc Mass Spectrom.* 1998; 9: 527-532.

32. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*. 1957;226:497-509.

33. Imig JD, Zhao X, Zaharis CZ, Olearczyk JJ, Pollock DM, Newman JW, Kim IH, Watanabe T, Hammock BD. An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. *Hypertension.* 2005; 46: 975-981.

34. Inceoglu B, Schmelzer KR, Morisseau C, Jinks SL, Hammock BD. Soluble epoxide hydrolase inhibition reveals novel biological functions of epoxyeicosatrienoic acids (EETs). *Prostaglandins Other Lipid Mediat.* 2007; 82: 42-49.

35. Inceoglu B, Jinks SL, Ulu A, Hegedus CM, Georgi K, Schmelzer KR, Wagner K, Jones PD, Morisseau C, Hammock BD. Soluble epoxide hydrolase and epoxyeicosatrienoic acids modulate two distinct analgesic pathways. *Proc Natl Acad Sci U S A.* 2008;105:18901-18906.

36. VanRollins, M., Baker, R.C., Sprecher, H., et al. Oxidation of docosahexaenoic acid by rat liver microsomes. *J. Biol. Chem*, 1984;259:5776-5783.

37. Oliw, E.H. and Sprecher, H.W. Metabolism of polyunsaturated (n-3) fatty acids by monkey seminal vesicles: Isolation and biosynthesis of ω-3 epoxides. *Biochim. Biophys. Acta*, 1991;1086:287-294.

38. Rodriguez-Leyva D, Dupasquier CMC, McCullough R, Pierce GN. The omega-3 fatty acid alpha linolenic acid within flaxseed provides beneficial cardiovascular effects. *Can J Cardiol*, 2010;26:489-496.

iii. Central Blood Pressure and Dietary Flaxseed Intervention

In the previous chapter, flaxseed effectively lowered brachial blood pressure in patients with peripheral artery disease and hypertension potentially through a modulation of oxylipins. However, central aortic blood pressure may be better than brachial blood pressure to predict future cardiovascular events. As a result, central aortic blood pressure was assessed in the FlaxPAD population. In addition, other mechanisms for the effects of flaxseed were investigated, including an alteration of arterial stiffnes or cardiac function. Through pulse wave analyses, central aortic blood pressure, arterial stiffness, and cardiac function were assessed non-invasively.

The following objectives will be assessed in the subsequent chapter:

1) Determine if flaxseed can influence central aortic blood pressure
2) Investigate the impact of flaxseed on arterial stiffness and cardiac function parameters
3) Determine the ability of plasma oxylipins to predict central aortic high blood pressure

Dietary flaxseed reduces central aortic blood pressure without cardiac involvement but through changes in plasma oxylipins

Submitted to: the Journal of the American College of Cardiology

Stephanie P. B. Caligiuri MSc[1-3] email: umcaligs@myumanitoba.ca

Delfin Rodriguez-Leyva MD, PhD[1-4] email: drodriguez@sbrc.ca

Harold M. Aukema PhD[1,5] email: harold.aukema@umanitoba.ca

Amir Ravandi MD, PhD[2-4] email: aravandi@sbrc.ca

Wendy Weighell RN[6] email: wweighell@sbgh.mb.ca

Randolph Guzman MD[6] email: rguzman@sbgh.mb.ca

Grant N. Pierce PhD, FACC[1-3] email: gpierce@sbrc.ca

[1]Canadian Centre for Agri-food Research in Health and Medicine, and [2]Institute of Cardiovascular Sciences, St. Boniface Hospital , [3]Department of Physiology and Pathophysiology, [4]Department of Internal Medicine, [5]Department of Human Nutritional Sciences, [6]Department of Surgery, University of Manitoba, Winnipeg, Manitoba, Canada;

Short Title: Flaxseed and central blood pressure

Abstract

Background: Dietary flaxseed generated a powerful reduction in brachial systolic blood pressure (SBP) and diastolic blood pressure (DBP) in patients with peripheral artery disease in the year long, randomized, double-blinded, controlled, FlaxPAD Trial.

Objectives: The current objectives were to: a) extend these data to central blood pressure (cBP), b) identify if changes in cardiac performance were involved in the BP changes, and c) identify the relationship of plasma oxylipins to cBP.

Methods: Radial tonometry and pulse wave analysis were used to non-invasively measure cBP and cardiac function parameters (n=62). Plasma oxylipins were analyzed with HPLC-MS/MS.

Results: After 12 months of dietary flaxseed intervention, average cSBP and cDBP decreased by 5 and 3 mmHg, respectively, from baseline ($p<0.05$). In a sub-group of patients with diagnosed hypertension, the average decrease in cSBP and cDBP versus control was 10 and 6 mmHg, respectively. Flaxseed did not lower blood pressure through changes in cardiac function or arterial elasticity. Flaxseed did not significantly impact heart rate, augmentation index, or other indices of cardiac function. Alternatively, the data support several specific oxylipins as potential mediators in the anti-hypertensive properties of flaxseed. For example, every 1 nM increase in plasma16-hydroxyeicosatetraenoic acid (HETE) increased the odds of higher cSBP and cDBP by 12-fold and 9-fold, respectively. In addition, every 1 nM increase in plasma thromboxane B_2 and 5,6-dihydroxyeicosatrienoic acid (DiHETrE) increased the odds of higher cBP by 33- and 9-fold, respectively. Flaxseed induced a decrease in many oxylipins which corresponded with a reduced risk of elevated cBP.

Conclusions: These data extend the anti-hypertensive properties of flaxseed to cBP and demonstrate these changes in blood pressure are without cardiac involvement. A strong relationship of plasma oxylipins to central hypertension was also identified. This may, therefore, identify new potential therapeutic targets for hypertension.

Key words: Hypertension, oxylipins, peripheral vascular disease, flaxseed, pulse wave analysis, central blood pressure, nutrition.

Abbreviations

CVD - cardiovascular disease, PAD - peripheral arterial disease, PG - prostaglandin, PWA - pulse wave analysis, PWV - pulse wave velocity, AI - augmentation index, BP - brachial blood pressure, SBP - brachial systolic blood pressure, DBP - brachial diastolic blood pressure, cBP - Central blood pressure, cSBP - central systolic blood pressure, cDBP - central diastolic blood pressure, cXBP - central mean blood pressure, TX – thromboxane, 6k - 6keto, ALA - alpha-linolenic acid, DiHDPA - dihydroxydocosapentanoic acid, DiHETrE - dihydroxyeicosatrienoic acid, DiHOME - dihydroxyoctadecenoic acid, HDOHE - hydroxydocosahexanoic acid, HEPE - hydroxyeicosapentanoic acid, HETE - hydroxyeicosatetraenoic acid, HETrE - hydroxyeicosatrienoic acid, HODE - hydroxyheptadecatrienoic acid, HOTrE - hydroxyoctadecatrienoic acid, oxoODE - oxooctadecadienoic acid, oxoOTrE - oxooctadecatrienoic acid, TriHOME - trihydroxyoctadecenoic acid

Introduction

In 2010 alone, hypertension accounted for 9.4 million deaths worldwide (1, 2) and represented an important economic burden (3). Dietary interventions represent an effective approach for the treatment of hypertension (4). Recently, in the Flax-PAD trial (5), dietary flaxseed was a powerful nutritional intervention that reduced brachial systolic blood pressure (SBP) and diastolic blood pressure (DBP) in patients with hypertension and peripheral artery disease (PAD) (5). A striking 15 and 7 mmHg reduction in SBP and DBP, respectively, was observed by 6 months. The α-linolenic acid (ALA) content of flaxseed was identified as the bioactive component responsible for the anti-hypertensive action of dietary flaxseed (5, 6). ALA directly inhibited the enzymatic activity of soluble epoxide hydrolase which reduced the concentrations of vasoconstrictive and pro-inflammatory oxylipins derived from soluble epoxide hydrolase in the plasma of the patients (6).

This previous work was done through conventional measurements of brachial blood pressure in patients. One of the aims of the present study was to investigate the capacity of flaxseed to influence central BP (cBP). Due to pulse pressure amplification, brachial BP may not correspond to cBP measured in arteries like the ascending aorta (8). Brachial BP is not always a good surrogate for the effect of anti-hypertensive drugs on arterial hemodynamics (8). Conversely, cBP is a better predictor of cardiovascular events and all-cause mortality in a wide range of populations (9). Another aim of the present study was to define the capacity of flaxseed to influence cardiac functional capacity and augmentation index (vascular stiffness) using pulse wave analysis (PWA) (10). It is possible that the changes in brachial BP induced by flaxseed may have occurred indirectly through a change in cardiac contractility or heart rate (7). However, this has not

been determined (5). Finally, it was important to determine if specific plasma oxylipins were associated with higher cBP. Dietary flaxseed decreased the concentration of pro-inflammatory and vasoconstricting oxylipins in the FlaxPAD Trial (6) but their relationship to cBP is unknown.

Methods

Experimental design: This clinical trial has been described previously (11) and is registered (NCT00781950) at clinicaltrials.gov. Briefly, 110 patients with PAD were recruited into this double blinded, controlled, randomized trial. Ethical approval was obtained as described (5, 11). The baseline characteristics of the Flax-PAD participants are found elsewhere (5, 11). The majority of these patients were on one or more medications to lower blood sugar, lipids, BP or thrombotic complications. The patients were provided a variety of foods (bagels, muffins, snack bars, buns, pasta, and tea biscuits) that contained 30 g of milled flaxseed or a control (wheat and mixed dietary oils) that resembled in appearance and texture the foods that contained flaxseed. One food product was ingested per day over one year. Details concerning the ingredients have been described previously (11-13).

Physiological Measurements: Radial applanation tonometry and the SphygmoCor PWV Medical software (Sydney, Australia) were used for generating and averaging the waveforms and pulse wave parameters. Values were used for analysis only if the operator index was ≥80, there was not an "inconclusive" result from the system, peripheral pulse maximum dP/dT was ≥300 mmHg/sec, ejection duration was ≥200 and ≤ 400 ms, and if the participant completed all time points with data meeting all quality criteria. The initial 110 patients at baseline was reduced to 62 patients for the current analysis due to attrition

(n=11 for control and n=13 for flax) and not meeting the above stated quality criteria for the PWA analyses (n=10 for control, n=14 for flax).

Plasma Oxylipins: Blood collected at baseline and 6 months was centrifuged at 4°C to isolate plasma in ethylenediaminetetraacetic acid tubes and stored at -80°C. Only plasma from baseline and 6 months were assessed for oxylipins due to an inadequate sample volume remaining for the 12 month period. Plasma oxylipins were analyzed by HPLC-MS/MS as previously described (6).

Statistical analysis: Statistical analyses were performed using SAS version 9.3 (Cary, NC, USA). Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene test, respectively. Pulse wave analysis parameters were analyzed using an advanced mixed model procedure with group as the between variable and time as the within variable. The Huynh-Feldt, compound symmetry, and unstructured models were run and the best model was chosen based on the Akaike, Bayesian, and Schwarz information criteria. To control for Type I error, a Bonferroni correction was applied. Associations of cBP to plasma oxylipins were assessed with logistic regression. Odds ratios were created with univariate logistic regression. Probability was assessed with the logit (p) equation produced from the logistic regression analyses. The binary division for logistic regression, or in other words, high cBP, was defined as 130/80 mmHg. As there are no indications to define high cBP, these BP limits were chosen in order to create a relatively equal population division in addition to providing the greatest power. All tests were set at a significance level of 0.05.

Results

The baseline characteristics of the patients from both groups in the FlaxPAD study as well as the CONSORT flow diagram have been published previously (5). Subject body weights, body mass index and waist circumference were not significantly different between flaxseed vs. control groups at any time point (15).

Central Blood Pressure

In this population (n=62), cSBP decreased in the flaxseed group by 3.4 and 4.9 mmHg at 6 and 12 months from baseline. The control group also exhibited a decrease in cSBP, but of a smaller magnitude with decreases of 1.7 and 3.3 mmHg at 6 and 12 months from baseline. The flaxseed and control groups were significantly different at all time points. CDBP decreased in the flaxseed group by 1.3 and 2.6 mmHg at 6 and 12 months from baseline. In contrast, the control group exhibited an increase in cDBP of 1.1 and 1.5 mmHg from baseline. CDBP was significantly different between the flaxseed and control group at 6 and 12 months (Figure 13).

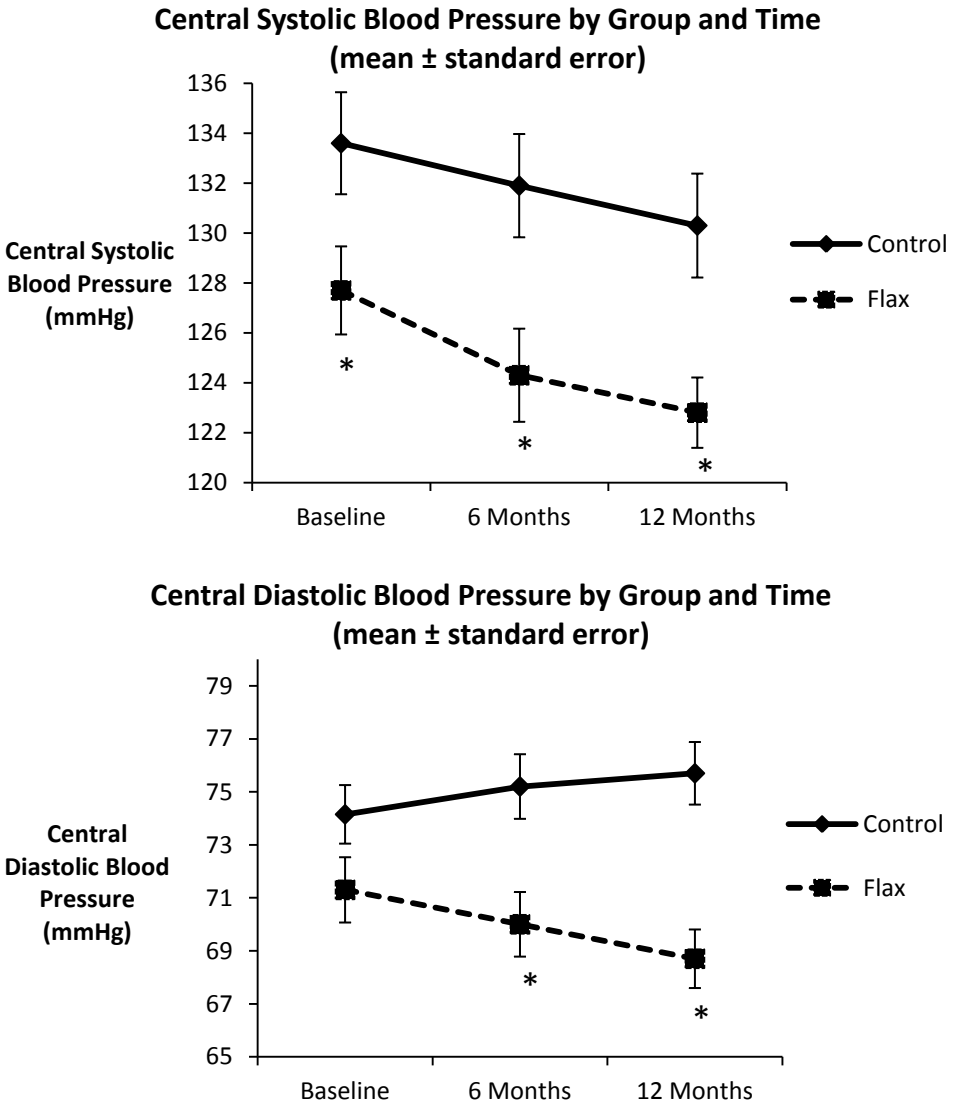


Figure 13. Central Systolic and Diastolic Blood Pressure by Group and Time (n=62). * p<0.05.

Factors to Determine Response to Flaxseed

Sub-group and logistic regression analyses were assessed in order to determine responders vs. non-responders. Therefore, cBP was analyzed in a subgroup of those diagnosed with hypertension at baseline (n=51). In patients with hypertension, the response to flaxseed was more prominent as the reduction in cSBP from baseline was 7.9 and 14.2 mmHg at 6 and 12 months, respectively. This was 6.3 and 9.7 mmHg lower than control at 6 and 12 months, respectively. At 12 months, cSBP was significantly different versus baseline and the control group ($p<0.05$). The reduction in cDBP from baseline in the flaxseed group was 5.3 and 8.7 mmHg at 6 and 12 months respectively. This was 1.7 and 5.5 mmHg lower than control at 6 and 12 months, respectively. CDBP in the flaxseed group at 12 months was significantly different from baseline ($p<0.05$), but not when compared to control ($p>0.05$) (Figure 14).

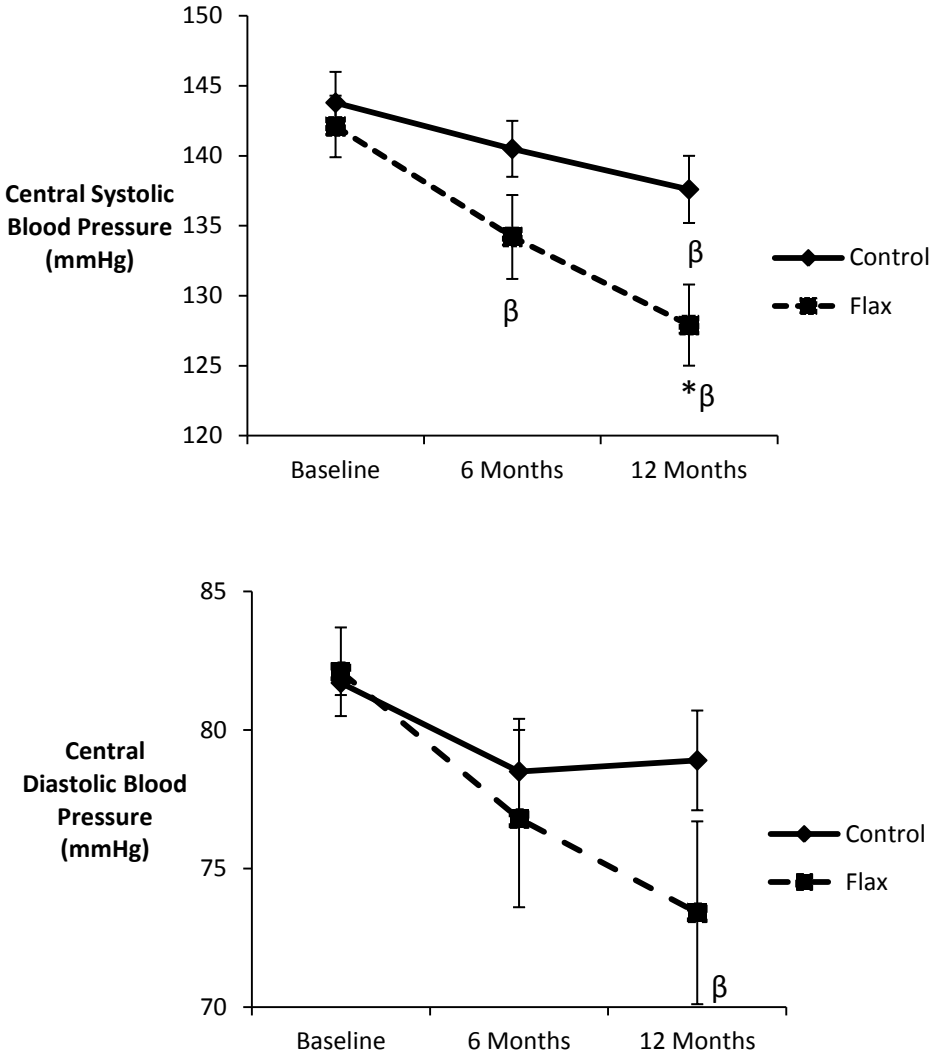


Figure 14. Central Systolic and Diastolic Blood Pressure by Group and Time in Those Diagnosed with Hypertension at Baseline (n=51). β - significantly different versus baseline ($p<0.05$). * - significantly different versus control ($p<0.05$).

Because ALA is the bioactive ingredient of flaxseed that was proposed to induce a reduction in brachial blood pressure (5), participants were sub-divided by change in plasma ALA from baseline. Participants were identified as either above average/average (≥61.0 μM) or below average (<61.0 μM) with respect to a change in plasma ALA. Participants with above average/ average changes in plasma ALA observed an average reduction from baseline of 8.2 mmHg in cSBP and 4.7 mmHg in cDBP by 12 months. In contrast, those in the below average change in plasma ALA group exhibited a decrease of 1.2 mmHg in cSBP and 0.8 mmHg in cDBP by 12 months (Figure 15).

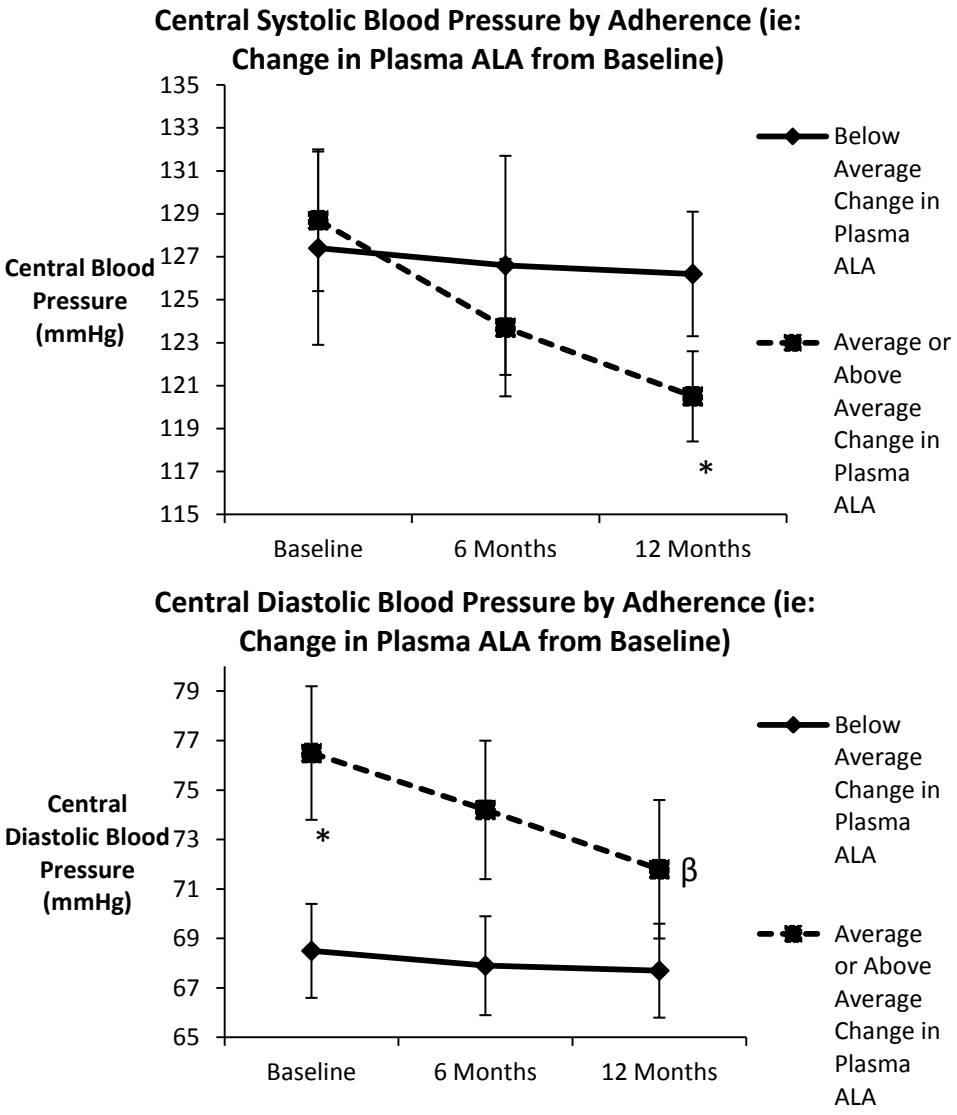


Figure 15. Central Systolic and Diastolic Blood Pressure by Change in Plasma ALA from Baseline. N = 18 for below average and n=12 for above average. β - significantly different versus baseline ($p<0.05$). * - significantly different versus control ($p<0.05$).

Utilizing multiple regression, every 1 μM increase in plasma ALA resulted in a decrease in cSBP and cDBP of 0.06 and 0.03 mmHg, respectively. Thirty percent of the patients in the flaxseed group exhibited an increase in plasma ALA greater than 100 μM. So for example, a patient with an increase in plasma ALA of 100 μM, would exhibit a decrease in cSBP and cDBP of 6 and 3 mmHg, respectively. Baseline plasma ALA may also be a predictor for blood pressure response as those in the above average/average group (ie: better responders) had a baseline plasma ALA concentration of 57 μM versus 89 μM in the below average group. Parameters such as age, gender, smoking status, diabetes mellitus, hyperlipidemia, body mass index, and waist circumference did not significantly influence blood pressure response to flaxseed.

Cardiac and Vascular Function

Indicators of cardiac function including heart rate, peripheral pulse dP/dt, ejection duration, central diastolic duration, and the central Buckberg subendocardial viability ratio, an index of myocardial oxygen supply and demand, did not change significantly over the 12 month intervention (Table 12). Some group*time interactions were significant at 12 months, however, this was due to both groups differing at baseline as well (Table 12).

Table 12 – Cardiac Function Measured with Pulse Wave Analysis by Group and Time

Parameter	Flaxseed (n=31) (mean ± SD)	Control (n=31) (mean ± SD)	P-Value *Group*	P-Value *Time*	P-Value *Group*Time*
Central End Systolic Pressure					
Baseline	112 ± 10.1	118 ± 13.4	0.020	0.063	0.089
6 Months	110 ± 12.0	117 ± 14.1			0.33
12 Months	109 ± 8.82	116 ± 15.4			0.017
Central Tension Time Index					
Baseline	2293 ± 421	2499 ± 317	0.0094	0.0013	0.024
6 Months	2245 ± 410	2412 ± 326			0.065
12 Months	2123 ± 313	2380 ± 323			0.0050
Central Diastolic Time Index					
Baseline	3343 ± 404	3410 ± 446	0.17	0.89	NS
6 Months	3279 ± 431	3475 ± 430			NS
12 Months	3313 ± 417	3473 ± 512			NS
Heart Rate					

Baseline	65.5 ± 10.0	71.8 ± 12.2	0.044	0.56	0.028
6 Months	66.2 ± 10.9	69.2 ± 11.7			0.29
12 Months	65.7 ± 10.1	71.9 ± 11.9			0.037
Peripheral Pulse Maximum dP/dT					
Baseline	1079 ± 381	1059 ± 313	0.69	0.26	NS
6 Months	1040 ± 360	993 ± 255			NS
12 Months	1047 ± 306	1029 ± 271			NS
Ejection Duration					
Baseline	314 ± 30.6	305 ± 27.3	0.28	0.00040	0.25
6 Months	312 ± 28.6	308 ± 34.5			0.62
12 Months	303 ± 29.7	294 ± 28.9			0.25
Central Diastolic Duration					
Baseline	625 ± 140	552 ± 113	0.035	0.66	0.025
6 Months	620 ± 138	581 ± 114			0.23
12 Months	632 ± 127	562 ± 122			0.031
Central Buckberg Sub-endocardial Viability Ratio					
Baseline	151 ± 36.8	139 ± 22.8	0.16	0.0023	0.10

6 Months	152 ± 35.3	146 ± 20.9			0.46
12 Months	160 ± 34.8	148 ± 28.8			0.13
Central Pulse Period					
Baseline	939 ± 155	858 ± 133	0.038	0.60	0.029
6 Months	932 ± 154	889 ± 141			0.25
12 Months	935 ± 145	856 ± 139			0.034
Central Ejection Duration/Period %					
Baseline	34.0 ± 4.29	36.1 ± 3.88	0.073	0.0048	0.043
6 Months	34.1 ± 4.35	35.1 ± 3.52			0.37
12 Months	32.8 ± 3.75	34.9 ± 4.42			0.045
Period Ejection Duration/Period %					
Baseline	66.0 ± 4.29	63.9 ± 3.88	0.071	0.0052	0.041
6 Months	65.9 ± 4.35	64.9 ± 3.52			0.37
12 Months	67.2 ± 3.75	65.1 ± 4.42			0.044

Data represents mean ± standard deviation and were analyzed using the advanced mixed model procedure. Group and time were analyzed and if significant results were rendered for either then the groups were assessed for statistical difference for each time period. NS: not significant. Post-hoc analyses were not included if the initial group and time analyses rendered non-significant results.

The augmentation index, as a measurement of arterial stiffness (16), did not significantly differ between the flaxseed and control group at 6 or 12 months. Augmentation load, central pulse height, start time of the reflected wave and radial/central pulse pressure amplification ratio were not significantly different between groups at 6 or 12 months (Table 13)

Table 13– Vascular Function Measured with Pulse Wave Analysis by Group and Time

Parameter	Flaxseed (n=31) (mean ± SD)	Control (n=31) (mean ± SD)	P-Value *Group*	P-Value *Time*	P-Value *Group*Time*
Central Augmentation/Pulse Height (%)					
Baseline	30.8 ± 8.81	30.7 ± 9.90	0.78	0.00010	0.99
6 Months	28.4 ± 8.93	30.3 ± 9.48			0.42
12 Months	26.4 ± 9.62	26.3 ± 9.96			0.96
Central Augmentation/Pulse Height Heart Rate Corrected (%)					
Baseline	14.1 ± 4.17	17.3 ± 6.23	0.032	0.00010	0.030
6 Months	12.5 ± 5.10	15.1 ± 6.21			0.069
12 Months	11.2 ± 5.43	13.4 ± 5.75			0.12
Augmentation Load					
Baseline	15.9 ± 3.99	15.9 ± 3.48	0.87	0.00010	0.78
6 Months	14.8 ± 4.12	15.4 ± 4.70			0.59
12 Months	13.3 ± 4.61	13.6 ± 4.67			0.86
Augmentation Time Index					

Baseline	136 ± 62.8	146 ± 52.5	0.51	0.00010	0.58
6 Months	123 ± 60.3	133 ± 60.8			0.51
12 Months	105 ± 57.1	113 ± 53.0			0.62
Central Pulse Height					
Baseline	57.2 ± 14.5	58.8 ± 16.2	0.68	0.048	0.67
6 Months	54.9 ± 15.0	56.3 ± 15.8			0.71
12 Months	53.9 ± 13.2	54.9 ± 14.6			0.77
Time of Start of Reflected Wave					
Baseline	132 ± 7.04	132 ± 8.80	0.65	0.0044	0.99
6 Months	134 ± 6.76	133 ± 7.74			0.63
12 Months	135 ± 6.78	133.7 ± 7.74			0.49
Pulse Pressure Amplification Ratio Radial/Central					
Baseline	124 ± 10.6	126 ± 12.8	0.75	0.00020	0.56
6 Months	128 ± 12.8	127 ± 14.5			0.71
12 Months	130 ± 13.5	132 ± 15.4			0.54
Central Augmented Pressure					
Baseline	17.5 ± 6.36	18.8 ± 8.12	0.39	0.00040	0.49

6 Months	15.7 ± 6.43	18.0 ± 9.00	0.24
12 Months	14.5 ± 7.08	15.2 ± 8.12	0.71

Data represents mean ± standard deviation and were analyzed using the advanced mixed model procedure. Group and time were analyzed and if significant results were rendered for either then the groups were assessed for statistical difference for each time period.

Relationship of plasma oxylipins to cBP

Forty oxylipins were identified and quantified in the plasma of the study participants. Of the 40 oxylipins, 19 were at least 1.4 fold higher in those with elevated cBP (≥130/80 mmHg). The oxylipins derived from docosahexanoic acid were generally elevated in those with higher cBP. Plasma maresin, the TriHOMEs, and 13-OxoDE were the only oxylipins that were lower in patients with elevated cBP (Figure 16).

Oxylipin	cSBP >130/ <130 mmHg	cDBP > 80/<80 mmHg	cXBP >100/<100 mmHg
Linoleic Acid Oxylipins			
9-HODE			
13-HODE			
9,10-DiHOME			
12,13-DiHOME			
13-OxoODE			
9,10,13 TriHOME			
9,12,13 TriHOME			
Dihomo γ-linolenic acid Oxylipins			
15 HETrE			
Alpha-Linolenic Acid Oxylipins			
9-HOTrE			
Arachidonic Acid Oxylipins			
11,12-DiHETrE			
14,15-DiHETrE			
5,6-DiHETrE			
8,9-DiHETrE			
5-HETE			
8-HETE			
11-HETE			
12-HETE			
15-HETE			
16-HETE			
17-HETE			
18-HETE			
6-k-PGF1α			
PGD2			
PGE2			
PGF2α			
TXB2			
Eicosapentanoic Acid Oxylipins			
5-HEPE			
12-HEPE			
18-HEPE			
Docosahexanoic Acid Oxylipins			
4-HDoHE			
7-HDoHE			
10-HDoHE			
11-HDoHE			
13-HDoHE			

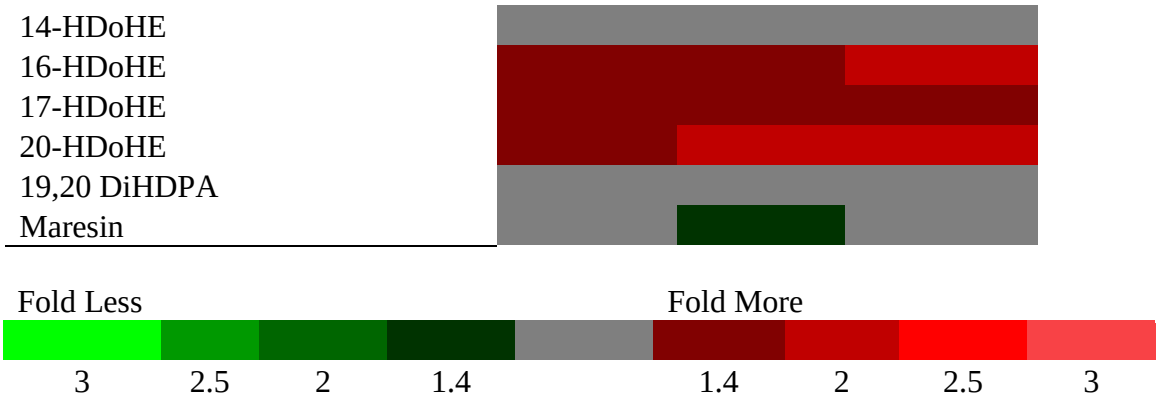


Figure 16. Heat map of average fold change in plasma oxylipins for higher versus lower central blood pressure.

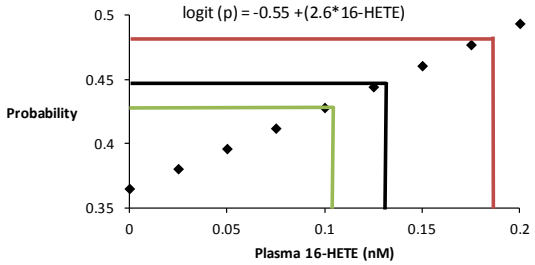
Study participants were divided into higher versus lower cSBP, cDBP, and cXBP. Higher blood pressure was chosen to be 130, 80, and 100 mmHg for cSBP, cDBP, and cXBP. The heat map was created by dividing the concentrations of oxylipins of the individuals in the higher blood pressure versus lower blood pressure category to achieve a fold difference. Abbreviations: dihydroxydocosapentanoic acid, DiHDPA; dihydroxyeicosatrienoic acid, DiHETrE; dihydroxyoctadecenoic acid, DiHOME; hydroxydocosahexanoic acid, HDOHE; hydroxyeicosapentanoic acid, HEPE; hydroxyeicosatetraenoic acid, HETE; hydroxyeicosatrienoic acid, HETrE; hydroxyheptadecatrienoic acid , HODE; hydroxyoctadecatrienoic acid, HOTrE; oxooctadecadienoic acid, oxoODE; oxooctadecatrienoic acid, oxoOTrE, trihydroxyoctadecenoic acid ; TriHOME.

Utilizing logistic regression analyses, eight plasma oxylipins were identified as significant predictors for higher cBP (Table 14). For example, every 1 nM increase in plasma thromboxane (TX) B_2, prostaglandin (PG) E_2 and 11,12-Dihydroxyeicosatrienoic acid (DiHETrE) increased the odds of higher cBP by 33-, 26-, and 9-fold, respectively. Because the logistic regression is indicated as per 1 nM increase, the concentration of oxylipins in the plasma must be taken into consideration. As a result, probability slopes by plasma oxylipin concentration are calculated in Figure 5. For example, within the concentration range of plasma 6ketoPGF$_{1\alpha}$ (6kPGF$_{1\alpha}$) in this population, the probability of high cBP ranged from 0 to 80%. 6kPGF$_{1\alpha}$ was the sole oxylipin identified as decreasing the odds of higher cDBP. The flax group at 6 months exhibited an increase in plasma 6kPGF$_{1\alpha}$ and a decrease in the probability of higher cDBP. In contrast, the control group exhibited a slight decrease in plasma 6kPGF$_{1\alpha}$ and an increase in the probability of higher cDBP (Figure 17). Plasma 16-HETE was a strong indicator of all 3 cBP parameters, cSBP, cDBP, and central mean BP (cXBP). Dietary flaxseed induced a reduction in plasma 16-HETE by 6 months (Figure 17). Plasma oxylipin concentrations by group and time have been previously published elsewhere (6). But of the oxylipins indicated in Figure 17, only 11,12-DiHETrE decreased in the flaxseed group and was significantly different versus control by 6 months (6).

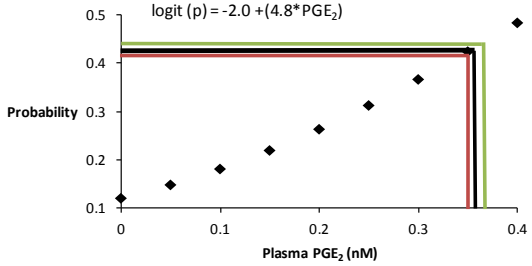
Table 14 – Odds Ratios of Higher Central Blood Pressure by Plasma Oxylipin

Variable	Odds Ratio	95% Wald Confidence Interval	p-value
Central Systolic Blood Pressure			
16-HETE	14.1	(13.1, 15.0)	0.0169
PGE_2	26	(14, 39)	0.00280
TXB_2	33.4	(30.3, 36.9)	0.0268
Central Diastolic Blood Pressure			
16-HETE	11.7	(11.0, 12.4)	0.0173
18-HETE	7.15	(6.78, 7.55)	0.0270
$6kPGF_{1\alpha}$	0.0870	(0.0810, 0.0940)	0.0364
Central Mean Arterial Pressure			
11,12-DiHETrE	8.61	(8.14, 9.12)	0.0190
16-HETE	7.72	(7.24, 8.23)	0.0495

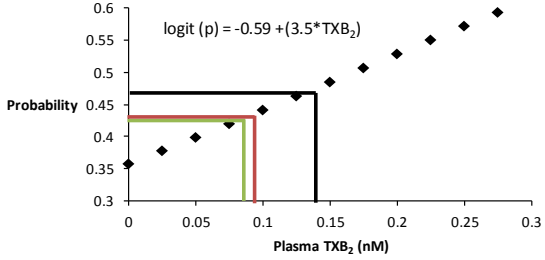
Probability of Higher Central Systolic Blood Pressure by Plasma 16-HETE Concentration



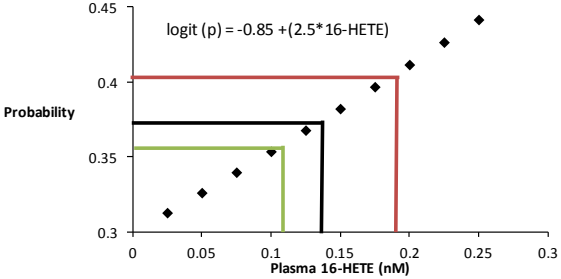
Probability of Higher Central Systolic Blood Pressure by Plasma PGE_2 Concentration



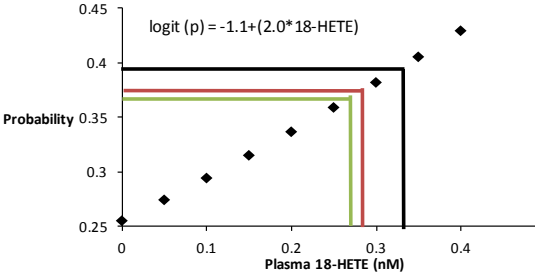
Probability of Higher Central Systolic Blood Pressure by Plasma TXB_2 Concentration



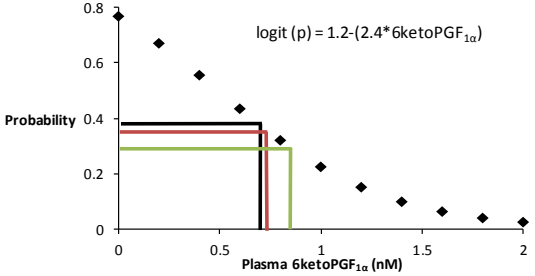
Probability of Higher Central Diastolic Blood Pressure by Plasma 16-HETE Concentration



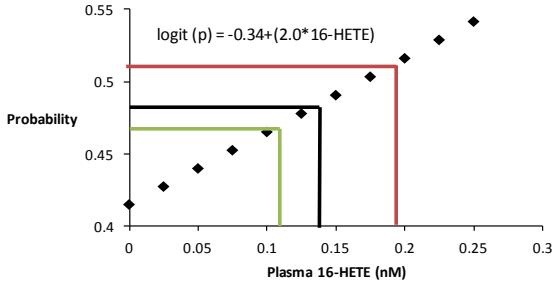
Probability of Higher Central Diastolic Blood Pressure by Plasma 18-HETE Concentration



Probability of Higher Central Diastolic Blood Pressure by Plasma 6ketoPGF$_{1\alpha}$ Concentration



Probability of Higher Mean Arterial Pressure by Plasma 16-HETE Concentration



Probability of Higher Mean Arterial Pressure by Plasma 11,12-DiHETrE Concentration

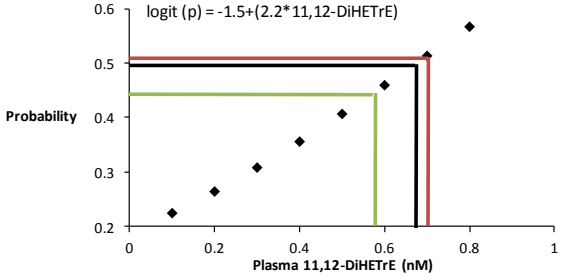


Figure 17. Predicting the probability of higher central blood pressure by plasma oxylipin concentration and the influence of flaxseed or control after 6 months.
Black line (baseline), red line (control at 6 months), and green line (flaxseed group at 6 months) indicates the average plasma oxylipin concentration and the associated probability of higher central blood pressure. Abbreviations: 6 keto prostaglandin $F_{1\alpha}$, 6ketoPG$F_{1\alpha}$; dihydroxyeicosatrienoic acid, DiHETrE; hydroxyeicosatetraenoic acid, HETE; prostaglandin E_2, PGE_2; thromboxane B_2, TXB_2.

Discussion

Dietary flaxseed can induce striking decreases in brachial BP (5) and in the present report, these decreases were also detected in cSBP and cDBP. An even more pronounced effect was observed in those patients diagnosed with hypertension at baseline. The reduction in cSBP (10 mmHg) and cDBP (6 mmHg) was similar to the average decrease in brachial SBP (15 mm Hg) and DBP (7 mm Hg) reported previously (5). In another trial, a 10 mmHg increase in cSBP was associated with a significant 8.8% increase in total cardiovascular events (cardiovascular death and nonfatal cardiovascular events - myocardial infarction, stroke, coronary artery re-stenosis) in patients with a variety of chronic vascular conditions (7). Therefore, with a similar decrease in cSBP, one could reasonably expect a 9% decrease in cardiovascular events.

Potential mechanisms of action

Identifying the mechanisms involved or, conversely, not responsible for the anti-hypertensive effects of flaxseed are essential for therapeutic development. Two factors can now be discounted as mechanistic factors involved in the antihypertensive action of flaxseed. First, given the stability of heart rate and the unchanged peripheral max dP/dt in both groups of patients, we can conclude that cardiac contractile function was not affected in the group of patients ingesting flaxseed. Peripheral max dP/dt calculations are correlated with invasive measurements of left ventricular dP/dt max (r=0.87) (18). Left ventricular dP/dt is an attractive, robust and sensitive indicator of changes in cardiac contractility (19). Left ventricular dP/dt can be influenced by heart rate but this was unchanged in the present study as was its load dependency (19). Thus, although changes in heart rate and cardiac contractile function can influence blood pressure (7), the data here suggest that it is unlikely that the antihypertensive actions of dietary flaxseed were

achieved through any alteration in cardiac function. Secondly, aortic stiffness has also been recognized as an independent predictor of cardiovascular and all-cause mortality in patients with essential hypertension (20). In the present trial, neither the augmentation index as a marker of arterial stiffness nor the augmentation pressure as a measure of the contribution that the wave reflection makes to the systolic arterial pressure (16) showed changes over the duration of the study. On this basis, it is unlikely that dietary flaxseed altered hypertension via changes in aortic stiffness. This may not be entirely unexpected. Arterial stiffness is a chronic process that evolves as a consequence of several structural alterations in the arterial wall including vascular hypertrophy and an increase in collagen content (21). To reverse these changes may require more time than 12 months of intervention in patients with PAD. Although ACE inhibitors, calcium channel blockers and nitrates can change arterial stiffness (22), effective anti-hypertensive strategies such as diuretics and β-blockers do not (22, 23).

The data above rule out several factors as being mechanistically involved in the anti-hypertensive actions of flaxseed. However, the present data add further support to the involvement of ALA and oxylipins in the anti-hypertensive action of flaxseed (5). The binary analysis of the change in plasma ALA demonstrated that those with an average/above change in plasma ALA exhibited larger reductions in blood pressure (8/5 mmHg) versus those with below average change in plasma ALA (1/1 mmHg). ALA can inhibit soluble epoxide hydrolase, which has resulted in decreased concentrations of pro-inflammatory and vasoconstrictive oxylipins in this population (6).

The role for oxylipins in BP regulation (6) has been strengthened in the present investigation by the demonstration of significant associations of novel plasma oxylipins

to elevated cSBP, cDBP and cXBP. Some of these effects were unexpected whereas others represent new information on the vascular role of oxylipins. For example, the association of thromboxane B_2, 6kPGF$_{1\alpha}$, and 11,12-DiHETrE to cBP was not unexpected. These oxylipins can regulate vascular tone (24-29). Previously, 11,12-DiHETrE had been positively associated with brachial blood pressure in the Flax-PAD trial (6). However, oxylipins reported as significant predictors of high cBP in this report including 16-HETE, thromboxane B_2, 6kPGF$_{1\alpha}$, and PGE_2 were not associated with brachial blood pressure previously (6). This could suggest that cBP is more susceptible to the impact of oxylipins than is brachial BP.

The observation that many of the novel docosahexanoic acid derived oxylipins, the hydroxydocosahexanoic acids (HDOHEs), were elevated in patients with higher cBP (Figure 4) was unexpected. For example, 17-HDOHE, is a precursor to the resolvins which are inflammatory resolving mediators (30). Inflammation is important in the etiology and progression of essential hypertension (31). However, the high concentration of HDOHEs in hypertension may instead reflect the augmented oxidative stress that is thought to play an important role in hypertension (31). HDOHEs are generated during oxidative stress as auto-oxidized products of docosahexanoic acid (32). In this population as well as in a healthy population, dietary flaxseed has lowered the concentration of plasma HDOHEs and pro-inflammatory oxylipins (6, 33). Thus flaxseed may lower blood pressure through the reduction of HDOHEs. 16-hydroxyeicosatetraenoic acid (HETE) increased the odds of high cSBP, cDBP and cXBP. Flaxseed induced a decrease in plasma 16-HETE. 16-HETE, therefore, may be an important diagnostic marker or therapeutic target for cBP. 16-HETE exhibits vasodilatory effects in rabbit renal arteries

(34) and is released by polymorphonuclear neutrophils *in vitro* (35). Because of its previously reported vasodilatory effects, it is possible that 16-HETE may be upregulated as a protective mechanism in patients with central hypertension.

Our study contains several limitations. Measurements of cBP were indirectly derived from PWA. However, PWA has been extensively validated and correlates strongly to direct measurement of cBP (36, 37). The number of patients included in each group was also relatively small (n=31/group). Nevertheless, as the first and largest study to evaluate the effects of dietary flaxseed on cBP, it is a reasonable sample size considering the interventional nature of the trial, its length (one year) and the tight controls used (double-blinded, controlled, and randomized). Further studies are warranted in view of the potential impact of our results on hypertension morbidity and mortality.

Conclusion

Dietary flaxseed induced a significant decrease in cBP in a patient population with hypertension. Control of hypertension is an important goal in PAD to reduce cardiovascular disease mortality, prevent functional declines and improve quality of life (38). This is important in the general population as well (2). Dietary flaxseed achieved these effects on BP without compromising cardiac contractile performance. Dietary flaxseed induced a significant reduction in left ventricle afterload over an extended period of time without introducing a deficit in the coronary oxygen supply/demand in PAD patients. This is evidenced by the improvement in systolic blood pressure and the unchanged central buckberg index (subendocardial viability ratio). These factors create a very favourable hemodynamic situation for CVD patients. It is important to remember that cBP may be a better predictor of strokes and myocardial infarctions than traditional

brachial BP (8). Finally, the data obtained on the role of oxylipins in essential hypertension may help to identify specific oxylipins as diagnostic markers of cBP or as therapeutic targets themselves.

Perspectives

Competency is Medical Knowledge

Dietary supplementation with flaxseed lowers brachial and cBP in patients with hypertension and PAD.

Competency in Patient Care

Patients with uncontrolled hypertension may benefit from adding 30 g of ground flaxseed/day to their diet.

Translational Outlook

Future research can investigate 1) the optimal flaxseed dose per patient and 2) other populations, such as those with metabolic syndrome, newly diagnosed hypertension, or CVD alone. Potential challenges may be participant recruitment and the cost associated with food preparation. A multi-centre trial may help to avoid these challenges.

Future research investigating the utility of the identified oxylipins for diagnostic or therapeutic purposes for central hypertension may prove to be of merit.

Acknowledgements

We are also thankful to Canada Bread (Toronto, Canada), the Canadian International Grain Institute (Winnipeg, Canada) and the Food Development Centre (Portage La Prairie, Canada) for preparing the foods for this trial. The flaxseed or wheat was provided by Glanbia Nutritionals Inc.

Disclosures

None.

Chapter II Section iii References

1. Chobanian AV, Bakris GL, Black HR, et al, Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High BP. National Heart, Lung, and Blood Institute; National High BP Education Program Coordinating Committee. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High BP. *Hypertension.* 2003;42:1206-52.
2. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*. 2012;380(9859):2224-60.
3. Qureshi AI, Suri MF, Kirmani JF, Divani AA. Prevalence and trends of prehypertension and hypertension in United States: National Health and Nutrition Examination Surveys 1976 to 2000. *Med Sci Monit*. 2005;11:CR403-9
4. James PA, Oparil S, Carter BL, et al. 2014 evidence-based guideline for the management of high BP in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). JAMA. 2014;311(5):507-520.
5. Rodriguez-Leyva D, Weighell W, Edel AL, et al. Potent antihypertensive action of dietary flaxseed in hypertensive patients. *Hypertension*. 2013;62:1081-9.
6. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, Pierce GN. Flaxseed consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via an α-linolenic acid-induced inhibition of soluble epoxide hydrolase. *Hypertension*. 2014;64:53-9.

7. Elliott WJ, Ram CV. Calcium channel blockers. *J Clin Hypertens (Greenwich)*. 2011; 13:687-9.
8. Williams B, Lacy PS, Thom SM, et al, CAFE Investigators; Anglo-Scandinavian Cardiac Outcomes Trial Investigators; CAFE Steering Committee and Writing Committee. et al. Differential impact of BP-lowering drugs on central aortic pressure and clinical outcomes: principal results of the Conduit Artery Function Evaluation (CAFE) study. *Circulation*. 2006;113:1213-25.
9. Vlachopoulos C, Aznaouridis K, O'Rourke MF, Safar ME, Baou K, Stefanadis C.
10. Prediction of cardiovascular events and all-cause mortality with central haemodynamics: a systematic review and meta-analysis. *Eur Heart J*. 2010;31:1865-71.
11. Adji A, Hirata K, O'Rourke MF. Clinical use of indices determined non-invasively from the radial and carotid pressure waveforms. *Blood Press Monit.* 2006;11:215–21.
12. Rodriguez-Leyva D, Zahradka P, Ramjiawan B, Guzman R, Aliani M, Pierce GN. The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with peripheral artery disease: rationale and design of the FLAX-PAD randomized controlled trial. *Contemp Clin Trials*. 2011;32:724-30.
13. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile of muffins and snack bars. *Food Res Int.* 2011;44:2489-2496.
14. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile and acceptability of bagels. *J Food Sci*. 2012;71:S62-S79.

15. Pickering TG, Hall JE, Appel LJ, et al. Recommendations for BP measurement in humans and experimental animals: part 1: BP measurement in humans: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High BP Research. *Circulation.* 2005;111,697-716.
16. Edel AL, Rodriguez-Leyva D, Maddaford TG, et al. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medication alone in patients with peripheral arterial disease. *J Nutr*. 2015 Apr;145(4):749-57.
17. Laurent S, Boutouyrie P, Asmar R, et al. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension*. 2001;37:1236-41.
18. Agabiti-Rosei E, Mancia G, O'Rourke MF, et al. Central BP measurements and antihypertensive therapy: a consensus document. *Hypertension*. 2007;50:154-60.
19. Brinton TJ, Cotter B, Kailasam MT, et al. Development and validation of a noninvasive method to determine arterial pressure and vascular compliance. *Am J Cardiol*. 1997;80:323-30.
20. Markert M, Trautmann T, Grob M, Ege A, Mayer K, Guth B. Evaluation of a method to correct the contractility index LVdP/dt(max) for changes in heart rate. *J Pharmacol Toxicol Methods*. 2012;66:98-105.
21. Fantin F, Mattocks A, Bulpitt CJ, Banya W, Rajkumar C. Is augmentation index a good measure of vascular stiffness in the elderly? *Age Ageing*. 2007;36:43-8.

22. Benetos A, Laurent S, Asmar RG, Lacolley P. Large artery stiffness in hypertension. *J Hypertens.*1997;15 (suppl 2):S89–S97.

23. Sohn SI, Kim CJ. Modulation of renin-angiotensin system and arterial stiffness: evidence from clinical trials. *Curr Hypertens Rev.* 2014;10:37-40.

24. Espinola-Klein C, Weisser G, Jagodzinski A, et al. β-Blockers in patients with intermittent claudication and arterial hypertension: results from the nebivolol or metoprolol in arterial occlusive disease trial. *Hypertension*. 2011;58:148-54.

25. Gebremedhin D, Ma YH, Imig JD, Harder DR, Roman RJ. Role of Cytochrome P-450 in Elevating Renal Vascular Tone in Spontaneously Hypertensive Rats. *J Vasc Res. 1*993; 30:53–60.

26. Ma YH, Harder DR, Clark JE, Roman RJ. Effects of 12-HETE on isolated dog renal arcuate arteries. *Am J of Physiol,*1991;261:H451-456.

27. Sudhahar V, Shaw S, Imig JD. Epoxyeicosatrienoic acid analogs and vascular function. *Curr Med Chem.* 2010;17:1181-1190.

28. Falck JR, Krishna UM, Reddy YK, et al. Comparison of vasodilatory properties of 14,15-EET analogs: structural requirements for dilation. *Am J Physiol Heart Circ Physiol.* 2003;284: H337-49.

29. McLaughlin VV, Genthner DE, Panella MM, Rich S. Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. N Engl J Med. 1998;338:273-7.

30. Cheng Y, Austin SC, Rocca B, *et al*. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science. 2002;296:539-41.

31. Serhan CN, Petasis NA, Resolvins and protectins in inflammation resolution. *Chem Rev.* 2011;111:5922-43.

32. Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. *Acta Cardiol.* 2000;55:221-232.

33. Reynaud D, Thickitt CP, Pace-Asciak CR. Facile preparation and structural determination of monohydroxy derivatives of docosahexaenoic acid (HDoHE) by alpha-tocopherol-directed autoxidation. *Anal Biochem*. 1993;214:165-70.

34. Caligiuri SP, Aukema HM, Ravandi A, Pierce GN. Elevated levels of pro-inflammatory oxylipins in older subjects are normalized by flaxseed consumption. *Exp Gerontol*. 2014; 59:51-57.

35. Carroll MA, Balazy M, Margiotta P, Huang DD, Falck JR, McGiff JC. Cytochrome P-450-dependent HETEs: profile of biological activity and stimulation by vasoactive peptides. *Am J Physiol – Regul, Integr, Comp Physiol*. 1996;27:R863-R869.

36. Bednar MM, Gross CE, Balazy MK, *et al*. 16(R)-hydroxy-5,8,11,14-eicosatetraenoic acid, a new arachidonate metabolite in human polymorphonuclear leukocytes. Biochem Pharmacol. 2000;60:447-55.

37. Chen CH, Nevo E, Fetics B, et al. Estimation of Central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. *Circulation.* 1997;95:1827-36.

38. Sharman JE, Lim R, Qasem AM, *et al*. Validation of a generalized transfer function to noninvasively derive central blood pressure during exercise. Hypertension. 2006;47:1203-8.

39. Murabito JM, Evans JC, Nieto K, Larson MG, Levy D, Wilson PW. Prevalence and clinical correlates of peripheral arterial disease in the Framingham Offspring Study. *Am Heart J*. 2002;143:961-5.

iv. Plasma Oxylipins and Cardiovascular Events in Patients with Peripheral Artery Disease

In the previous sections, plasma oxylipins were implicated in the blood pressure lowering mechanism of dietary flaxseed. Next, we hypothesized that plasma oxylipins were also implicated in cardiovascular events in this patient population. Due to the role of oxylipins in inflammation and vasoconstriction, it was hypothesized that pro-inflammatory and vasoconstrictive oxylipins would increase the odds of cardiovascular events. The objectives of the current study included:

1) Quantify the precursors to oxylipins, (ie: polyunsaturated fatty acid plasma profile)
2) Determine any relationship between plasma fatty acid and oxylipins to cardiovascular events.

The following study was designed in order to assess the above objectives.

Beyond Conventional Risk Factors: Specific Plasma Oxylipins Increase the Odds of Cardiovascular and Cerebrovascular Events in Patients with Peripheral Artery Disease

Under Review: JACC – Basic Translational Research

Stephanie P.B. Caligiuri M.Sc,[1-3] Harold M. Aukema Ph.D,[1,4] Amir Ravandi M.D. Ph.D,[2,3,5] Renée Lavallée B.Sc[1,2], Randy Guzman M.D,[6] and Grant N. Pierce Ph.D[1-3]

[1]Canadian Centre for Agri-food Research in Health and Medicine (CCARM) and [2]the Institute of Cardiovascular Sciences, St. Boniface Hospital Research Centre, and [3]the Departments of Physiology, [4]Human Nutritional Sciences, [5]Internal Medicine, and [6]Surgery, University of Manitoba, Winnipeg, Canada

Abstract

Objectives: To determine if plasma oxylipins and fatty acids can influence the odds of cardiovascular/cerebrovascular events in patients with peripheral artery disease (PAD).

Background: Oxylipins and fatty acids may be novel therapeutic targets for cardiovascular and cerebrovascular disease because they can modulate inflammation and vascular tone.

Methods: The prevalence of cardiovascular and cerebrovascular events in patients with PAD was recorded. Plasma fatty acids and oxylipins were analyzed with gas chromatography and HPLC-MS/MS, respectively. Wilcoxin Rank Sum tests and logistic regression were utilized to identify potential relationships between clinical events and plasma fatty acids and oxylipins.

Results: In 98 patients with PAD, the prevalence of transient ischemic attacks, cerebrovascular accidents, stable angina and acute coronary syndrome was n= 16, 10, 16, and 24, respectively. Risk factors such as diagnosed hypertension, diabetes mellitus, and hyperlipidemia were not associated with events. Twenty-four fatty acids were quantified of which none were associated with events. In contrast, 39 plasma oxylipins were quantified, of which 8 were significantly associated with events. These 8 oxylipins are known regulators of vascular tone. For example, every 1 unit increase in Thromboxane B_2/Prostaglandin $F_{1\alpha}$ and every 1 nM increase in plasma 16-hydroxyeicosatetraenoic acid (16-HETE), Thromboxane B_2, or 11,12-dihydroxyeicosatrienoic acid (DiHETrE) increased the odds of having had 2 or more events versus no event ($p<0.05$).

Conclusion: In the presence of well controlled risk factors, specific oxylipins were highly associated with clinical events. Therefore, oxylipins should be investigated as specific biomarkers and/or therapeutic targets in clinical trials of a larger scale.

Abbreviations: 6 keto (6k), acute coronary syndrome (ACS), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DiHETrE), dihydroxyoctadecenoic acid (DiHOME), HETrE (hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid, (HDOHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETrE), hydroxyoctadecadienoic acid (HODE), hydroxyoctadecatrienoic acid (HOTrE), oxooctadecadienoic acid (OXOODE), prostaglandin (PG), standard error of the mean (SEM), transient ischemic attack (TIA), trihydroxyoctadecenoic acid (TriHOME), thromboxane (TX)

Introduction

Oxylipins are an intriguing group of molecules due to their ability to regulate inflammation and vascular tone (1). There are three oxylipin subclasses: eicosanoids, octadecanoids and docosanoids. The eicosanoids are the most characterized in terms of their physiological and pathophysiological effects. Due to recent advancements in detection technology, many novel oxylipins within the octadecanoid and docosanoid subclasses have now also been identified (1). The substrate of origin and often the enzyme that produces these novel oxylipins are known, but the role for many of these molecules in physiology and disease remains unclear. Some oxylipins play a fundamental role in vascular tone by acting as vasorelaxing or vasoconstricting factors (2, 3). Oxylipins also regulate inflammation (4, 5). It is generalized that most omega 6 fatty acid-derived oxylipins are more pro-inflammatory and vasoconstrictive than those produced from omega 3 fatty acids (1). Changes in vascular tone and inflammation have been implicated in endothelial dysfunction, hypertension, and atherosclerosis. These processes play a fundamental role in the etiology and progression of both cerebrovascular accidents and myocardial infarction (6-8). It is plausible, therefore, that plasma oxylipins may act as significant mediators in the progression of these events.

A patient population that is at a high risk for cardiovascular and cerebrovascular events are those living with peripheral artery disease (PAD). PAD is defined as chronic arterial occlusion typically from atherosclerosis to the arteries exclusive of intracranial and coronary circulation, ie: typically in the arms and legs (9). However, patients with PAD often progress to coronary artery disease or cerebral disease. In fact, patients with PAD have a 35% lower survival rate and a higher incidence of myocardial infarctions and cerebrovascular accidents than matched patients with coronary artery disease alone (10).

For this reason, a patient population living with PAD was chosen for the current investigation. The hypothesis of the current study was oxylipins derived from omega-6 fatty acids will increase the odds of cardiovascular and cerebrovascular events in patients with PAD. In order to assess this hypothesis, the prevalence of angina, myocardial infarctions, transient ischemic attacks and cerebrovacular accidents in patients living with PAD were assessed with regard to their relationship to coventional risk factors, plasma fatty acid and oxylipin concentrations.

Material and Methods

Baseline data from patients enrolled in the randomized, double-blinded, control controlled FlaxPAD trial were included in this analysis (11). The trial was approved and in accordance with the University of Manitoba Research Ethics Boards, Health Canada Natural Health Product Directorate, and the St. Boniface Hospital Research Review Committee. All participants provided written consent. The total sample size for the current study was 98 participants. Further details of the patient population have been published elsewhere (11, 12).

Plasma fatty acids were detected and quantified using gas chromatography and indicated as ng/mL. Details of methodology for fatty acid extraction and quantification are as previously described (13). An expansive lipidomics analysis searching for over 100 pro-inflammatory and pro-resolving mediators were included in this study. Plasma oxylipins were extracted, detected, and quantified as previously described (12). Briefly, plasma oxylipins were extracted with solid phase extraction, detected with multiple reaction monitoring on the QTRAP 4000 HPLC-MS/MS system, and quantified with the stable isotope dilution method. Oxylipins are represented as nM.

Accurate coronary syndrome (ACS) was defined by S-T elevation myocardial infarction, non S-T Elevation myocardial infarction, and unstable angina. These were defined according to the Third Universal definition (14), ie: myocardial necrosis due to acute myocardial ischemia. ACS was defined by detecting a change of cardiac biomarkers with one biomarker greater than the 99^{th} percentile upper reference limit in addition to a minimum of one of the following: ischemic symptoms, new ST changes, new left bundle branch block, pathological Q waves, new loss of healthy myocardium, left ventricular wall abnomality, and/or intracoronary thrombus.(14). Stable angina pectoris was defined as symptoms of chest discomfort without elevated cardiac biomarkers or evidence of other extraneous plausible causes (eg: gastroesophageal reflux) upon exertion.(14). A cerebrovascular accident (CVA) was defined in accordance with the American Heart Association and American Stroke Association guidelines and diagnosed with a computed tomography scan (15). A transient ischemic attack (TIA) was defined as an event without permanent cerebral damage but rather a brief period of focal cerebral impedance of blood flow that resulted in temporary neurological dysfunction (16). This data was collected from patient medical history.

Statistical analyses were performed using SAS version 9.3 (Cary, NC, USA). Logistic regression with score selection was utilized to determine the three most significantly associated oxylipins or fatty acids with patient events. Univariate and multivariate forward selection logistic regression were utilized to determine the association of oxylipins and fatty acids to events while controlling for hypertension, diabetes mellitus, hyperlipidemia, age, gender, smoking status and medications. Bootstrap analysis was utilized for distribution estimation. Probability was assessed with

the logit (p) equation produced from the logistic regression analyses. The Wilcoxin-Rank Sum test was utilized for 2 group comparisons due to the non-Gaussian distribution with a post-hoc Bonferroni correction. All tests were set at a significance level of 0.05.

Results

Prevalence of Cardiovascular and Cerebrovascular Events

Figure 18 illustrates the prevalence of cardiovascular and cerebrovascular events in the study population. ACS affected the population studied here more than any other clinical event (n=24 patients). No patients presented with all 4 events. Four patients presented with 3 of the 4 possible events, 17 patients had 2 events, 20 patients presented with 1 event, and 57 patients had no cardiovascular or cerebrovascular event.

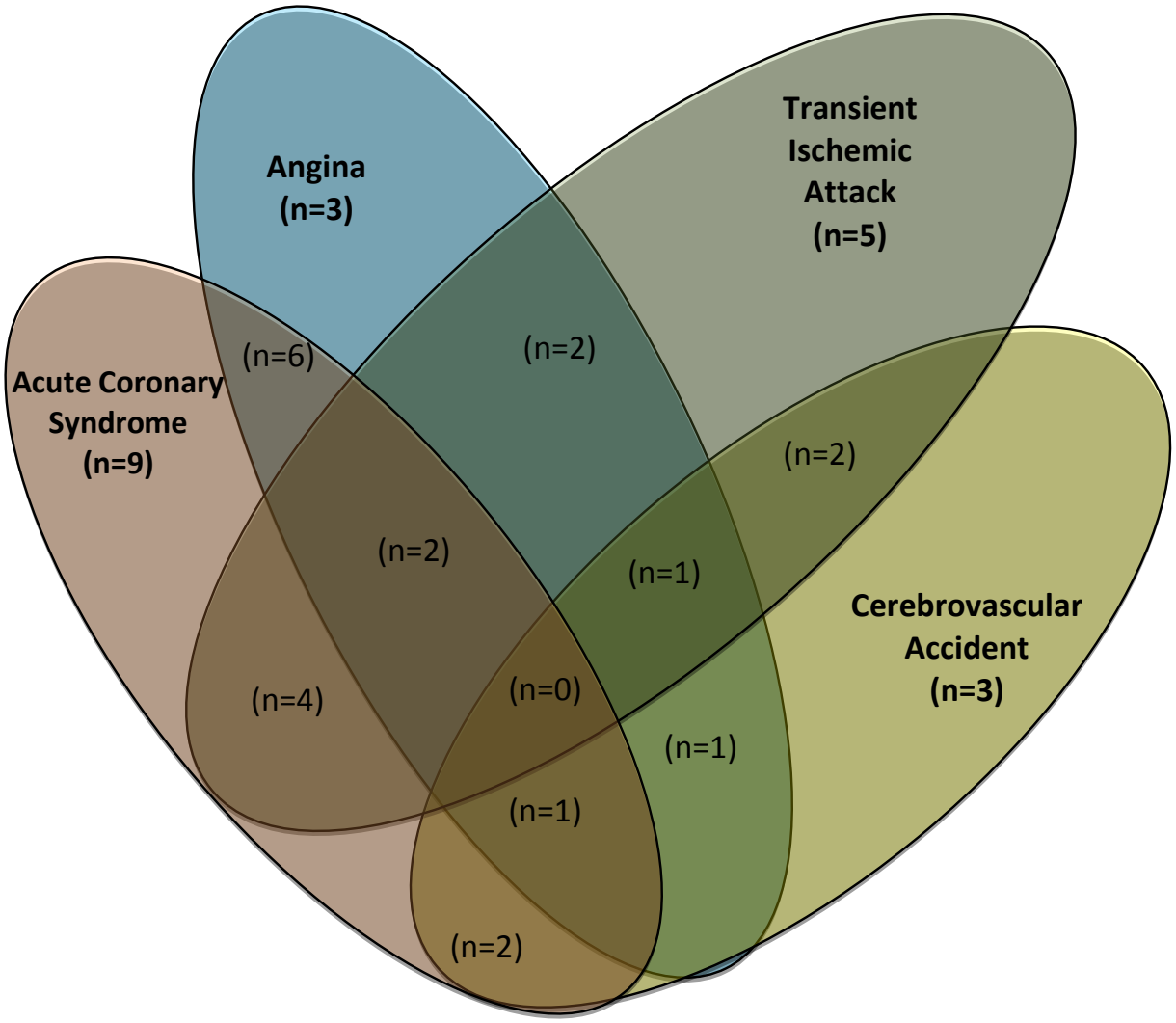


Figure 18: Venn Diagram of Cardiovascular and Cerebrovascular Event Prevalence in 98 Patients With Peripheral Arterial Disease

Plasma Fatty Acid Concentrations by Presence of Event

Because polyunsaturated fatty acids are the substrates to the bioactive oxylipins, plasma fatty acid concentrations were compared by presence of event. Twenty-four plasma fatty acids were detected. Twleve of these were polyunsaturated fatty acids. Plasma fatty acid concentrations (ng/mL) did not differ significantly among patients for presence of angina, ACS, TIA or CVA (Table 15 and 16), nor were plasma fatty acids significant predictors of events in logistic regression models.

Table 15: Plasma fatty acid concentrations (µg/mL) by presence of angina or myocardial infarction

Fatty Acid	No Angina (n=82)	SEM	Yes Angina (n=16)	SEM	No Myocardial Infarction (n=74)	SEM	Yes Myocardial Infarction (n=24)	SEM
C12:0	0.7	0.3	0.3	0.3	0.5	0.3	1.0	0.6
C14:0	24.7	1.7	23.2	4.6	25.3	2.0	22.1	2.2
C14:1	1.7	0.3	1.5	0.8	1.5	0.3	2.0	0.7
C16:0	640.6	25.9	578.8	65.0	642.6	29.1	593.2	40.6
C16:1	59.9	4.2	56.8	17.2	62.1	5.5	50.9	5.9
C18:0	189.0	5.4	170.7	12.7	188.0	5.9	179.7	9.4
C18:1 Oleic	703.4	26.6	705.3	72.2	707.2	28.9	692.9	51.4
C18:1 Vaccenic	45.9	1.7	44.5	5.6	46.7	2.0	42.6	3.2
C18:2	702.3	21.3	637.2	52.0	703.3	22.4	655.7	41.7
C20:0	5.5	0.3	4.4	0.8	5.6	0.3	4.7	0.6
C18:3 DGLA	13.2	0.8	12.2	1.4	12.9	0.8	13.5	1.1
C20:1	3.4	0.3	4.0	0.7	3.6	0.3	3.2	0.5
C18:3 ALA	18.2	1.2	22.9	4.1	18.4	1.2	20.7	3.0
C20:2	3.6	0.3	3.9	0.7	3.6	0.4	3.8	0.6
C22:0	15.9	0.7	12.8	1.5	15.9	0.8	13.8	1.2
C20:3	36.5	1.4	33.4	3.7	36.8	1.5	33.3	2.4
C20:4	212.0	6.6	194.3	14.7	210.3	7.1	205.4	11.8
C22:2	0.1	0.0	0.3	0.3	0.1	0.1	0.2	0.2
C24:0	11.9	0.5	9.2	1.1	11.9	0.5	10.3	0.8
C20:5	20.8	1.2	20.4	2.1	20.7	1.3	20.8	2.0
C24:1	24.2	0.8	22.1	2.9	24.4	0.9	22.3	1.9
C22:4	3.9	0.3	3.1	0.8	3.9	0.4	3.5	0.6
C22:5	13.3	0.6	13.5	1.3	13.2	0.7	13.7	1.1
C22:6	41.6	2.2	40.3	3.6	42.7	2.4	37.5	2.5

Abbreviations: acute coronary syndrome (ACS), alpha-linolenic acid (ALA), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), transient ischemic attack (TIA), standard error of the mean (SEM)

Table 16: Plasma fatty acid concentrations (µg/mL) by presence of transient ischemic attack (TIA) and cerebrovascualar accident (CVA)

Fatty Acid	No TIA (n=82)	SEM	Yes TIA (n=16)	SEM	No CVA (n=88)	SEM	Yes CVA (n=10)	SEM
C12:0	0.7	0.3	0.3	0.3	0.6	0.3	0.7	0.5
C14:0	25.2	1.9	20.8	2.1	24.2	1.7	27.0	4.4
C14:1	1.8	0.3	0.9	0.5	1.6	0.3	1.7	0.9
C16:0	636.0	27.4	602.5	45.4	627.1	26.0	660.9	61.2
C16:1	60.9	5.1	51.6	7.9	58.8	4.8	64.7	11.8
C18:0	186.5	5.6	183.1	11.5	185.2	5.4	192.9	12.8
C18:1 Oleic	705.6	27.8	693.7	58.4	699.4	27.0	741.3	63.5
C18:1 Vaccenic	45.6	1.9	46.2	3.9	45.1	1.7	51.1	6.4
C18:2	684.2	21.2	730.2	54.0	688.1	21.1	722.7	58.7
C20:0	5.3	0.3	5.6	0.8	5.3	0.3	5.5	0.7
C18:3 DGLA	12.9	0.7	13.9	1.7	12.7	0.7	16.8	2.0
C20:1	3.5	0.3	3.8	0.7	3.4	0.3	4.9	0.7
C18:3 ALA	18.3	1.3	22.4	2.8	18.9	1.3	19.2	2.5
C20:2	3.6	0.3	4.1	0.8	3.5	0.3	4.8	0.9
C22:0	15.4	0.7	15.0	2.0	15.4	0.7	14.8	2.2
C20:3	36.2	1.4	34.7	3.1	35.5	1.4	40.0	3.0
C20:4	204.9	6.6	230.7	14.2	205.5	6.1	240.6	24.1
C22:2	0.1	0.1	0.0	0.0	0.1	0.1	0.0	0.0
C24:0	11.4	0.5	11.7	1.1	11.4	0.5	11.9	1.3
C20:5	20.0	1.2	24.0	2.5	20.4	1.2	22.8	2.9
C24:1	23.7	0.8	24.9	2.7	23.9	0.8	24.3	3.5
C22:4	3.7	0.4	4.3	0.6	3.6	0.3	5.6	1.0
C22:5	13.2	0.6	14.2	1.2	13.1	0.6	15.7	2.0
C22:6	39.7	1.6	50.1	8.8	41.0	2.1	45.4	4.5

Abbreviations: acute coronary syndrome (ACS), alpha-linolenic acid (ALA), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), transient ischemic attack (TIA), standard error of the mean (SEM)

Plasma Oxylipin Concentrations by Presence of Event

Thirty-nine plasma oxylipins were quantified in the current study population living with peripheral artery disease. Using the Wilcoxin-Rank sum test followed by a Bonferroni correction, four plasma oxylipins were significantly different between the presence of an event or absence (Figure 19). For example, plasma 16-hydroxyeicosatetraenoic acid (HETE) was more than 4 times higher in patients that suffered from a CVA versus patients that did not (Figure 19).

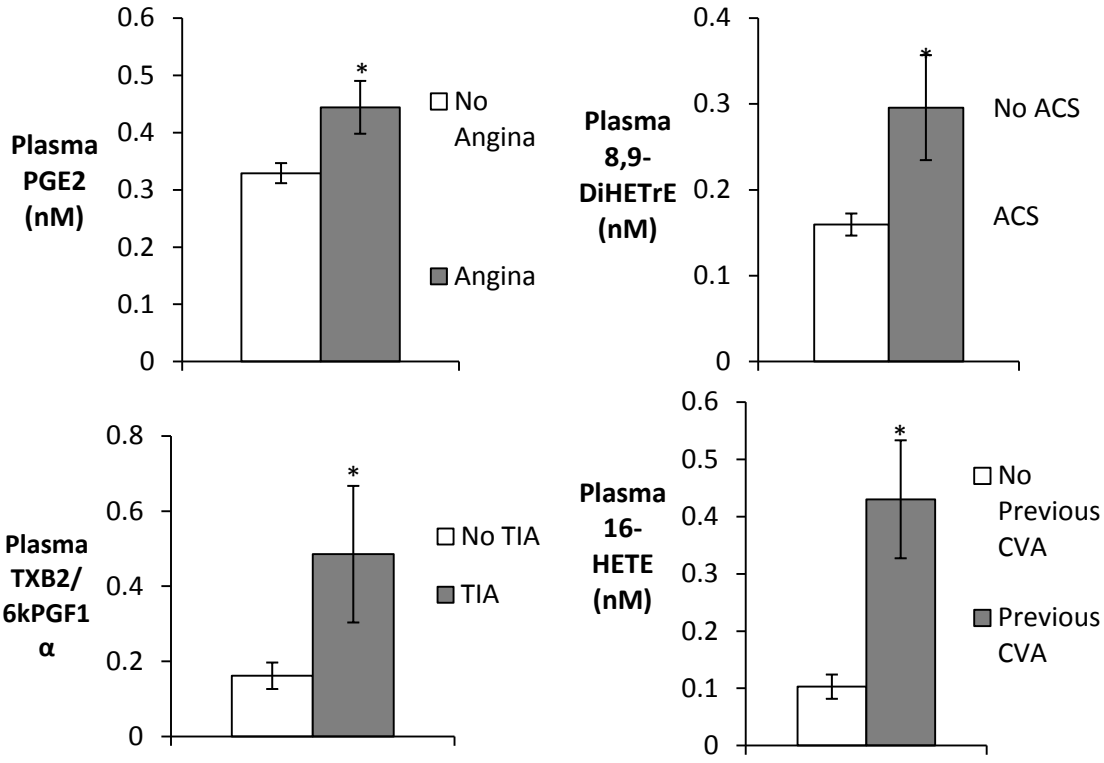


Figure 19: Concentration of Plasma Oxylipins by Presence of Cardiovascular/Cerebrovascular Outcomes

* denotes p-value <0.05. Abbreviations: 6-keto (6k), acute coronary syndrome (ACS), cerebrovacular accident (CVA), dihydroxyeicosatrienoic acid (DiHETrE), hydroxyeicosatetranoic acid (HETE), prostaglandin (PG), transient ischemic attack (TIA), thromboxane (TX).

Using the score selection method of logistic regression, the oxylipins most significantly associated with an event were identified. Two plasma oxylipins significantly increased the odds of angina (Table 17), 16-hydroxyeicosatetraneoic acid (HETE) and prostaglandin (PG) E_2. 16-HETE and PGE_2 were 200% and 33% higher, respectively, in those with past or current angina versus those without (Table 19). Plasma 8,9-dihydroxyeicosatrienoic acid (DiHETrE) and 18-hydroxyeicosapentaenoic acid (HEPE) significantly increased the odds of past myocardial infarctions (Table 18). 18-HEPE and 8,9-DiHETrE were 200% and 88% higher, respectively, in those with a past myocardial infarction versus those without a past myocardial infarction (Table 19). Only 6 keto (6k)-$PGF_{1\alpha}$ resulted in an odds ratio less than 1 for the prevalence of TIAs, indicating a protective effect (Table 21). $6kPGF_{1\alpha}$ was 17% lower in those who suffered from a TIA. This contributed to the ratio of $TXB_2/6kPGF_{1\alpha}$ significantly increasing the odds of a TIA by 3.8-fold for every 1 unit increase (Table 21). The ratio of $TXB_2/6kPGF_{1\alpha}$ was 200% greater in patients with a past TIA versus those with no TIA. Plasma 16-HETE and $PGF_{2\alpha}$ increased the odds of past cerebrovascular accidents (Table 22). Patients with past cerebrovascular accidents had on average 300% and 200% higher concentrations of 16-HETE and $PGF_{2\alpha}$ versus those without a prior cerebrovascular accident (Table 20).

It was important to take into account the physiological concentrations of the plasma oxylipins, as logistic regression is per 1 nM increase. Therefore, Figure 20 illustrates the probability of an event by plasma oxylipin concentration observed in the current population ($p<0.05$). 16-HETE resulted in the greatest increase in the probability of angina and cerebrovascular accidents based on the observed physiological

concentration range (Figure 20). However, 8,9-DiHETrE had the largest slope at 4.53 (as indicated by b in the y = a + bx equation) for prediction of ACS prevalence.

Table 17: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Angina

Variable	Cases	Controls	Univariate OR (95% CI)	p-value	Multivariate OR (95% CI)	p-value
Plasma Oxylipins Score	43	N/A				
Selection						
PGE_2			80 (71, 90)	0.022	80 (71, 90)	0.022
16-HETE			9.1 (8.5, 9.7)	0.025		
Other Characteristics						
Age ≥65 years	65	33	1.8 (0.54, 6.2)	0.33		
Female Gender	25	73	0.63 (0.16, 2.4)	0.50		
Smoking Status (current)	25	73	0.40 (0.083, 1.9)	0.24		
Hypertension	74	24	2.2 (0.47, 11)	0.31		
Diabetes Mellitus	33	65	1.9 (0.62, 5.8)	0.27		
Hyperlipidemia	78	20	3.9 (0.49, 32)	0.20		
Medications						
Beta Blocker	33	65	3.1 (1.0, 9.3)	0.043		
Ca Channel Blocker	27	71	0.33 (0.07, 1.5)	0.16		
ACE inhibitor	47	51	1.6 (0.56, 4.8)	0.37		
ARB	15	83	1.4 (0.33, 5.4)	0.68		
Diuretic	39	39	0.89 (0.30, 2.7)	0.84		
Aspirin	71	27	0.65 (0.21, 2.0)	0.45		
Statin	70	28	2.5 (0.52, 12)	0.26		

Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), Hydroxyeicosatetraenoic acid (HETE), Prostagladin (PG)

Table 18: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Myocardial Infarctions

Variable	Cases	Controls	Univariate OR (95% CI)	p-value	Multivariate OR (95% CI)	p-value
Plasma Oxylipins Score	43	N/A				
Selection						
8,9-DiHETrE			92.4 (82.0, 104)	0.0192	92.4 (82.0, 104)	0.0192
18-HEPE			4.1 (4.0, 4.3)	0.045		
Other Characteristics						
Age ≥65 years	65	33	1.1 (0.44, 3.0)	0.78		
Female Gender	25	73	0.71 (0.23, 2.2)	0.55		
Smoking Status	25	73	1.0 (0.35, 3.0)	0.97		
(current)						
Hypertension	74	24	4.4 (0.96, 21)	0.057		
Diabetes	33	65	1.6 (0.60, 4.0)	0.36		
Hyperlipidemia	78	20	2.0 (0.52, 7.4)	0.32		
Medications						
Beta Blocker	33	65	8.8 (3.1, 25)	<0.00010	12 (3.6, 40)	<0.0001
Ca Channel Blocker	27	71	0.44 (0.14, 1.4)	0.18		
ACE inhibitor	47	51	1.6 (0.62, 3.9)	0.35		
ARB	15	83	0.43 (0.090, 2.0)	0.29		
Diuretic	39	39	1.7 (0.69, 4.4)	0.24		
Aspirin	71	27	0.62 (0.23, 1.6)	0.33		
Statin	70	28	3.1 (0.82, 11)	0.094		

Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), (DiHETrE), hydroxyeicosapentanoic acid (HEPE), hydroxyeicosatetranoic acid (HETE), prostaglandin (PG), thromboxane (TX).

Table 19: Plasma Oxylipin Concentration (nM) by Presence of Event

Oxylipin	No Angina (n=82)	SEM	Yes Angina (n=16)	SEM	No ACS (n=74)	SEM	Yes ACS (n=24)	SEM
10-HDOHE	0.4	0.1	0.9	0.5	0.4	0.1	0.9	0.4
11,12-DiHETrE	0.6	0.0	0.8	0.1	0.6	0.0	0.8	0.1
11-HDOHE	0.8	0.1	1.2	0.4	0.8	0.1	1.3	0.4
11-HETE	0.8	0.1	1.2	0.5	0.7	0.0	1.3	0.4
12-HETE	3.8	0.6	4.1	0.9	4.0	0.6	3.4	0.7
12,13-DiHOME	3.4	0.4	2.5	0.3	3.3	0.4	3.0	0.3
12-HEPE	0.8	0.1	1.3	0.4	0.8	0.2	1.1	0.3
13-HODE	10.1	1.0	9.2	1.0	10.2	1.0	9.3	0.9
13-HDOHE	0.3	0.1	0.5	0.3	0.2	0.0	0.6	0.3
13-OXOODE	9.0	2.2	6.7	0.8	9.3	2.5	6.4	0.7
14,15-DiHETrE	0.8	0.0	0.8	0.1	0.8	0.0	0.9	0.1
14-HDOHE	1.4	0.2	1.9	0.5	1.5	0.2	1.5	0.3
15-HETrE	0.5	0.1	0.9	0.4	0.5	0.1	1.0	0.3
15-HETE	1.1	0.1	1.5	0.4	1.1	0.1	1.5	0.4
16-HDOHE	0.3	0.1	0.6	0.4	0.2	0.0	0.7	0.3
16-HETE	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1
17-HDOHE	0.9	0.1	1.3	0.8	0.7	0.1	1.5	0.6
18-HEPE	0.1	0.0	0.3	0.2	0.1	0.0	0.3	0.2
18-HETE	0.3	0.0	0.5	0.3	0.3	0.0	0.5	0.2
19,20-DiHDPA	1.5	0.1	1.6	0.2	1.5	0.1	1.5	0.1
20-HDOHE	0.4	0.1	0.7	0.4	0.3	0.0	0.6	0.3
4-HDOHE	0.6	0.1	0.8	0.3	0.5	0.1	1.0	0.4
5-HETE	1.3	0.1	1.9	0.7	1.2	0.1	2.1	0.6
5,6-DiHETrE	0.3	0.0	0.3	0.1	0.3	0.0	0.3	0.1
5-HEPE	0.6	0.1	1.1	0.5	0.5	0.1	1.1	0.4
6keto-$PGF_{1\alpha}$	0.7	0.0	0.7	0.0	0.7	0.0	0.7	0.0
7-HDOHE	0.2	0.1	0.5	0.4	0.1	0.0	0.6	0.3
8,9-DiHETrE	0.2	0.0	0.3	0.1	0.2	0.0	0.3	0.1
8-HETE	0.7	0.1	1.0	0.4	0.6	0.1	1.1	0.3
9-HODE	8.8	0.9	8.4	1.2	8.9	1.0	8.2	0.9
9,10,13-TriHOME	5.5	2.1	2.1	0.3	5.9	2.3	2.0	0.2
9,10-DIHOME	3.7	0.4	3.0	0.5	3.7	0.5	3.1	0.4
9,12,13-TriHOME	3.2	1.1	1.6	0.3	3.4	1.2	1.6	0.2
9-HOTrE	0.8	0.1	0.9	0.2	0.7	0.1	1.0	0.2
Maresin	27.6	4.4	20.6	6.1	26.5	4.6	26.3	6.6
PGD_2	10.4	3.7	4.6	1.1	27.5	17.9	8.6	3.1
PGE_2	0.3	0.0	0.4	0.0	0.3	0.0	0.4	0.0
$PGF_{2\alpha}$	0.3	0.1	0.1	0.1	0.3	0.0	0.3	0.1
TXB_2	0.1	0.0	0.2	0.1	0.1	0.0	0.2	0.1

Table 20: Plasma Oxylipin Concentration (nM) by Presence of Event

Oxylipin	No TIA (n=82)	SEM	Yes TIA (n=16)	SEM	No CVA (n=88)	SEM	Yes CVA (n=10)	SEM
10-HDOHE	0.5	0.1	0.4	0.1	0.5	0.1	0.9	0.4
11,12-DiHETrE	0.6	0.0	0.8	0.1	0.7	0.0	0.7	0.1
11-HDOHE	0.9	0.1	0.8	0.3	0.8	0.1	1.5	0.6
11-HETE	0.8	0.1	0.8	0.1	0.8	0.1	1.3	0.6
12-HETE	3.9	0.6	3.5	0.8	3.9	0.5	4.0	1.1
12,13-DiHOME	3.3	0.4	2.7	0.2	3.2	0.3	3.1	0.5
12-HEPE	0.9	0.2	0.8	0.3	0.9	0.1	1.3	0.5
13-HODE	9.8	0.9	10.8	1.3	10.1	0.9	9.4	1.4
13-HDOHE	0.3	0.1	0.2	0.1	0.3	0.1	0.5	0.3
13-OXOODE	9.0	2.2	6.9	1.1	8.9	2.1	5.9	1.0
14,15-DiHETrE	0.8	0.0	0.8	0.1	0.8	0.0	0.9	0.1
14-HDOHE	1.5	0.2	1.5	0.5	1.5	0.2	1.8	0.5
15-HETrE	0.6	0.1	0.5	0.1	0.6	0.1	0.9	0.4
15-HETE	1.2	0.1	1.2	0.1	1.1	0.1	1.8	0.5
16-HDOHE	0.4	0.1	0.3	0.1	0.3	0.1	0.6	0.3
16-HETE	0.1	0.0	0.2	0.0	0.1	0.0	0.4	0.1
17-HDOHE	0.9	0.2	1.0	0.4	0.9	0.2	1.4	0.8
18-HEPE	0.1	0.0	0.1	0.1	0.1	0.0	0.4	0.2
18-HETE	0.3	0.1	0.3	0.1	0.3	0.1	0.5	0.2
19,20-DiHDPA	1.5	0.1	1.7	0.2	1.5	0.1	1.8	0.2
20-HDOHE	0.4	0.1	0.5	0.2	0.4	0.1	0.6	0.2
4-HDOHE	0.6	0.1	0.5	0.1	0.5	0.1	1.3	0.7
5-HETE	1.4	0.2	1.4	0.3	1.3	0.1	2.3	0.9
5,6-DiHETrE	0.3	0.0	0.2	0.0	0.3	0.0	0.3	0.1
5-HEPE	0.7	0.1	0.7	0.2	0.6	0.1	1.4	0.6
6keto-$PGF_{1\alpha}$	0.7	0.0	0.6	0.1	0.7	0.0	0.7	0.1
7-HDOHE	0.3	0.1	0.2	0.1	0.2	0.1	0.6	0.4
8,9-DiHETrE	0.2	0.0	0.2	0.0	0.2	0.0	0.3	0.1
8-HETE	0.8	0.1	0.7	0.2	0.7	0.1	1.3	0.5
9-HODE	8.6	0.9	9.2	1.2	8.8	0.8	8.1	1.4
9,10,13-TriHOME	5.4	2.1	2.6	0.4	5.3	1.9	2.2	0.4
9,10-DIHOME	3.6	0.4	3.5	0.6	3.6	0.4	3.5	0.7
9,12,13-TriHOME	3.1	1.1	2.0	0.3	3.1	1.0	1.7	0.3
9-HOTrE	0.8	0.1	0.9	0.2	0.8	0.1	0.8	0.3
Maresin	26.2	4.4	27.8	6.9	26.2	4.0	28.5	12.0
PGD_2	26.2	16.1	5.7	1.0	23.9	15.0	13.9	7.3
PGE_2	0.3	0.0	0.4	0.1	0.3	0.0	0.4	0.0
$PGF_{2\alpha}$	0.3	0.1	0.3	0.1	0.2	0.0	0.6	0.3
TXB_2	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1

Abbreviations: 6 keto (6k), acute coronary syndrome (ACS), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DiHETrE), dihydroxyoctadecenoic acid (DiHOME), HETrE (hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid, (HDOHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETrE), hydroxyoctadecadienoic acid (HODE), hydroxyoctadecatrienoic acid (HOTrE), oxooctadecadienoic acid (OXOODE), prostaglandin (PG), standard error of the mean (SEM), transient ischemic attack (TIA), trihydroxyoctadecenoic acid (TriHOME), thromboxane (TX).

Table 21: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Transient Ischemic Attacks

Variable	Cases	Controls	Univariate OR (95% CI)	p-valu	Multivariate OR (95% CI)	p-value
Plasma Oxylipins Score	43	N/A				
Selection						
$TXB_2/6kPGF_{1\alpha}$			3.8 (3.6, 3.9)	0.0048	3.8 (3.6, 3.9)	0.019
$6kPGF_{1\alpha}$			0.066 (0.061, 0.072)	0.040		
Other Characteristics						
Age ≥65 years	65	33	1.8 (0.54, 6.2)	0.33		
Female Gender	25	73	0.37 (0.077, 1.7)	0.21		
Smoking Status (current)	25	73	0.36 (0.076, 1.7)	0.20		
Hypertension	74	24	1.3 (0.33, 5.0)	0.71		
Diabetes	33	65	0.96 (0.30, 3.1)	0.95		
Hyperlipidemia	78	20	0.97 (0.24, 3.8)	0.96		
Medications						
Beta Blocker	33	65	3.1 (1.0, 9.3)	0.043		
Ca Channel Blocker	27	71	1.7 (0.57, 5.4)	0.33		
ACE inhibitor	47	51	0.90 (0.31, 2.6)	0.85		
ARB	15	83	1.3 (0.33, 5.4)	0.68		
Diuretic	39	39	0.64 (0.20, 2.0)	0.45		
Aspirin	71	27	0.47 (0.16, 1.4)	0.18		
Statin	70	28	0.95 (0.62, 1.5)	0.83		

6-keto (6k), Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), Prostagladin (PG), Thromboxane (TX)

Table 22: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Cerebrovascular Accidents

Variable	Cases	Controls	Univariate OR (95% CI)	p-value	Multivariate OR (95% CI)	p-value
Plasma Oxylipins Score	43	N/A				
Selection						
16-HETE			55 (51, 60)	0.0016	55 (51, 60)	0.0016
$PGF_{2\alpha}$			3.3 (3.2, 3.5)	0.047		
Other Characteristics						
Age ≥65 years	65	33	0.33 (0.86, 1.3)	0.10		
Female Gender	25	73	2.1 (0.55, 8.3)	0.28		
Smoking Status (current)	25	73	0.81 (0.16, 4.2)	0.80		
Hypertension	74	24	1.1 (0.21, 5.7)	0.91		
Diabetes	33	65	0.97 (0.23, 4.1)	0.96		
Hyperlipidemia	78	20	0.84 (0.16, 4.4)	0.83		
Medications						
Beta Blocker	33	65	1.4 (0.36, 5.2)	0.66		
Ca Channel Blocker	27	71	0.63 (0.13, 3.2)	0.58		
ACE inhibitor	47	51	3.1 (0.75, 13)	0.12		
ARB	15	83	<0.001 (<0.001, >999)	0.96		
Diuretic	39	39	4.1 (0.99, 17)	0.052		
Aspirin	71	27	0.38 (0.10, 1.4)	0.15		
Statin	70	28	1.1 (0.29, 4.4)	0.86		

Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), Hydroxyeicosatetraenoic acid (HETE), Prostagladin (PG)

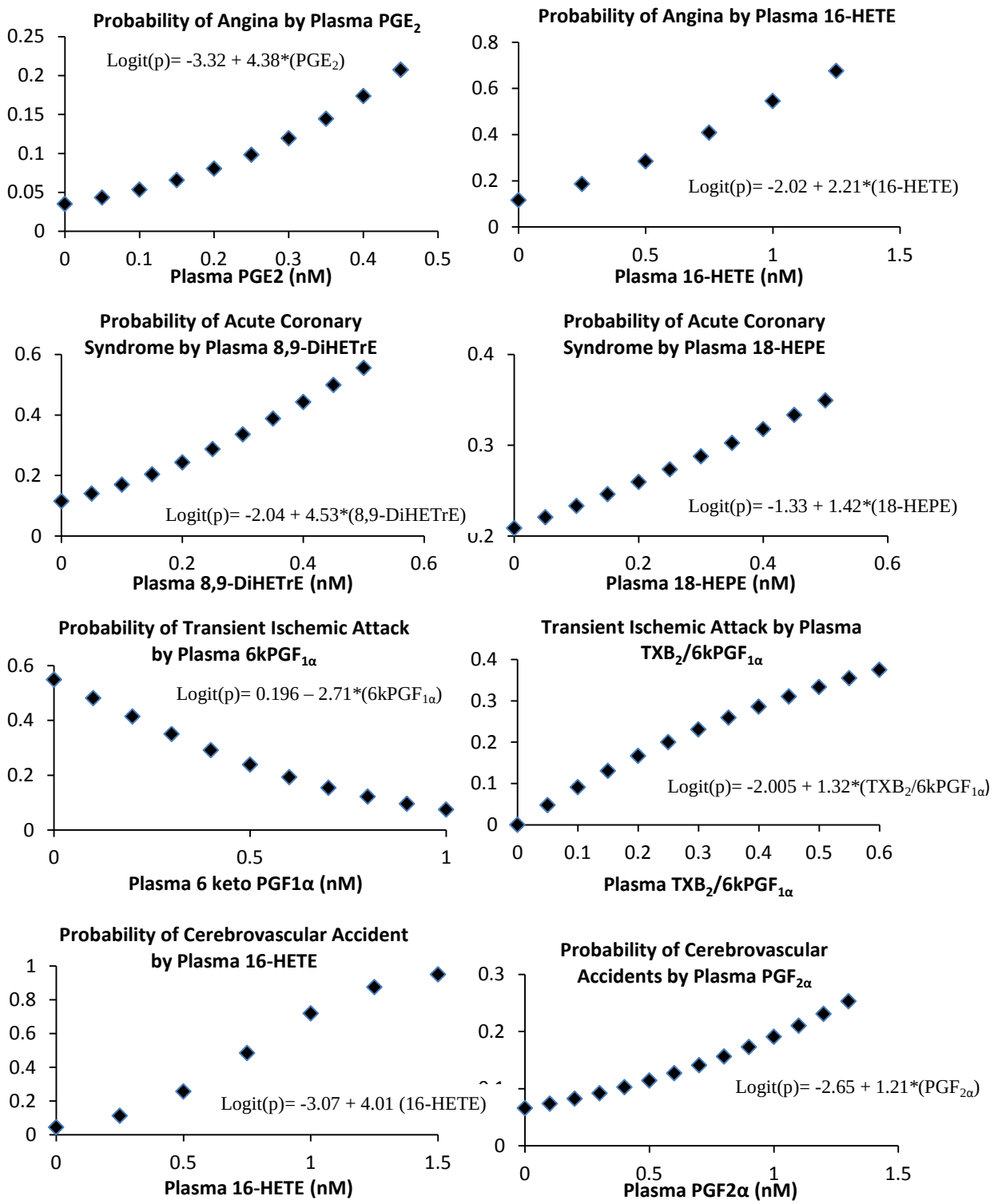


Figure 20: Probability of Cardiovascular and Cerebrovascular Events by Plasma Oxylipin Concentrations (nM).

Oxylipins were selected based on the score selection method of logistic regression to determine the strongest predicting oxylipins. Probabilities were obtained by taking the EXP of logit (p) to first obtain the odds. Probability was calculated by odds/(1+odds).

Abbreviations: 6k – 6 keto, DiHETrE – dihydroxyeicosatrienoic acid, HEPE – hydroxyeicosapentanoic acid, HETE – hydroxyeicosatetraenoic acid

PG – prostaglandin, TX – thromboxane

The plasma oxylipin profiles were also compared between those with multiple events and those with no event. 16-HETE (OR: 16.1 (14.9, 17.3)), thromboxane B_2 (OR: 10.5 (9.77, 11.3), and 11,12-DiHETrE (OR: 10.1 (9.52, 10.7)), all increased the odds of having had multiple events (ie: 2 or 3 events) versus no events. The relationship of hypertension, diabetes, hyperlipidemia, oxylipins, age, gender, smoking status, and medications to events was assessed using multivariate logistic regression. β-blockers were significantly associated with the prevalence of angina, ACS and TIAs but not CVAs. Age, gender, smoking status, hyperlipidemia, hypertension, diabetes mellitus, and other medications were not significantly associated with any events. In the presence of these factors, 16-HETE and 8,9-DiHETrE, still significantly influenced the odds of events.

The ratio of TXB_2 to $6kPGF_{1\alpha}$ has previously held predictive value for cardiovascular disease and therefore was assessed as a predictor of events in this population (3, 17). $TXB_2/6kPGF_{1\alpha}$ increased the odds of a TIA with an odds ratio of 3.79 (3.66, 3.93; p-value = 0.019) as well as having multiple events (ie: 2 or 3 events) versus no event OR: 5.13 (4.90, 5.36; p-value = 0.024). Adding all four DiHETrEs together provided predictive value for having any event OR: 2.0 (2.0, 2.1) p-value = 0.034 as well as an ACS OR: 1.95 (1.05, 3.64) p-value = 0.035. Adding the concentration of HETEs had no predictive value for events. Likewise, summation of the oxylipins by their respective enzyme (cyclooxygenase, lipoxygenase, and cytochrome P450) held no predictive value.

Logistic regression was also performed comparing patients with only one event, as indicated in Figure 1, versus the rest of the population without that particular event. Because this decreased the number of cases to less than 10, this reflected an exploratory analysis only. In these assessments, every 1 nM increase in plasma 8,9-DiHETrE increased the odds

of an ACS by 454-fold (377, 547) with a p-value of 0.041. Every 1 nM increase of plasma 16-HETE increased the odds of a CVA by 25-fold (22, 27) with a p-value of 0.043. There were no significant predictors for angina or TIA in these exploratory models.

Discussion

Oxylipins may have a role in cardiovascular and cerebrovascular events because of their actions on immunity, inflammation and vascular tone (4, 5). Patients with PAD are at a higher risk of myocardial infarctions and cerebrovacular accidents versus a healthy population or even patients with coronary artery disease (10). For this reason, patients with PAD presented an important population to investigate. In this population, typical risk factors such as hypertension, diabetes, and hyperlipidemia did not influence the risk of events. This could be due to the fact that hypertension, hyperlipidemia, and diabetes mellitus were well controlled in the population. Despite this, the oxylipins which are markers for vascular tone and inflammation were significantly associated to events. Oxylipins may present a new era of risk markers/therapeutic targets particulary in the presence of well controlled risk factors.

This is the first study to observe the relationship of cardiovascular/cerebrovascular events to a comprehensive plasma lipidomics profile in patients with PAD. There was no significant relationship between fatty acid profiles and cardiovascular events; however their products, plasma oxylipins, did significantly influence the presence of events. A similar observation was observed in an obese animal model provided diets of varying fatty acid levels. Renal phospholipid fatty acid concentrations did not differ among groups but renal oxylipins significantly differed (18). Similarly in the current study, patients with past clinical events did not have significantly different concentrations of omega-6 fatty acids versus patients with no events. However, patients with events did have significantly higher concentrations of many pro-inflammatory and vasoconstrictive oxylipins produced from

omega 6 fatty acids. In contrast, one omega-6 derived oxylipin, $6kPGF_{1\alpha,}$ had a protective odds ratio of 0.066 against transient ischemic attacks. $6kPGF_{1\alpha}$ is the stable product of prostacyclin (PGI_2), which is an anti-aggregatory, endothelial-derived vasodilator (19). PGI_2 counteracts the aggregatory and vasoconstrictive effects of thromboxane A_2 (19). This was supported by our finding that the ratio of TXB_2 to $6kPGF_{1\alpha}$ held predictive value for patients having multiple events or a TIA alone.

16-HETE, thromboxane B_2 and 11,12-DiHETrE are all produced from arachidonic acid and all significantly increased the odds of having had multiple cardiovascular events versus no events. 16-HETE is produced by polymorphonuclear leukocytes *in vitro* (20) and is released upon angiotensin II stimulation which may explain its positive relationship to CVAs and angina. However, the exact mechanism for the relationship with cardiovascular events has yet to be determined and represents an intriguing area for future research. Less surprising was the positive relationship of TXB_2 with events because TXB_2 is the stable product of the potent vasoconstrictor thromboxane A_2. Similarly, thromboxane B_2 is present in significantly higher concentrations in the coronary circulation in patients with angina versus healthy subjects and during times of chest pain versus no pain (21). 11,12-DiHETrE is a product of 11,12-epoxyeicosatrienoic acid (EpETrE). When 11,12-EpETrE is converted by soluble epoxide hydrolase to 11,12-DiHETrE, it causes a significant loss of vasodilation (22, 23). Therefore, higher concentrations of 11,12-DiHETrE have been implicated in hypertension (12). It may be precisely for this reason that 11,12-DiHETrE was associated with an increased odds of events in the present study.

Oxylipins produced from other fatty acid precursors were also associated with clinical cardiovascular events in the present cohort of patients. For example, the positive relationship of plasma 18-HEPE to ACS prevalence was identified. This relationship was

unexpected. 18-HEPE is a pre-cursor to the E-series resolvins which regulate resolution of inflammation and, therefore, are generalized as protective (24, 25). It is possible that following ACS, plasma 18-HEPE levels increase as a protective adaptive mechanism to reduce inflammation. Future research is required to determine if 18-HEPE can improve cardiac remodelling post-MI.

It is also important to note that the largest slopes in the probability analysis shown in Figure 3 belonged to 8,9-DiHETrE and 16-HETE. This indicates that the odds of events changed more significantly with smaller changes in the plasma concentration of these two oxylipins versus other oxylipins. 8,9-DiHETrE and 16-HETE were also significant predictors of ACS and CVAs, respectively, in the multivariate logistic regression as well as the exploratory analysis of patients with only one event. As a result, these two molecules may prove to be particularly powerful targets for therapeutic investigation or risk marker assessment of ACS and CVAs in the future.

Relationship of other factors to events

The relationship of β-blockers to events was significant for angina, ACS, and TIAs. This analysis does not have the ability to indicate if this relationship was causative or a consequence of β-blockers. β-blockers are a first-line therapy for hypertension and angina, and are often prescribed to prevent a secondary myocardial infarction (26). For these reasons, it likely explains the relationship between this class of medications and events. It was an unexpected finding that no significant relationship existed between prevalence of events and gender, smoking status, or age. It is well documented that the prevalence of events increases with age and smoking (27). However, that relationship was not observed in the current study.

Limitations

It is important to note that several limitations exist within this study. First, because oxylipins were elevated in patients following an event, it is diffcult to ascertain if they are a cause or a consequence of the event. Because of their physiological role (ie: vasoconstricting or inflammatory), it is reasonable to propose that either or both roles are valid. Secondly, the length of time from the clinical event to the measurement of the oxylipins in this study was variable and may have influenced the oxylipin concentrations. Third, information regarding the type of CVA or ACS and the diagnostic method to detect the events was not standardized in the medical records used. Future prospective studies following a large cohort of at risk patients may resolve these questions by obtaining plasma oxylipin profiles prior to and immediately after an event.

Conclusion

In conclusion, this research supports a significant relationship for specific plasma oxylipins to cardiovascular and cerebrovascular events in the presence of well controlled risk factors. Oxylipins may have the potential to serve as new risk markers/therapeutic targets beyond conventional standard of care practice. This was not a phenomenon generalized to all lipids as plasma fatty acid concentrations were not associated with events in the current study. It is important to note that the oxylipin concentrations observed here may increase the risk of a clinical event or be important mediators in the healing process following an event. This study has identified specific oxylipins that are attractive targets to investigate for their involvement in cardiovascular and cerebrovascular events. Therefore, these oxylipins may be used as targets in future clinical or experimental investigations utilizing a lipidomics approach.

Clinical Competencies

Medical Knowledge

The aim of the current study was to utilize samples from a clinical trial to provide a foundational knowledge base for future diagnostic and therapeutic development. Current needs for physicians include: 1) better diagnostic tools for earlier diagnosis or prediction of cardiovascular and cerebrovascular events, and 2) improved therapies beyond conventional risk factor management for the prevention of events or for improved outcomes following an event. The investigators have utilized an advanced targeted lipidomics methodology to investigate oxylipins. Specific oxylipins can regulate vascular tone, inflammation, and an immune response. All three of these processes are critical in the pathophysiology of clinical events and healing following an event. The investigators have identified a limited number from over 100 specific oxylipins that are significantly associated with clinical cardiovascular events in patients with peripheral artery disease. These data provide information on potentially important targets for therapeutic interventions that may become clinically useful in the near future.

Translational Outlook

Future Research Directions

By identifying novel markers of clinical events using the latest lipidomics analyses, it may be possible to further elucidate the potential of these specific oxylipins in the diagnosis and treatment of clinical events in carefully designed clinical trials. This can only be accomplished by collaboration with researchers and clinicians. Future research can quantitate plasma oxylipin levels in a multi-center, large population of high risk patients before and after an event. It would be important to extend this beyond patients living with PAD. In pre-clinical work, animal studies that target oxylipin receptors or the enzymes that

metabolize these oxylipins would be invaluable to understand the influence of these factors on the incidence and severity of cardiovascular events, as well as the post event healing processes.

Impediments and Challenges

The challenges include: 1) the extensive technology required (ie: solid phase extraction, HPLCS-MS/MS, stable isotope dilution quantitation) to quantitate plasma oxylipin levels. A more efficient technology will need to be developed once a specific oxylipin is targeted if this is to achieve clinical utility; 2) The need for a large high risk population in a multi-center trial to assess the validity of using these oxylipins as diagnostic markers.

Implications

This translational biomedical research provides information for the creation of diagnostics and therapeutic techniques. This study will hopefully start in earnest a wave of treatment beyond the management of conventional risk factors. In addition, currently plasma markers for transient ischemic attacks and cerebrovascular accidents do not exist. There is an intriguing potential for these molecules to act as diagnostic markers due to their relationship to events observed here. Secondly, these particular oxylipins may serve as therapeutic targets due to their role in vascular tone and inflammation and the observed relationship to events in this population. This may reveal novel directions to impact the way we diagnose and treat cardiovascular and cerebrovascular events in the future.

Disclosures

The authors have no conflicts of interest or disclosures.

Acknowledgements/Funding Sources

We would like to thank the following agencies for funding support: Canadian Institutes for Health Research, Flax 2015, Agri-Food Research Development Initiative, Western Grains Research Foundation and St. Boniface Hospital Foundation. Stephanie Caligiuri was funded by the Canadian Institutes of Health Research Patient Oriented Research Doctoral Scholarship and a Research Manitoba Doctoral Scholarship.

Chapter II Section iv References

1. Gabbs M, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in our understanding of oxylipins derived from dietary PUFAs. Adv Nutr. 2015;6:513-40.

2. Eskildsen MP, Hansen PB, Stubbe J, *et al*. Prostaglandin I2 and prostaglandin E2 modulate human intrarenal artery contractility through prostaglandin E2-EP4, prostacyclin-IP, and thromboxane A2-TP receptors. Hypertension. 2014;64:551-6.

3. Nishimaki S, Seki K. An imbalance between prostacyclin and thromboxane in relation to cerebral blood flow in neonates with maternal preeclampsia. Prostaglandins Other Lipid Mediat. 1999;58:43-9.

4. Norris PC, Dennis EA. A lipidomic perspective on inflammatory macrophage eicosanoid signaling. Adv Biol Regul. 2014;54:99-110.

5. Dennis EA, Norris PC. Eicosanoid storm in infection and inflammation. Nat Rev Immunol. 2015;15:511-23.

6. Puddu P, Puddu GM, Zaca F, Muscari A. Endothelial dysfunction in hypertension. Acta Cardiol. 2000;55:221-32.

7. Montezano AC, Touyz RM. Molecular mechanisms of Hypertension—Reactive oxygen species and antioxidants: A basic science update for the clinician. Canadian Journal of Cardiology. 2012;28:288-95.

8. Shearer GC, Newman JW. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. Curr Atheroscler Rep. 2009;11:403-10.

9. Ouriel K. Peripheral arterial disease. Lancet. 2001;358:1257-64.

10. Welten GM, Schouten O, Hoeks SE, *et al*. Long-term prognosis of patients with peripheral arterial disease: A comparison in patients with coronary artery disease. J Am Coll Cardiol. 2008;51:1588-96.

11. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013;62:1081-1089.

12. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, Pierce GN. Flaxseed consumption reduces blood pressure in patients with hypertension by altering circulating oxylipins via an alpha-linolenic acid-induced inhibition of soluble epoxide hydrolase. Hypertension. 2014;64:53-59.

13. Austria JA, Richard MN, Chahine MN, *et al*. Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. J Am Coll Nutr. 2008;27:214-21.

14. Thygesen K, Alpert JS, Jaffe AS, *et al*. Third universal definition of myocardial infarction. Circulation. 2012;126:2020-35.

15. Sacco RL, Kasner SE, Broderick JP, *et al*. An updated definition of stroke for the 21st century: A statement for healthcare professionals from the american heart Association/American stroke association. Stroke. 2013;44:2064-89.

16. Easton JD, Saver JL, Albers GW, *et al*. Definition and evaluation of transient ischemic attack: A scientific statement for healthcare professionals from the american heart Association/American stroke association stroke council; council on cardiovascular surgery and anesthesia; council on cardiovascular radiology and intervention; council on cardiovascular nursing; and the interdisciplinary council on peripheral vascular disease. the american academy of neurology affirms the value of this statement as an educational tool for neurologists. Stroke. 2009;40:2276-93.

17. de Leval X, Hanson J, David JL, Masereel B, Pirotte B, Dogne JM. New developments on thromboxane and prostacyclin modulators part II: Prostacyclin modulators. Curr Med Chem. 2004;11:1243-52.

18. Caligiuri SP, Love K, Winter T, *et al*. Dietary linoleic acid and alpha-linolenic acid differentially affect renal oxylipins and phospholipid fatty acids in diet-induced obese rats. J Nutr. 2013;143:1421-31.

19. Cheng Y, Austin SC, Rocca B, *et al*. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science. 2002;296:539-41.

20. Bednar MM, Gross CE, Balazy MK, *et al*. 16(R)-hydroxy-5,8,11,14-eicosatetraenoic acid, a new arachidonate metabolite in human polymorphonuclear leukocytes. Biochem Pharmacol. 2000;60:447-55.

21. Tada M, Kuzuya T, Inoue M, *et al*. Elevation of thromboxane B2 levels in patients with classic and variant angina pectoris. Circulation. 1981;64:1107-15.

22. Sudhahar V, Shaw S, Imig JD. Epoxyeicosatrienoic acid analogs and vascular function. Curr Med Chem. 2010;17:1181-90.

23. Falck JR, Krishna UM, Reddy YK, *et al*. Comparison of vasodilatory properties of 14,15-EET analogs: Structural requirements for dilation. Am J Physiol Heart Circ Physiol. 2003;284:H337-49.

24. Oh SF, Pillai PS, Recchiuti A, Yang R, Serhan CN. Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. J Clin Invest. 2011;121:569-81.

25. Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. Chem Rev. 2011;111:5922-43.

26. Bradley EH, Herrin J, Mattera JA, *et al*. Quality improvement efforts and hospital performance: Rates of beta-blocker prescription after acute myocardial infarction. Med Care. 2005;43:282-92.

27. Danaei G, Ding EL, Mozaffarian D, *et al*. The preventable causes of death in the united states: Comparative risk assessment of dietary, lifestyle, and metabolic risk factors. PLoS Med. 2009;6:e1000058.

v. Investigating Dietary Flaxseed in Patients with Newly Diagnosed Hypertension

The chapters previous illustrated the significant impact dietary flaxseed had on a population of patients with peripheral artery disease already taking anti-hypertensive medications. However, many questions arose as a result of these investigations.

1) Can flaxseed lower blood pressure in patients without peripheral artery disease?
2) Can flaxseed lower blood pressure in patients not on anti-hypertensive medications?
3) Can flaxseed replace the need for anti-hypertensive medications?

The next chapter outlines the study protocol for the HyperFlax clinical trial that will answer the 3 above questions. The University of Manitoba Research Ethics Board Approval for this clinical trial is found in Appendix 1. The informed consent document is located in Appendix 2. The Health Canada Clinical Trial Application can be found in Appendix 3.

The HYPERFlax Trial: Determining the anti-HYPERtensive effects of dietary Flaxseed in newly diagnosed Stage 1 hypertensive patients using a parallel, phase II, randomized, controlled, double-blinded clinical trial

Published in: *Trials.* 2014 Jun 18;15:232.

Stephanie P.B. Caligiuri,[1,2,3] email: umcaligs@myumanitoba.ca

Brian Penner[4] email: bpenner@cc.umanitoba.ca

Grant N. Pierce[1,2,3] email: gpierce@sbrc.ca

[1]Canadian Centre for Agri-food Research in Health and Medicine (CCARM) and [2]the Institute of Cardiovascular Science, St. Boniface Hospital Research Centre, 351 Taché Avenue, Winnipeg, MB R2H 2A6, and the [3]Departments of [3]Physiology and [4]Internal Medicine, University of Manitoba, 745 Bannatyne Avenue, Winnipeg, MB R3E 0J9

Abstract

Background: The World Health Organization in 2013 deemed hypertension as a global crisis as it is the leading risk factor attributed to global mortality. Therefore, there is a great need for effective alternative treatment strategies to combat a condition that affects 40% of adults worldwide. Recently, the FlaxPAD Trial observed a significant reduction in systolic and diastolic blood pressure in hypertensive patients with peripheral arterial disease that consumed 30 g of milled flaxseed per day for one year. However, these patients were already on anti-hypertensive medication. Therefore, there is a need to assess if dietary flaxseed can effectively reduce blood pressure in the absence of peripheral arterial disease and anti-hypertensive medication in newly diagnosed hypertensive patients.

Methods/Design: The HYPERFlax Trial is a parallel, superiority, phase II, randomized, double-blinded, controlled clinical trial. St. Boniface Hospital and the Health Sciences Centre of Winnipeg, Canada, will recruit 100 participants newly diagnosed with Stage 1 hypertension who have yet to be administered anti-hypertensive medication. Participants will be randomly allocated with a 1:1 ratio into a flaxseed or control group and provided food products to consume daily for 6 months. At baseline, 2, 4, and 6 months, participant assessments will include the primary outcome measure, averaged automated blood pressure and secondary measures: 24-hour food recall, international physical activity questionnaire, anthropometrics, and blood and urine sampling for biochemical analysis. Plasma will be assessed for lipids, metabolomics profiling, and molecules that regulate vascular tone. Urine will be collected for metabolomics profiling. With an estimated drop-out rate of 20%, the trial will have a power = 0.80 to detect differences between groups and across time of an effect size = 0.7 SD at an α level = 0.05.

Discussion: This trial will determine if dietary flaxseed is efficacious over 6 months as an anti-hypertensive therapy in subjects newly diagnosed with hypertension. If flaxseed can

effectively reduce blood pressure as a monotherapy, then flaxseed will provide individuals on a global basis with a cost-effective food-based strategy to control hypertension.

Trial Registration Number: NCT01952340

List of Abbreviations

Alpha-linolenic acid (ALA)

Body Mass Index (BMI)

Flaxseed for Peripheral Arterial Disease (FlaxPAD)

anti-HYPERtensive effects of dietary Flaxseed (HYPERFlax)

Background

The World Health Organization in 2013 highlighted that high blood pressure is a global crisis (1). Hypertension was identified as the number one risk factor attributable to most deaths worldwide and accounted for 16.5% of deaths (1). Globally, in 2008, 40% of adults aged ≥25 years had hypertension (1). Many individuals live with undiagnosed, untreated, or uncontrolled hypertension and this can lead to cerebrovascular disease, dementia, stroke, retinopathy, myocardial infarctions, heart failure, coronary artery disease, renal disease, vision impairment and peripheral arterial disease (1). Therefore, it is imperative to identify more effective and desirable treatment options to reduce the burden of hypertension globally.

Dietary interventions have been suggested as a preferential complimentary strategy to current pharmacological strategies to control blood pressure (2). Flaxseed is one dietary intervention that has been used recently to reduce the risk of cardiovascular disease. Flaxseed has exhibited cardioprotective effects in animal models pre-disposed to cardiovascular disease by reducing atherogenicity (3, 4), (5), plasma cholesterol (4), (6), plasma glucose (6), plasma trans fats (5), and blood pressure (7).

In humans, patients with peripheral arterial disease (75% hypertensive) were administered 30 g of milled flaxseed per day for 6 months. Flaxseed consumption resulted in a large decrease in systolic (-10 mmHg) and diastolic blood pressure (-7 mmHg) that was statistically different from the control group (8). Blood pressure was inversely associated with plasma concentrations of two flaxseed bioactives, alpha linolenic acid (ALA) and enterolignans (8).

Despite the significant anti-hypertensive effects demonstrated in the FlaxPAD Trial, the results contained some important limitations. Firstly, participants of the FlaxPAD Trial were already on anti-hypertensive medication yet still exhibited poorly controlled blood

pressure (8). Therefore, it is not possible to conclude if flaxseed can reduce blood pressure as a monotherapy or if it is only effective in conjunction with anti-hypertensive medication. Secondly, it remained unclear if flaxseed could effectively reduce blood pressure in hypertensive patients without co-existing peripheral arterial disease. Therefore, despite evidence showing the potential of flaxseed as a potent cardioprotective functional food, the efficacy of flaxseed as an independent treatment to reduce blood pressure in newly diagnosed patients with primary hypertension has yet to be investigated.

The hypothesis to be tested in the present study is: dietary flaxseed will reduce blood pressure in those patients with newly diagnosed hypertension. In order to test this hypothesis, a phase II, randomized, double-blinded, controlled clinical trial called HYPERFlax has been designed. The Acronym for the trial, HYPERFlax, stands for the anti-HYPERtensive effects of dietary Flaxseed. Parameters such as blood pressure, plasma and urine metabolomics, plasma lipid profiling, and plasma vascular tone regulators will be analyzed to assess the efficacy and mechanisms of action of dietary flaxseed in hypertension management.

The aims of the HYPERFlax Trial are:

1. Determine if consuming 30 g of milled flaxseed daily can effectively reduce blood pressure over 6 months in newly diagnosed Stage 1 primary hypertensive participants.
2. Determine if dietary flaxseed is efficacious as a monotherapy.
3. Determine if the dosage of anti-hypertensive medication can be reduced through the use of dietary flaxseed.
4. Identify the mechanisms of action responsible for the potential anti-hypertensive effects of flaxseed.

Methods/Design

Design Overview

The HyperFlax trial is a parallel, phase II, randomized, superiority, double-blinded, controlled study with an allocation ratio of 1:1. The participants, study coordinator (Stephanie Caligiuri), nurse, overseeing medical doctor (Dr. Brian Penner), Principal Investigator (Grant Pierce), and researchers will be blinded to the group designation. One individual that dispenses the food products will be aware of the group designation. The trial sponsor is St. Boniface Hospital. The trial has received approvals from the Health Canada Natural Health Products Directorate, the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. The trial is registered at clinicaltrials.gov (NCT01952340). http://www.clinicaltrials.gov/ct2/show/NCT01952340?term=grant+pierce+flax&rank=2

Settings and Participants

The trial will take place at the Health Sciences Centre Hospital and St. Boniface Hospital in Winnipeg, Manitoba, Canada. The aim is to enroll 100 participants. Based on the previous FlaxPAD trial (8), the expected attrition rate is 20%, which still allows for a power = 0.80 to detect differences between groups and across time of an effect size = 0.7 standard deviation at an α level = 0.05. Participants will be recruited through doctor referral or in response to public advertisements. The inclusion and exclusion criteria below fit the criteria of individuals who are not recommended to be administered anti-hypertensive medication immediately according to the Canadian Hypertension Education Program Guidelines (9). These individuals do not have a systolic/diastolic blood pressure >160/100 mmHg and do not have diabetes or macrovascular target organ damage. The exclusion criteria, therefore, allow a period of time for lifestyle intervention, including new therapeutic strategies like dietary flaxseed, in low-risk hypertensive individuals.

Inclusion Criteria

1. Stage 1 essential hypertension (average automated systolic blood pressure of 135-160 mmHg OR diastolic blood pressure of 85-100 mmHg) (9)
2. Newly diagnosed. Defined as being clinically diagnosed within the last 6 months. This includes being diagnosed at the screening examination.
3. Either gender.
4. Untreated by medications for hypertension.
5. 18-85 years old and able to provide informed consent.
6. Females who are not pregnant and not planning on becoming pregnant during the course of the trial. A pregnancy test will not be administered for the trial.
7. Subjects administered anti-platelet therapy must be on a stable dose for 3 months prior to the study.
8. Subjects taking lipid lowering drugs must be on a stable dose for 3 months prior to the study.
9. Subjects must have access to freezer space in their residence to hold up to one month of frozen food products associated with this study.

Exclusion Criteria

1. Patients with ischemic pain at rest in limbs, ulceration, or gangrene.
2. Patient has undergone percutaneous coronary angioplasty, has had coronary bypass within the last 6 months.
3. Known secondary hypertension of any etiology.

4. Patients with confirmed and clinically significant renal or hepatic abnormalities (creatinine > 0.130 mM or creatinine clearance < 45ml/min, AST 2-3x normal, ALT > 2-3x normal) and/or electrolyte imbalance serum K+ < 3.5 or > 5.5 mM.
5. History of major bleeding.
6. Patients with diabetes mellitus, bowel disease (including Crohn's disease, celiac disease, colitis, peptic ulcer disease, irritable bowel syndrome and diverticulosis) or other diseases such as active systemic lupus erythematosus, cancer, or end stage respiratory disease.
7. Patients with macrovascular target organ damage, including: cerebrovascular disease, stroke, dementia, hypertensive retinopathy, left ventricular dysfunction, myocardial infarction, renal disease, and peripheral artery disease.
8. Patients with clinical evidence of heart failure or an estimated life expectancy less than 2 years or with high baseline cardiac risk (post ischemic or diabetic cardiomyopathy with an ejection fraction < 40%, Canadian Cardiovascular Society Class 3 or 4 angina or need for coronary revascularization procedures).
9. Subjects who are on supplements other that those prescribed by their clinician for the entire duration of the study. Please see point 10 below.
10. Subjects ingesting more than 2 servings of fish per week, taking omega-3 fatty acid supplements, and/or consuming milled flaxseed or flax oil on a regular basis (ie: ≥1 tablespoon of milled flaxseed or 1 teaspoon of flax oil per week). If the participant is willing, a 4-week washout period eliminating these supplements may be allowed before entry into the trial.
11. Patients having participated in an investigational drug program in the preceeding 30 days or unable or unwilling to comply with the protocol.

12. Subjects with allergies to any ingredient in the study product or control foods (including gluten).
13. Patients who will undergo surgery or intend to move outside Winnipeg during the trial period.

Consent

Interested individuals will contact the study coordinator by telephone upon which a pre-screening will be conducted to exclude participants based on the criteria above that can be determined over the phone. If the first criteria are met, a meeting will be scheduled to go through the consent and a secondary screening process will take place. The study coordinator will again go through the exclusion and inclusion criteria to see if the participants are eligible for the study. Next, all study details will be outlined and the participants will be given a chance to ask any questions. If they consent to the study, the participant's blood pressure will be measured 6 times within 10-15 minutes using a BPTru automated blood pressure machine to test if the participant has elevated blood pressure and meets the study criteria. The health care professional will also perform a brachial blood pressure measurement with a sphygmomanometer just as typically done at the doctor's office. At this time, they will also see the lead medical doctor, Dr. Brian Penner, at the hypertension clinic at Health Sciences Centre, for an examination to exclude any macrovascular target organ damage. If the participants meet the inclusion and exclusion criteria, they will be notified by telephone of their acceptance into the trial if they still choose to participate. Figure 21 details the study overview and design.

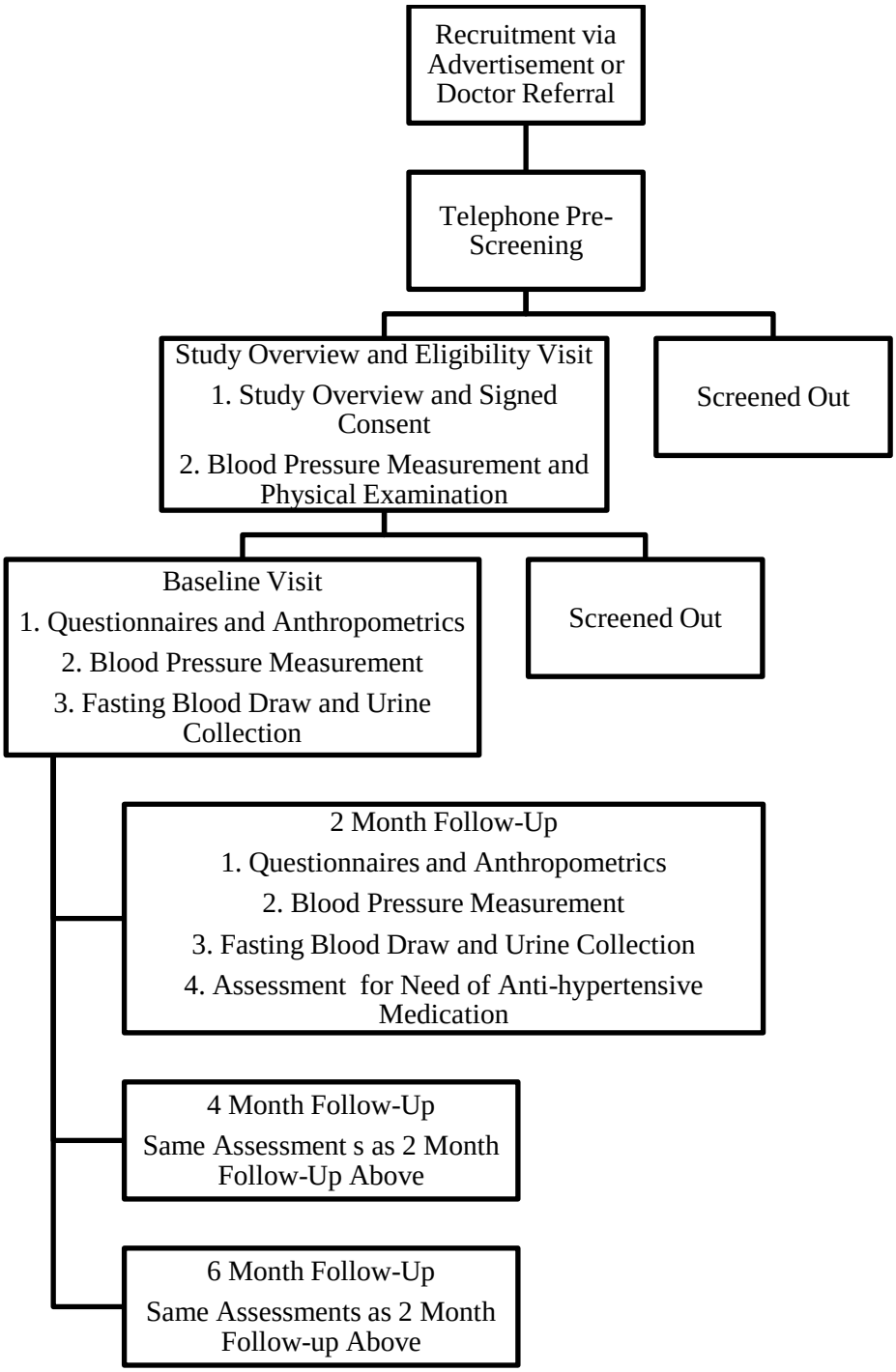


Figure 21: Study Design Overview

Randomization and Interventions

Upon acceptance into the trial, all participants will be educated on lifestyle management of hypertension. These strategies include the Dietary Approaches to Stop Hypertension, limiting alcohol and salt intake, limiting or stopping cigarette smoking, increasing physical activity, and losing weight if the individual is overweight or obese.

Immediately after the baseline assessements, subjects will be randomized to one of two possible groups by a non-restricted computer generated randomization schedule. The allocation ratio will be 1:1. The study coordinator will enroll the participants and the individual in charge of food delivery will be in charge of participant allocation. The allocation order will be in sequential, opaque, sealed envelopes. At this point all will be blinded to the group allocation excluding the individual responsible for food delivery.

The participants will be provided either the control or flaxseed containing food products such as muffins, bagels, snack bars, and milled seeds to consume once a day for six months. A variety of flavours will be provided to assure the participants do not tire of the foods. In addition, participants will be encouraged to remain in the study and be adherent by describing to them the importance of their contribution to new treatment strategies for hypertension. The control food products will contain a combination of mollasses, wheat and wheat bran to replace the flaxseed. This combination of ingredients for the control food product will and has allowed for the best concealment and abillity for a blinded trial. The formulation and flavour profiling of the flaxseed food products has been previously published (10, 11).

Participant Assessments

Averaged Automated Blood Pressure

The primary outcome measure is blood pressure. Blood pressure will be measured at a total of 5 visits: screening, baseline, 2, 4, and 6 months. A BPTru machine will be utilized to measure blood pressure 6 times within 10-15 minutes. The first reading will be discarded and the remaining 5 will be averaged. This measurement will take place in a quiet room while the participant is in a seated position and arm rested on an arm rest at heart level. A blood pressure reading with a sphygmomanometer and stethescope will also be performed to ensure the congruency between this measurement and the automated measurement.

Need for Anti-hypertensive Medication

One of the secondary outcome measures is the need for anti-hypertensive medication. In the first two months of the trial, the participants will not be prescribed anti-hypertensive medication, as this is the lifestyle intervention period. A meta-analysis published in 2008 concluded the administration of a control to patients with hypertension for 4-8 weeks provided no objective reason against safety (12). At months, 2, 4, and 6, the participants will have a blood pressure follow-up. The participants will be assessed for need of anti-hypertensive medication. If deemed necessary, they will be prescribed anti-hypertensive medication according to the Canadian Hypertension Education Program guidelines. If prescribed anti-hypertensive medication, the type and dose of anti-hypertensive medication will be recorded. If a follow-up with the participants sooner than the 2 month interval is required, the patient will be asked to return to the clinic for more visits as standard of care.

Anthropometics and Questionnaires

Secondary outcome measures also include anthropometrics, food intake, and physical activity. At baseline, the participants will be asked to fast (9-12 hour overnight fast) for blood and urine collection. The blood and urine will be analyzed utilizing techniques

including metabolomics and lipid profiling. Biological specimens will be coded with an anonymous patient code and locked in a -80°C freezer. Height, weight and waist circumference will be measured in order to calculate body mass index (BMI) and assess if the intervention may cause any changes in weight or waist circumference. They will also be asked to fill out a 24-hour food recall with the study coordinator using the Multiple-Pass Method as established by the United States Department of Agriculture (13) and the standardized international physical activity questionnaire (short) (14). These two surveys are used to assess the participants' typical food intake and physical activity habits. These assessments will be repeated at month 2, 4, and 6, to assess any changes in diet or physical activity over time, as detailed in Figure 21.

Biochemical Analysis

Secondary analysis includes biochemical analysis of plasma or blood. Biochemical analysis also will be performed to determine potential anti-hypertensive mechanisms of action and adherence to intervention. Plasma samples from baseline, 2, 4, and 6 months will be analyzed for lipid profiles, metabolomics profile, and for any circulating plasma component that may influence blood pressure regulation. Plasma analysis of alpha-linolenic acid and enterolignans will act as adherence markers. Urine samples from baseline, 2, 4, and 6 months will be analyzed for metabolomics profiling as well.

Statistical Analysis

All data will be stored without identifiable participant information in a password protected electronic document or locked cabinet. The final trial data set will be accessible only to researchers involved in the trial and any medical review board that requires access for safety purposes. In the likelihood of an unbalanced data set, absolute values will be analyzed with a mixed 2-way repeated measures model with group and time as the between

and within factor, respectively. A post-hoc comparison of the least squared means with a Tukey's adjustment will be utilized to determine where the specific differences lie. Absolute change will also be calculated only for participants who provided clinical information and samples at all time points. Absolute change can be analyzed with ANOVA/Kruskal-Wallis or multiple t-tests/Mann-U-Whitney with a false discovery rate correction. Multiple regression may be used to determine the influence of flax on blood pressure while including factors such as age, BMI, gender, diet, or physical activity. Correlations will be utilized to determine relationships between biochemical markers and blood pressure. Sub-group analyses may also be performed, for example, dichotomization of change in biochemical markers and observing subsequent change in blood pressure. All statistical tests will be set at a significance level of 0.05. Data will be published in peer-reviewed journals and discussed in public forums such as radio and television broadcasts.

Reporting and Evaluation of Serious Adverse Events

No adverse effects are expected based upon published literature and our past experience with dietary flaxseed supplementation for one year (8). Flaxseed has been granted Generally Recognized as Safe status by the Food and Drug Act and viewed as safe to consume for the general public (15). Overall, toxicology and safety data in both human and animal trials have concluded flaxseed as safe to consume (16-23). The food products provided to the participants will be prepared by companies that follow Good Manufacturing Practice. The perishable food products will be prepared, frozen, and stored frozen in a food warehourse. They will then be delivered frozen to the participants monthly.

Due to the ability of flaxseed to increase stool bulk and frequency of defecation, patients with a history of bowel obstruction, irritable bowel syndrome, or diverticular disease will be excluded. When large amounts of dietary fibre are consumed, gastrointestinal

discomfort may occur. Results from our lab have indicated that the gastrointestinal discomfort and flatulence disappear within a few weeks once the participants have become accustomed to the fibre load. In addition, gradual addition of flaxseed or wheat/wheat bran (control) throughout the first month allow the participants to become easiliy accustomed to the increase in fibre intake.

However, adverse events, as identified by the World Health Organization scale, will be followed up, if medically indicated, with relevant laboratory investigations under the direction of the study medical monitor. At this point, the medical board independent from the sponsor will be unblinded to the participant group allocation. Research staff will record the final outcome and the resolution date of the event wherever possible. If deemed necessary the medical board will have the final say if the trial should be ended.

All serious adverse events (representing a significant health hazard to the participant) will be reviewed by the medical monitor within 24 hours of becoming aware of the events. The monitor will notify the Ethics Review Board within 7-14 days of the event. The University of Manitoba and St. Boniface Hospital have Research Ethics Boards that review all clinical trials for safety and ethical considerations. Health Canada, the University of Manitoba, or St. Boniface Hospital may conduct audits at random to ensure adherement of guidelines. If any changes are required to be made to the protocol, the above parties will be notified.

Discussion and Implications

The HyperFlax trial will be the first study to investigate flaxseed as a therapeutic strategy for the reduction and management of blood pressure in patients newly diagnosed with hypertension who are yet to receive anti-hypertensive medication. Dietary flaxseed can reduce blood pressure in peripheral arterial disease patients already administered anti-hypertensive medication (8). Therefore, the next logical step was to evaluate flaxseed as a

monotherapy rather than in combination with anti-hypertensive medication and without the added complication of peripheral arterial disease. The need for therapeutic strategies to reduce the prevalence and incidence of hypertension is necessary. Therefore, this investigation aims to provide essential knowledge on an alternative treatment strategy for hypertension management.

Chapter II Section v References

1. World Health Organization. A global brief on hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization, 2013.

2. Bazzano LA, Green T, Harrison TN, Reynolds K. Dietary approaches to prevent hypertension. Curr Hypertens Rep. 2013.

3. Dupasquier CM, Weber AM, Ander BP, *et al*. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. Am J Physiol Heart Circ Physiol. 2006;291:H2987-96.

4. Dupasquier CM, Dibrov E, Kneesh AL, *et al*. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am J Physiol Heart Circ Physiol. 2007;293:H2394-402.

5. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN. The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. Am J Physiol Heart Circ Physiol. 2011;301:H2220-6.

6. Tomaz Pacheco J, Beltrame Daleprame J, Teles Boaventura G. Impact of dietary flaxseed (linum usitatissimum) supplementation on biochemical profile in healthy rats. Nutr Hosp. 2011;26:798-802.

7. Park JB, Velasquez MT. Potential effects of lignan-enriched flaxseed powder on bodyweight, visceral fat, lipid profile, and blood pressure in rats. Fitoterapia. 2012;83:941-6.

8. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013.

9. Hypertension Canada. The 2013 CHEP recommendations: 2013. http://guidelines.hypertension.ca/

10. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile and acceptability of bagels. J Food Sci. 2012;77:S62-70.

11. Aliani M, Ryland D, Pierce GN. Effect of flax addition on the flavor profile of muffins and snack bars. Food Res Int. 2011;44:2489.

12. DeFelice A, Willard J, Lawrence J, *et al*. The risks associated with short-term placebo-controlled antihypertensive clinical trials: A descriptive meta-analysis. J Hum Hypertens. 2008;22:659-68.

13. Raper N, Perloff B, Ingwersen L, Steinfeldt L, Jaswinder A. An overview of USDA's dietary intake data system. J Food Comp Anal. 2004;17:545.

14. Kim Y, Park I, Kang M. Convergent validity of the international physical activity questionnaire (IPAQ): Meta-analysis. Public Health Nutr. 2013;16:440-52.

15. Food and Drug Act. GRAS notice 000280: Whole and milled flaxseed. 2009.

16. Shultz TD, Bonorden WR, Seaman WR. Effect of short-term flaxseed consumption on lignan and sex hormone metabolism in men. Nutr Res. 1991;11:1089-100.

17. Frische EJ, Hutchins AM, Martini MC, Thomas W, Slavin JL. Effect of flaxseed and wheat bran on serum hormones and lignan excretion in premenopausal women. J Am Coll Nutr. 2003;22:550-4.

18. Faintuch J, Horie LM, Barbeiro HV, *et al*. Systemic inflammation in morbidly obese subjects: Response to oral supplementation with alpha-linolenic acid. Obes Surg. 2007;17:341-7.

19. Dodin S, Lemay A, Jacques H, Legare F, Forest JC, Masse B. The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: A randomized, double-blind, wheat germ placebo-controlled clinical trial. J Clin Endocrinol Metab. 2005;90:1390-7.

20. Bloedon LT, Balikai S, Chittams J, *et al*. Flaxseed and cardiovascular risk factors: Results from a double blind, randomized, controlled clinical trial. J Am Coll Nutr. 2008;27:65-74.

21. Orcheson LJ, Rickard SE, Seidl MM, Thompson LU. Flaxseed and its mammalian lignan precursor cause a lengthening or cessation of estrous cycling in rats. Cancer Lett. 1998;125:69-76.

22. Chen J, Tan KP, Ward WE, Thompson LU. Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. Exp Biol Med (Maywood). 2003;228:951-8.

23. Hemmings SJ, Barker L. The effects of dietary flaxseed on the fischer 344 rat: I. development, behaviour, toxicity and the activity of liver gamma-glutamyltranspeptidase. Cell Biochem Funct. 2004;22:113-21.

vi. HyperFlax Trial Progress

To date, the HyperFlax clinical trial is ongoing for recruitment of participants. In the next chapter, the data from the various recruitment methods are presented. Throughout the recruitment period, a hypertension awareness campaign was created in order to 1) screen for potential participants and 2) translate knowledge about hypertension and to increase awareness of hypertension to the public. Information such as the prevelance of different high blood pressure categories in Winnipeg and populations to focus upon are discussed in the following section.

High Blood Pressure Prevalence in Manitoba

Stephanie PB Caligiuri, S. Brian Penner, Alejandro Austria, Grant N. Pierce

In Preparation for the Canadian Journal of Physiology and Pharmacology

Abstract

Introduction: Hypertension is the leading risk factor attributed to death in the world. Clinical research pertaining to current status of hypertension prevalence and new treatments are of paramount importance. Mobile clinics may be effective means in order to assess hypertension prevalence, increase hypertension awareness, and allow for the recruitment of clinical trials.

Methods: A mobile clinic called the Hypertension Awareness Campaign was organized by nurses, students, and dietitians in Winnipeg, Manitoba. The purpose of the clinic was to measure blood pressure and to provide individualized nutrition/lifestyle education for hypertension management. Participants were categorized by hypertension category and eligibility for the HyperFlax clinical trial.

Results: From July 2014 to March 2016, 1005 adults were screened through means of an automated blood pressure measurement and questionnaire. Forty-nine percent of participants presented with normal or high-normal blood pressure and 29% were prescribed anti-hypertensive medications. Fifteen percent of participants had blood pressure within the Stage 1 range and without blood pressure medications. In addition, 2% of participants presented with a hypertensive urgency/emergency.

Conclusion: Mobile clinics are an excellent strategy to increase awareness of hypertension and to recruit participants for clinical trials. The abundance of individuals with untreated hypertension, in particular, hypertensive emergencies, are an essential population to target and treat in order to prevent cardiovascular events.

Introduction

Hypertension has a staggering impact on the quality of life and risk of morbidity and mortality worldwide. The lifetime risk of hypertension is 90% (1) and hypertension alone contributes to 7.5 million deaths per year (2). Hypertension is attributed to approximately 54% of cerebrovascular accidents and 47% of ischemic heart disease cases every year (3). As a result, hypertension in 2013 was deemed the number one risk factor associated with mortality in the world (4). Statistics such as this have led to the World Health Organization to declare hypertension as a global crisis.

The HyperFlax Trial aims to determine if dietary flaxseed can lower blood pressure in patients with Stage 1 Hypertension that have yet to be prescribed anti-hypertensive medications (5). This trial was designed for many reasons. 1) Flaxseed previously lowered blood pressure by 15 and 7 mmHg for systolic and diastolic blood pressure, respectively in patients with hypertension and peripheral artery disease (6); 2) It was unclear if flaxseed could replace anti-hypertensive medications in those with hypertension but without peripheral artery disease; 3) 51% of patients are low-adherers to their anti-hypertensive medications (7); 4) 73% of Americans believe that food and nutrition play a great role in maintaining and improving health (8); 5) 87% of Americans are interested in learning about foods with health benefits (8). As a result, a clinical trial was designed to identify a potentially desirable and accessible treatment strategy for patients with hypertension. Throughout the recruitment phase of the trial, a hypertension awareness campaign was created which in itself generated important data. The objectives of the hypertension awareness campaign were to: 1) translate scientific knowledge to the community; 2) determine the prevelance of individuals with high blood pressure yet to start anti-hypertensive medications; 3) serve as a method of recruitment for the HyperFlax Trial.

Methods and Results

The eligibility criteria and study design for the HyperFlax Trial have been previously published (5). Briefly, the objectives of the trial include: 1) to determine if dietary flaxseed can lower blood pressure in patients newly diagnosed with hypertension, 2) to determine if flaxseed can replace the need for anti-hypertensive medications, and 3) further elucidate the potential mechanisms of action. The investigators were to recruit 100 participants with newly diagnosed Stage 1 Hypertension that have yet to take anti-hypertensive medications. Individuals deemed at high risk such as those with diabetes mellitus, Stage 2 hypertension, peripheral artery disease, pregnant/breastfeeding women, have cancer, lung failure, renal failure, hepatic failure, or previous cardiovascular events are excluded from the trial. The trial is randomized, double-blinded, and controlled. The duration is 6 months with a total of 5 visits. As a result, the trial is designed to investigate the effectiveness of flaxseed rather than the efficacy. The investigators are not only answering if flaxseed can physiologically lower blood pressure, but are also investigating if dietary flaxseed can be a treatment that patients will desire, and to which they will be compliant.

In order to recruit the 100 participants, the investigators created a five-arm recruitment approach:

1) Family physician referral – letters sent to physicians with study posters and contact information to provide to patients;
2) Dr. Brian Penner (Hypertension Specialist) referral – referral directly to study coordinator;
3) Public advertisements (posters, magazine, radio, television, social media);
4) Recruitment from other clinical trials – permission provided from participants that they can be contacted about other trials. If blood pressure was elevated in other clinical trials, study coordinator would contact the participant;

5) Public screening – workplaces, colleges, and community sites and events.

The majority of participants have been screened through the public screening recruitment method (**Figure 22**). Public screening is carried out through means of the hypertension awareness campaign. During the campaign participants have their blood pressure measured through auscultatory and/or oscillometric methods after sitting at rest for a few minutes. Blood pressure measurements were performed in quiet and private locations whenever possible. Following the blood pressure measurement, the participants met with a dietitian or nurse to discuss ways to help manage or prevent hypertension. The campaign was carried out at workplaces, shopping centres, colleges, and community events. The characteristics of the population assessed were vast due to the different locations. Therefore, men and women of different ethnic backgrounds, occupations, socioeconomic status, and age (18+ years) were included in the hypertension awareness campaign.

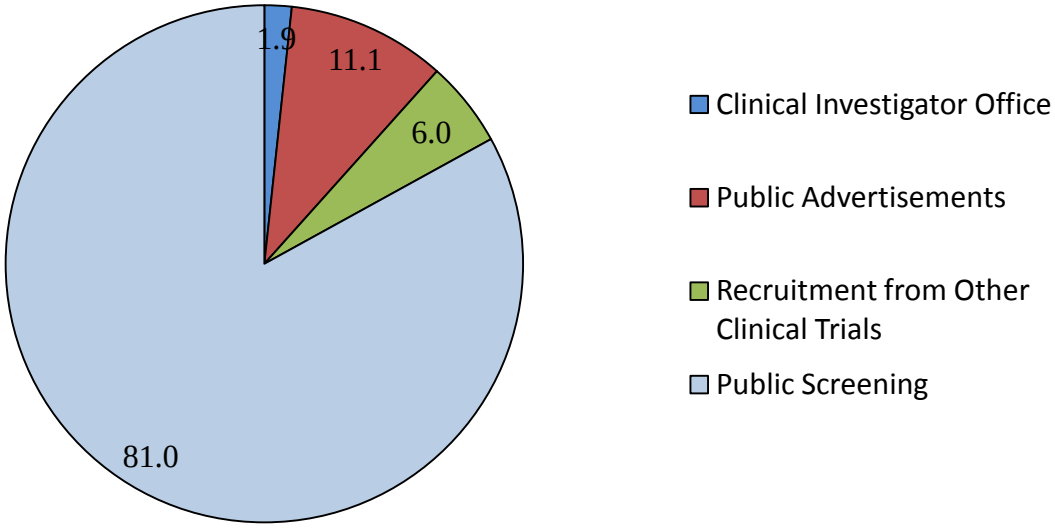


Figure 22: Proportion of Pre-Screened Individuals by Recruitment Method
Note: Family physician is not indicated in the pie chart as 0% of participants were screened/recruited from this recruitment method.

From July 2014 to March 2016, 1116 participants were pre-screened in Winnipeg, Manitoba. Of the 1116 people screened, 1005 were screened through means of a blood pressure measurement by personnel on the research team. Of the 1005 participants screened from a mobile clinic, 49% had normal or high-normal blood pressure and 29% were already taking anti-hypertensive medications. Fifteen percent of participants were eligible based on the pre-screen **(Figure 23)**. However, only approximately half of those eligible were interested or able to take part in a full screening appointment to determine eligibility for the HyperFlax clinical trial**.** Surprisingly, during the hypertension awarenss campaign, 2% of participants that had their blood pressure measured, presented with a hypertensive urgency/emergency. Two-thirds of the individuals with an urgency/emergency were previously diagnosed with hypertension but were not taking their anti-hypertensive medications. The remaining one-third were less than 40 years of age with no prior diagnosis of hypertension.

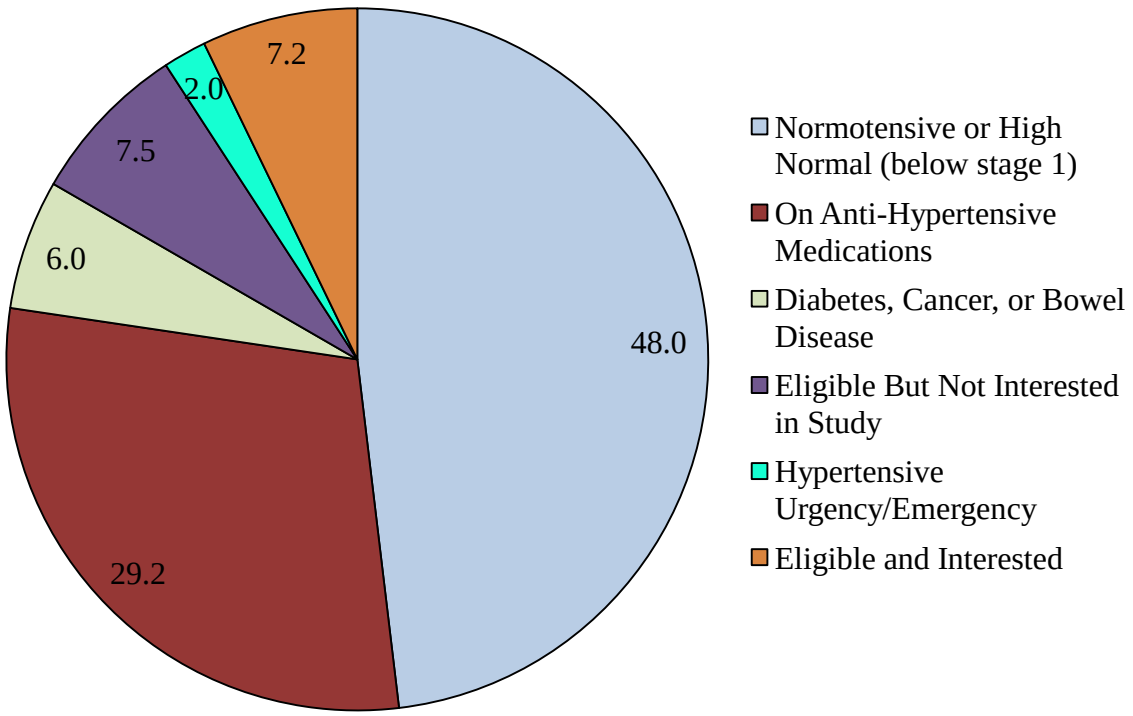


Figure 23: Percentage of Pre-screened Individuals by Category of Eligibility.

Sample size included 1005 individuals that came through the mobile hypertension awareness clinic.

Discussion

The data collected during the recruitment phase of the HyperFlax clinical trial on the prevalence of hypertension in Winnipeg are different than previously reported national and provincial reports. The Canadian average for the prevalence of hypertension, (ie: on anti-hypertensive medications or blood pressure ≥140/90 mmHg), was reported to be 22.6% in adults (9). In Manitoba, the prevalence of diagnosed hypertension was similar to the national average - 20.3% (10). In our survey of the Winnipeg population, the prevalence of hypertension/high blood pressure was much higher at 52%. Of the 52%, the majority (57 %) were already on anti-hypertensive medications. These data are quite different when compared to the national and provincial prevalences of hypertension at 20-23%. The differences in findings could be explained by means of categorization. Data was not collected based on medical records, but rather on blood pressure readings that day. Therefore, the data in the current report illustrate those with high blood pressure (≥140/90 mmHg).

Differences in the current data compared to national standards could also be explained by the differences in data collection. The national statistics were based on 3 different databases (9): 1) Canadian Health Measures Survey, 2) the National Population Health Survey and the Canadian Community Health Survey, and 3) the Canadian Chronic Disease Surveillance System. The means by which these 3 surveys collected data were different. The methods included 1) self-reported diagnosis or prescription of anti-hypertensive drugs, 2) blood pressure measurement using a BPTru machine at mobile clinics, 3) a database that determined a diagnosis of hypertension with 2 physician claims within 2 years or an in-patient hospitalization (9). The data collected in the Hypertension

Awareness Campaign was through mobile clinics only. It is arguable if the data collected from mobile clinics is more accurate than self-reporting or physician claims in a database. What is different about mobile clinics versus physician claims in a database is the mobile clinics are limited to individuals interested in having their blood pressure measured. Therefore, it can be concluded that of the individuals interested in having their blood pressure checked, approximately half are diagnosed with hypertension or have high blood pressure.

This limitation to the data can be alternatively veiwed as a strength. Individuals diagnosed with a condition may be more interested in their health or more willing to sign up/approach mobile health clinics than individuals that are not diagnosed with a condition. However, it is the individuals that are unknowing that should be targeted for health awareness. Therefore, techniques to communicate effectively to this population are important. For example, seven individuals under the age of 40 years old exhibited hypertensive emergencies during the screening campaign. The only location where these particular individuals were seen was at their workplace. Workplace mobile health clinics appear to be a very effective way to reach people who may otherwise be too busy or not have access to a physician. This has large implications not only for proper estimates of hypertension prevalence but also for hypertension and health awareness.

This data has implications for medical research advancement. (1) Recruitment of participants through mobile clinics is an effective strategy for clinical trial recruitment, and particularly for reaching individuals who otherwise would not see a family physician or participate in a clinical trial. (2) The mobile clinics offer a strategy for knowledge translation to the public. (3) Information about areas that require further health research or care may become apparent. For example, through the hypertension awareness campaign, it was

identified that hypertensive emergencies were still prevalent in people without any symptoms.

Conclusion

The hypertension awareness campaign was created with the purposes: of (1) determining the frequency of hypertension in a population from Winnipeg; (2) increasing hypertension awareness to the public and (3) to identify eligible participants for the HyperFlax clinical trial. The mobile clinics were not only an effective means of recruiting participants for the clinical trial, but the campaign provided awareness to populations unaware of impending medical need. The data also showed that approximately half of the people interested in having their blood pressure measured do indeed have high blood pressure or are already diagnosed. Clinical trials can benefit from adding mobile screening clinics as a recruitment method to the study design.

Chapter II Section vi References

1. Vasan RS, Beiser A, Seshadri S, *et al*. Residual lifetime risk for developing hypertension in middle-aged women and men: The framingham heart study. JAMA. 2002;287:1003-10.

2. World Health Organization. Global health observatory data - raised blood pressure. 2016.

3. Lawes CM, Vander Hoorn S, Rodgers A, International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. Lancet. 2008;371:1513-8.

4. World Health Organization. A global brief on hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization, 2013.

5. Caligiuri SP, Penner B, Pierce GN. The HYPERFlax trial for determining the anti-HYPERtensive effects of dietary flaxseed in newly diagnosed stage 1 hypertensive patients: Study protocol for a randomized, double-blinded, controlled clinical trial. Trials. 2014;15:232,6215-15-232.

6. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013.

7. Mazzaglia G, Ambrosioni E, Alacqua M, *et al*. Adherence to antihypertensive medications and cardiovascular morbidity among newly diagnosed hypertensive patients. Circulation. 2009;120:1598-605.

8. International Food Information Council Foundation. 2011 functional Foods/Foods for consumer trending survey. 2011.

9. Padwal RS, Bienek A, McAlister FA, Campbell NR, Outcomes Research Task Force of the Canadian Hypertension Education Program. Epidemiology of hypertension in canada: An update. Can J Cardiol. 2015.

10. Robitaille C, Dai S, Waters C, *et al*. Diagnosed hypertension in canada: Incidence, prevalence and associated mortality. CMAJ. 2012;184:E49-56.

Chapter III - Discussion

Implications of Dietary Flaxseed for Hypertension Standard of Care

More people will die from cardiovascular disease than any other disease in the world. More specifically, patients will suffer from a cerebrovascular accident or myocardial infarction (1). The number one risk factor for these events is hypertension (2). The unfortunate reality is that the majority of patients, 52%, are poor adherers to their anti-hypertensive medication routine (3). This greatly contributes to the risk of hypertensive emergencies as observed in the hypertension awarenss campaign. Hypertensive emergencies or uncontrolled hypertension significantly increase the risk of cardiovascular events (4). As a result, research to discover therapeutic strategies to which patients can easily adhere is vital for lowering the risk of cerebrovascular accidents and myocardial infarctions. The research data gathered in this thesis investigated the effectiveness of flaxseed as an anti-hypertensive strategy. When combined with anti-hypertensive medications, flaxseed dramatically lowered systolic and diastolic blood pressure in patients with PAD (the FlaxPAD trial). The mechanism by which flaxseed accomplished this may be in part through soluble epoxide hydrolase inhibition, and the subsequent reduction of inflammation and vasoconstriction.

Flaxseed induced a significant decrease in several pro-inflammatory oxylipins in healthy older adults (eg: 5-HETE and the TriHOMEs). This stimulated the next hypothesis that flaxseed may have reduced brachial blood pressure in patients with hypertension and PAD by reducing pro-inflammatory and vasoconstrictive oxylipins. It was later observed that flaxseed induced a decrease in plasma oxylipins produced by sEH in patients with hypertension and PAD. Because sEH is a current pharmacological target for hypertension, this data further supports sEH and its oxylipins as therapeutic targets in hypertension. This

research also provides information on a new inhibitor of sEH, ALA, that can be further investigated. Because hypertension is a risk factor for cardiovascular events, central aortic blood pressure and cardiovascular events themselves were investigated for their relationship to flaxseed and/or oxylipins. It was uncovered that central aortic blood pressure also decreased with flaxseed consumption and many oxylipins could predict high central aortic blood pressure. Cardiovascular events were also strongly related to particular plasma oxylipins.

As a result of this exciting data, some questions arose. 1) Can flaxseed lower blood pressure in patients without PAD? 2) Is flaxseed effective in patients not on anti-hypertensive medications? 3) Can flaxseed essentially replace the need for anti-hypertensive medications? In order to investigate this, the HyperFlax Clinical Trial was designed.

The HyperFlax Clinical trial is a 6 month randomized, double-blinded, controlled clinical trial. The purpose of the study is to investigate if 30 g of ground flaxseed can lower blood pressure and prevent the need for anti-hypertensive medications in patients newly diagnosed with hypertension. It is hypothesized that flaxseed will lower blood pressure into the normal blood pressure range and thus negating the need for blood pressure medications. Thus far, 26 participants have been recruited in the clinical trial. The implications of flaxseed for hypertension could be better control of hypertension and a decreased risk of cardiovascular events.

Many other benefits of dietary flaxseed for hypertension management exist. Benefits for flaxseed range from improvement in other risk factors to cost effectiveness.

Cost effective - Almost 80 million anti-hypertensive prescriptions are provided to Canadians every year. This equated to an expense of $3 billion in 2006 alone (5). The expense for ground flaxseed for 1 month at 30 g/day would be approximately $15 maximum

based on current consumer prices in North America. To compare, a prescription for anti-hypertensive medication can cost $42-45 in Canada and the United States per month (5, 6). In addition, the majority of patients are prescribed more than one anti-hypertensive medication, which can increase the expense even further. For those patients on multiple anti-hypertensive medications who may have resistant hypertension, flaxseed offers a potential solution when all other strategies have been inadequate (7).

Accessible – A significant implication of flaxseed for hypertension is its wide acessiblity. Despite hydrochlorothiazide, enalapril, bisoprolol, and amolodipine being on the World Health Organization's list of essential medicine, accessibility in remote areas still may be compromised (8). Developing countries may not have access to anti-hypertensive medications (8). By contrast, flaxseed is grown in North America, Asia, Europe, Africa, Australia, Central America, and South America. In addition, many of the countries that grow flaxseed are low to middle income (9). This is a critical point, for 80% of the deaths from cardiovascular disease occur in low and middle income countries. The communities living in developing countries have the greatest need for hypertension management (10). As of 2011, flaxseed is grown in 54 countries (9) and according to the World Bank income classification (11), 50% of the flax growing countries are low or middle income regions. Flaxseed, therefore, may aid in hypertension management for communities living with poor access to anti-hypertensive medications.

Fewer Side Effects Plus Additional Benefits - As indicated in previous chapters, the prevalence of side effects for anti-hypertensive medications can be up to 10% of patients. The only known side effects of flaxseed are mild gastrointestinal upset, flatulence, and bloating. This is related to the high fibre content of flaxseed. These side effects can be avoided with slow introduction of flaxseed into the diet. This was accomplished in the

FlaxPAD trial (7) and is being implemented in the current HyperFlax trial. For example, for the first week of study participation, participants would consume 1/3 of a food product every day for 1 week. The second week, they were asked to consume 2/3 of the food product/day for a week. By the third week, the participants were asked to consume the entire product that contained 30 g of flaxseed or control ingredients. Fewer side effects compared to anti-hypertensive drugs may increase the desirability of flaxseed for some. In addition, flaxseed has other health benefits such as reducing cholesterol (12), improving laxation and glycemic response (13).

The impact of oxylipins in standard of care

The data pertaining to the impact of flaxseed on oxylipins and the relationship of oxylipins to blood pressure can lead to successful therapies. Currently, research is being conducted on pharmaceutical inhibitors of soluble epoxide hydrolase (14-18). The data herein illustrate an important relationship between soluble epoxide hydrolase derived oxylipins and blood pressure. A new category of anti-hypertensive medications may improve control of blood pressure and adherence. The data herein also illustrates an important relationship between central aortic blood pressure and oxylipins. There is potential for particular oxylipins that were strongly associated with central aortic blood pressure, such as 16-HETE, to act as a diagnostic marker of central hypertension. Of interest, certain oxylipins were associated with only central blood pressure but not brachial blood pressure. The data herein has narrowed down a large list of oxylipins to a particular few that may be important to target for diagnostic potential and therapeutic development.

Oxylipins were also strongly related to cardiovascular events in patients, ie: stable angina, acute coronary syndrome, transient ischemic attack, and cerebrovascular accidents. The ability of these oxylipins to act as a marker of cerebrovascular accidents is particularly intriguing. Currently, a diagnostic marker for CVAs in the blood does not exist. To diagnose

a CVA, a CT Scan or MRI is required. These methods of detection have many limitations including length of time and access. A diagnostic marker in the blood would facilitate a much more rapid diagnosis and therefore more rapid treatment and preservation of neuronal cells. With a faster implementation of treatment, one would expect a lower fatality rate and a better quality of life for patients. The research in this thesis lays the foundation for oxylipins to be further investigated in a more specific and larger population to assess the capacity of these molecules to act as diagnostic markers.

Challenges with the HyperFlax Clinical Trial

Through the challenges of the HyperFlax Clinical trial, a great amount of information can be shared in order to assist other investigators with predictions for recruitment and study design. The ability to recruit the required number of participants into a clinical trial within a short time period places a significant barrier to the success of clinical trials. Slow recruitment rates can lead to more costly trials and can impact the integrity of samples and results. Inadequate recruitment can be a common cause for the failure of clinical trials and the inability to translate basic research into a clinical setting. This may also result in longer clinical trial duration than anticipated or incompletion of trials. This is supported by the data output by clinicaltrials.gov. In 2010, 101,163 clinical trials were registered. Five years later when results would be anticipated from the majority of trials, only 20,468 trials obtained results (19).

Strategizing eligibility criteria and recruitment methods to enhance recruitment rates should be a high priority for all clinical trials. Strategies to ensure adequate participant recruitment include electronic health record clinical trial alert systems. These systems should be instituted in Canada in order to stimulate clinical research and to improve standard of care, not to mention the added benefit of the additional medical attention patients would receive when participating in a clinical trial. Projected recruitment rates as in this scenario,

are often overestimated. It was our hope that benefits of participation such as free food every day for 6 months, access to the province's hypertension specialist (Dr. Brian Penner), and personalized nutrition information/education would entice individuals to enroll in the clinical trial. However, it is not patient interest that is the largest barrier to recruitment. Interest in the HyperFlax trial was relatively high. The greatest difficulty to patient recruitment was in finding individuals that were generally healthy, not yet on anti-hypertensive medications, but exhibited Stage 1 hypertension. The hope is that by sharing the challenges and recommendations, other researchers may be able to establish successful clinical trials in order to advance patient standard of care.

In terms of study participant recruitment, of the 1116 participants screened, 5.6% of individuals were eligible at pre-screen and interested in coming in for a full screen. This equated to 63 individuals being contacted to be scheduled for a full screening appointment. Of the 63, after resting auscultatory and oscillemetric blood pressure measurement, serum analysis, urine analysis, EKG, and physical exam, 26 individuals were eligible and recruited for the trial. This resulted in a recruitment rate of 2.3%, or in other words, 1.4 people per month. This recruitment rate is well below what was anticipated. The majority of patients recruited into the trial came from the public screening and referral from Dr. Penner (Hypertension Specialist). Because of the large number of people pre-screened from the public screening method, referral from a hypertension specialist appears to be the most effective recruitment strategy. This is likely because these patients are formally diagnosed with hypertension at this point. Throughout the process of study design, ethics board approvals, and recruitment, the investigators met many challenges and learned of ways to meet those challenges. Below are a list of five challenges and recommendations to overcome the obstacles.

Challenge #1

Limited to a finite population. Whenever a food or nutritional product is being investigated to replace a medication in a long term trial, the investigators are limited to recruiting a narrow population for safety reasons. In this regard, participants with diabetes mellitus, Stage 2 Hypertension, those diagnosed with hypertension > 6 months ago, those on anti-hypertensive medication prior cannot stop medications for the trial, or those with macrovascular target organ damage were excluded. As a result, a significant number of individuals were screened out.

Recommendations:

1) To include not only those with Stage 1 Hypertension but also individuals with high-normal blood pressure.
2) Design a shorter-term clinical trial (ie: 4 weeks) to assess efficacy. In this regard, the exclusion criteria would be less strict.

Challenge #2

The control group must be an active comparator. Because some effective treatment strategies exist for hypertension management, it is difficult for new treatments to enter the market. For this reason, superiority trials are designed. With these trial designs, an active comparator group acts as the control group. In the case of hypertension clinical trials, the "control" group could be receiving a standard of care anti-hypertensive medication (20) or in our case, nutrition counseling. In this regard, the therapeutic of interest should be superior to the current treatments in order to be considered in standard of care.

Recommendation:

1) Investigate the therapeutic as an addition to the new prescription of anti-hypertensive medications. Ie: both groups are prescribed an anti-hypertensive medication; but one group has the new therapeutic + anti-hypertensive medication.

2) A short term trial looking at the acute effects of a therapeutic is less likely to require an active comparator, ie: studying the efficacy rather than effectiveness.

Challenge #3

The variability of blood pressure. Patients that were previously diagnosed with hypertension by a physician were required to go through a screening visit. This screening visit included 2 auscultatory readings per arm, followed by 6 oscillimetric readings 2 minutes apart on the arm with the higher reading. The oscillimetric readings were taken while the patient sat quietly in the room alone. A number of patients that were diagnosed with hypertension or had high readings in their physician's office previously, would have normal blood pressure readings during the screening visit. As a result, people that were potentially hypertensive were excluded from the trial based on one day's reading.

Recommendation:

1) To expand the inclusion of participants based on high readings in the family physician's office, use 24 hour monitorinf of blood pressure, or at home blood pressure readings. Do not base the participant eligibility on office blood pressure readings at the screening visit alone.

Challenge #4

Lack of referral from family physicians. The method of communication to family physicians was through Dr. Brian Penner, the province's hypertension specialist. A personal letter was sent out to more than 25 general practitioners requesting assistance with recruitment for the trial. A poster that included the study coordinator's contact information was provided along with a study protocol. Surprisingly, zero referrals occurred as a result of this strategy. Perhaps this form of communication was not ideal. Logistically it may have

been difficult for physicians to refer their patients to the trial, or perhaps some physicians did not see the value of the trial.

Recommendation:

1) Utilize an electronic health record clinical trial alert system. A system like this will alert physicians if their patient is eligible based on their health record and will allow the physician to confidentially send the patient's contact information to the study coordinator directly using the system (21).

Challenge #5

The anticipated recruitment rate based on national statistics was overestimated. The most recent reports on the prevalence of hypertension in Canada were published by Padwal et al in 2015 (22). The national average for the prevalence of hypertension, ie: on anti-hypertensive medications or blood pressure ≥140/90 mmHg, was reported to be 22.6% in adults. Within this hypertensive population, 84% were officially diagnosed with hypertension, 80% were currently being treated for hypertension, and 68% had blood pressure within goal (22). This meant that 3.6% of adults in Canada have high blood pressure and yet to be diagnosed and 4.5% have yet to be treated for hypertension. In another report by Robitaille et al in 2012 (23), the prevalence of diagnosed hypertension among adults aged 20 years and older in Canada was 19.6% with an incidence of 2.4 per 100 people per year. In Manitoba, the prevalence of diagnosed hypertension was close to the national average with a prevalence of 20.3% (23).

Because of these statistics, we anticipated that approximately 5-7% of adults would meet the inclusion criteria. It was also anticipated that through the different recruitment methods, approximately 30 people would be referred and/or screened per month, with a recruitment rate of 5 people per month. For example, one source reported that on average,

with 114 physicians contacted to refer patients to a clinical trial, 6 patients were referred per month by the physicians (21). It was anticipated that with the addition of the other 4 recruitment methods, it would be feasible to screen 30 potential candidates/month. However, in reality, the actual screening rate was almost double at 59 people per month and the recruitment rate was much lower at 1.4 people per month.

Recommendation:

1) Underestimate the recruitment rate if based on population prevalence;

2) Communicate with other investigators that have recruited a similar patient population to estimate the recruitment rate;

3) Plan and have the ethics board approve several recruitment strategies that can be employed if necessary to increase the recruitment rate;

4) Design a multi-centre trial in which multiple cities are recruiting simultaneously.

Despite the slower than anticipated recruitment rate, the public screening also referred to as the Hypertension Awareness Campaign, became a large part of the current thesis. Valuable information was obtained from this campaign.

Implications of the Hypertension Awareness Campaign

The implications of the hypertension awareness campaign are numerous. Firstly, the campaign provided evidence that awareness should be a pillar in all research projects. Being in the community and working with patients helps researchers to understand the important questions. The most important aspect of medical research is to ask the right questions. This is why the flaxseed research is underway - to find a desirable treatment strategy to which patients can easily adhere. Secondly, the prevalence of hypertensive emergencies in the community was not anticipated and raises an important area of concern. Individuals having a hypertensive emergency are at an incredibly high risk of organ damage and cardiovascular

events. In these individuals, no signs or symptoms were apparent. More widespread awareness campaigns are needed to help identify individuals at risk because an annual check-up with a family physician may not catch the variability in blood pressure. Home monitoring and machines in pharmacies have assisted in raising awareness; yet the data presented here clearly illustrates that additional strategies for hypertension awareness are essential. Thirdly, the obstacles to healthy eating and lifestyle changes became very apparent after speaking with many individuals. The barriers responsible for the lack of adherence to anti-hypertensive therapies needs to be understood and researched to improve standard of care in the future.

Future Directions

Determining which participants respond to flaxseed intervention will be an essential aspect for introducing flaxseed into standard of care. Data from the HyperFlax trial provide this. For example, differences in gender, age, body weight, and diet can be assessed for their impact on response to flaxseed.

The therapeutic dose of flaxseed still has to be identified. The therapeutic dose of flaxseed was selected as 30 g/day due to elevated levels of plasma ALA with flaxseed ingestion. A dose dependency study was investigated in healthy individuals (24). Doses of 10 g, 20 g, 30 g, and 40 g were studied. Significantly higher plasma ALA concentrations were exhibited in those consuming 30 g versus 20 and 10 g. However, the plasma ALA concentrations were the same between the 30 g and 40 g groups (24). Because 30 g would be easier to incorporate into a food product versus 40 g, the 30 g dose was chosen. However, the lowest possible dose that exerts anti-hypertensive effects has yet to be investigated.

During the process of the HyperFlax clinical trial, many participants of a smaller stature and body weight had some difficulty consuming a food product containing all 30 g of

ground flaxseed. By contrast, it could be argued that a dose per kg of body weight is justified. A functional food is not the same as a medication even though it may have therapeutic effects. Because flaxseed is a food, the dose should be considered as a food. A 120 kg individual requires more calories, fat, protein, and carbohydrates, than an individual that weighs 50 kg. In the HyperFlax trial, considering body weight as a determinant of blood pressure response to flaxseed would provide insight into this possibility. Instead, a flaxseed dose per kg of body weight could be used. For example, 0.45 g of flaxseed per kg of body weight per day could be proposed. For a 70 kg individual, a dose of 31.5 g of ground flaxseed per day would be recommended whereas a 50 kg individual would require 22.5 g/day and a 115 kg individual would require 52 g of flaxseed per day.

For oxylipins, many future directions exist. Many oxylipins can be directly tested for their effect on blood pressure, CVA incidence, or ACS incidence in animal models. For example, the spontaneously hypertensive stroke prone model or the thromboemoblic model could be intravenously injected with 16-HETE to determine the effect on blood pressure and CVA occurrence or severity. Many of the oxylipins that strongly correlated to blood pressure or stroke could be investigated in this manner. In an opposing manner, inhibitors or receptor blockers of the oxylipins could be investigated for the influence on blood pressure and CVA severity in animal models as well.

To investigate the diagnostic potential of oxylipins, an observational clinical trial could be designed. Blood samples from individuals who are hospitalized for transient ischemic attacks or cerebrovascular accidents could be assessed for levels of oxylipins in the blood. This could be compared to a population that has not suffered from a CVA to determine the potential of these markers for diagnosis. This could also be assessed at the cellular level. Cultured human brain tissue can be exposed to hypoxia as in an ischemic

cerebrovascular accident. To determine if oxylipins are released, increased, or altered at all during hypoxia could provide insight into the pathophysiological processes occurring in the brain of patients suffering a CVA.

Conclusion

The current thesis highlights the need for improved hypertension management. This can be achieved by investigating functional foods and natural health products as effective therapies. One therapy that stands out in particular is flaxseed due to its significant impact on blood pressure in patients with peripheral artery disease and hypertension. Flaxseed lowered blood pressure likely through means of soluble epoxide hydrolase inhibition and the reduction of pro-inflammatory and vasoconstrictive oxylipins. Particular plasma oxylipins in this patient population were associated with a change in brachial blood pressure, central aortic blood pressure, and cardiovascular events. New research will investigate if flaxseed can lower blood pressure in patients newly diagnosed with hypertension. The hope is to generate research that can lead to effective therapies for patients worldwide in order to reduce the burden of cardiovascular disease on patients and their families.

Chapter III References

1. The World Health Organization. Top 10 causes of death. fact sheet N°310. 2014.

2. World Health Organization. Global health observatory data - raised blood pressure. 2016.

3. Mazzaglia G, Ambrosioni E, Alacqua M, *et al*. Adherence to antihypertensive medications and cardiovascular morbidity among newly diagnosed hypertensive patients. Circulation. 2009;120:1598-605.

4. Sokol MC, McGuigan KA, Verbrugge RR, Epstein RS. Impact of medication adherence on hospitalization risk and healthcare cost. Med Care. 2005;43:521-30.

5. Jackevicius CA, Cox JL, Carreon D, *et al*. Long-term trends in use of and expenditures for cardiovascular medications in canada. CMAJ. 2009;181:E19-28.

6. Drugs: Savings from generic drugs purchased at retail pharmacies. 2009. Accessed April 2 2016. http://www.fda.gov/drugs/resourcesforyou/ucm134205.htm

7. Rodriguez-Leyva D, Weighell W, Edel AL, *et al*. Potent antihypertensive action of dietary flaxseed in hypertensive patients. Hypertension. 2013.

8. World Health Organization. WHO model lists of essential medicines. 2016. http://www.who.int/selection_medicines/committees/expert/20/EML_2015_FINAL_amended_AUG2015.pdf?ua=1

9. Food and Agriculture Organization of the United Nations. Linseed. 2014. http://faostat3.fao.org/faostat-gateway/go/to/search/flaxseed/E

10. World Health Organization. A global brief on hypertension: Silent killer, global public health crisis. WHO/DCO/WHD/2013.2 ed. Geneva, Switzerland: World Health Organization, 2013.

11. The World Bank: Income levels. 2013. Accessed April 1 2016. https://wdronline.worldbank.org/worldbank/a/incomelevel

12. Edel AL, Rodriguez-Leyva D, Maddaford TG, *et al*. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease. J Nutr. 2015;145:749-57.

13. Dahl WJ, Lockert EA, Cammer AL, Whiting SJ. Effects of flax fiber on laxation and glycemic response in healthy volunteers. J Med Food. 2005;8:508-11.

14. Honetschlagerova Z, Kitada K, Huskova Z, *et al*. Antihypertensive and renoprotective actions of soluble epoxide hydrolase inhibition in ANG II-dependent malignant hypertension are abolished by pretreatment with L-NAME. J Hypertens. 2013;31:321-32.

15. Kato Y, Fuchi N, Saburi H, *et al*. Discovery of 2,8-diazaspiro[4.5]decane-based trisubstituted urea derivatives as highly potent soluble epoxide hydrolase inhibitors and orally active drug candidates for treating hypertension. Bioorg Med Chem Lett. 2013;23:5975-9.

16. Varcabova S, Huskova Z, Kramer HJ, *et al*. Antihypertensive action of soluble epoxide hydrolase inhibition in ren-2 transgenic rats is mediated by suppression of the intrarenal renin-angiotensin system. Clin Exp Pharmacol Physiol. 2013;40:273-81.

17. Imig JD, Walsh KA, Hye Khan MA, *et al*. Soluble epoxide hydrolase inhibition and peroxisome proliferator activated receptor gamma agonist improve vascular function and decrease renal injury in hypertensive obese rats. Exp Biol Med (Maywood). 2012;237:1402-12.

18. Kopkan L, Huskova Z, Sporkova A, *et al*. Soluble epoxide hydrolase inhibition exhibits antihypertensive actions independently of nitric oxide in mice with renovascular hypertension. Kidney Blood Press Res. 2012;35:595-607.

19. US National Institutes of Health. Clinical trials.gov - trends, charts, and maps. 2016. https://clinicaltrials.gov/ct2/resources/trends#RegisteredStudiesOverTime

20. Stanton A, Jensen C, Nussberger J, O'Brien E. Blood pressure lowering in essential hypertension with an oral renin inhibitor, aliskiren. Hypertension. 2003;42:1137-43.

21. Embi PJ, Jain A, Clark J, Bizjack S, Hornung R, Harris CM. Effect of a clinical trial alert system on physician participation in trial recruitment. Arch Intern Med. 2005;165:2272-7.

22. Padwal RS, Bienek A, McAlister FA, Campbell NR, Outcomes Research Task Force of the Canadian Hypertension Education Program. Epidemiology of hypertension in Canada: An update. Can J Cardiol. 2016 May;32(5):687-94.

23. Robitaille C, Dai S, Waters C, *et al*. Diagnosed hypertension in Canada: Incidence, prevalence and associated mortality. CMAJ. 2012;184:E49-56.

24. Edel AL, Patenaude AF, Richard MN, *et al*. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults. Eur J Nutr. 2016;55:651-63.

Chapter IV - Appendices

Appendix 1 – Research Ethics Board Approval for the HyperFlax Clinical Trial

P126 - 770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Telephone 204-789-3255
Fax 204-789-3414

UNIVERSITY of MANITOBA | BANNATYNE CAMPUS Research Ethics Boards

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)

CERTIFICATE OF FINAL APPROVAL FOR NEW STUDIES

Full Board Review

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. G. Pierce	St. Boniface Hospital Research Centre / Physiology and Pharmacology	B2013:079
BREB MEETING DATE:	**APPROVAL DATE:**	**EXPIRY DATE:**
June 24, 2013	December 18, 2013	June 24, 2014

STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (if applicable):

PROTOCOL NUMBER: NA

PROJECT OR PROTOCOL TITLE: The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Agriculture Canada and Canola Council of Canada

Submission Date(s) of Investigator Documents:	REB Receipt Date(s) of Documents:
June 6, undated, December 12, 2013	June 7, October 11 and December 17, 2013

THE FOLLOWING ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol:		
Protocol		6/6/2013
Consent and Assent Form(s):		
Research Participant Information and Consent Form	2.0	December 12, 2013
Other:		
Screening criteria Advertisement received June 7, 2013 Radio or Telephone Advertisement received June 7, 2013 Appendices received June 7, 2013 including: - 24-hour Food Recall Multiple Pass Method - 24-hour Food Recall Recipe - International Physical Activity Questionnaire Investigator's Brochure for Flaxseed	1	6/6/2013 June 6, 2013

CERTIFICATION

The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the research study/project named on this ***Certificate of Final Approval*** at the ***full board meeting*** date noted above and was found to be acceptable on ethical grounds for research involving human participants. The study/project and documents listed above was granted final approval by the Chair or Acting Chair, UM BREB.

Appendix 2 – Informed Consent Document for the HyperFlax Clinical Trial

Hôpital St-Boniface Hospital
RECHERCHE • RESEARCH

Health Sciences Centre
Winnipeg

PARTICIPANT INFORMATION AND CONSENT FORM

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

TITLE OF STUDY: A double-blinded, randomized, controlled trial for the assessment of dietary flaxseed on reducing blood pressure in newly diagnosed hypertensive patients.

List of investigators

Principal Investigator — Grant Pierce, PhD
St. Boniface Hospital Research Centre
351 Taché Avenue
Winnipeg, MB R2H 2A6
(204) 235-3414 gpierce@sbrc.ca

Qualified Investigator — Brian Penner, MD
Health Sciences Centre Hospital
707 Mcdermot Avenue - GF329 General Centre
Winnipeg, MB, R3A 1R9
204-787-2684 bpenner@cc.umanitoba.ca

Associate Investigators — Amir Ravandi, MD PhD
351 Taché Avenue
Winnipeg, MB R2H 2A6
(204) 235-3325 aravandi@sbrc.ca

Initial: _____

Michel Aliani, PhD

351 Taché Avenue

Winnipeg, MB R2H 2A6

(204) 474-8070 maliani@sbrc.ca

Delfin Rodriguez-Leyva, MD, PhD

St. Boniface Hospital Research Centre

351 Taché Avenue

Winnipeg, MB R2H 2A6

(204) 235-3855 drodriguez@sbrc.ca

Study Coordinator/ Student Investigator

Stephanie Caligiuri, M.Sc., PhD student

St. Boniface Hospital Research Centre

351 Taché Avenue

Winnipeg, MB R2H 2A6

(204) 801-8593 umcaligs@myumanitoba.ca

Sponsor

St. Boniface Hospital Research Centre

351 Taché Avenue

Winnipeg, MB R2H 2A6

You are being asked to participate in a clincal trial (a human research study). Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends, and family before you

Initial: _____

make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

This study will investigate if adding flaxseed to the diet will lower blood pressure in people who have recently (within the last 6 months) been found to have high blood pressure (hypertension) by a medical doctor. These individuals with hypertension (Automated systolic blood pressure 135-160 OR diastolic blood pressure 85-100) will be provided foods to examine if the addition of flaxseed to the diet will prevent or reduce the need for anti-hypertensive medication (drugs). Finally, the study will assess how flaxseed may influence blood pressure at a biochemical level.

You are being asked to take part in this study because you have been diagnosed with hypertension within the last 6 months by a medical doctor and have yet to take anti-hypertensive medication. If you meet all criteria below you may be a participant in the trial.

Costs

All clinic and professional fees, diagnostic and laboratory tests which will be performed as part of this study are provided at no cost to you. A travel reimbursement of $10 will be provided to you for each of the visits to the Hospital as part of this trial, if required.

Initial: _____

The study institution will receive financial support through the SaskFlax, Canada Bread, St. Boniface Hospital Foundation, and the Canadian Institutes of Health Research to conduct this study.

Inclusion Criteria

1) Essential hypertension (average automated systolic blood pressure of 135-160 AND/OR diastolic blood pressure of 85-100)
2) Either gender
3) Untreated for hypertension
4) 18-85 years old and able to provide informed consent.
5) Females who are:
6) highly unlikely to conceive due to surgical sterilization
7) postmenopausal female with >2 years since last menses
8) or non-sterilized, pre-menopausal female who agrees to: 1. Use an adequate method of contraception to prevent pregnancy (such as a double-barrier method or hormonal); 2. Abstain from heterosexual activity for study period; or 3. Only engage in heterosexual activity with surgically sterilized male partner(s) and not planning on becoming pregnant during the study.
9) Subjects taking anti-platelet therapy must be on a stable dose for 3 months prior to the study.
10) Subjects taking lipid lowering drugs must be on a stable dose for 3 months prior to the study.
11) Subjects must have access to freezer space in their residence to hold up to one month of frozen food products associated with this study.

Initial: _____

Exclusion Criteria

1) Patients with ischemic pain at rest in limbs, ulceration, or gangrene.
2) Clinical evidence of peripheral artery disease, previous myocardial infarction, or stroke.
3) Patient has undergone percutaneous coronary angioplasty, has had coronary bypass within the last 6 months, or has unstable angina.
4) Known secondary hypertension of any etiology.
5) Patients with confirmed and clinically significant renal or hepatic abnormalities (creatinine > 0.130 mM or creatinine clearance < 45ml/min, AST 2-3x normal, ALT > 2-3x normal) and/or electrolyte imbalance serum K+ < 3.5 or > 5.5 mM.
6) History of major bleeding.
7) Patients with diabetes mellitus, bowel disease (including Crohn's disease, celiac disease, colitis, peptic ulcer disease, irritable bowel syndrome and diverticulosis) or other diseases such as active systemic lupus erythematosus, cancer, or end stage respiratory disease.
8) Patients with macrovascular target organ damage, including: cerebrovascular disease, stroke, dementia, hypertensive retinopathy, left ventricular dysfunction, angina pectoris, myocardial infarction, renal disease, and peripheral artery disease.
9) Patients with clinical evidence of heart failure or an estimated life expectancy less than 2 years and with high baseline cardiac risk (post ischemic or diabetic cardiomyopathy with an ejection fraction < 40%, Canadian Cardiovascular Society Class 3 or 4 angina or need for coronary revascularization procedures).
10) Subjects that are on supplements other that those prescribed by their clinician for the entire duration of the study. Please see point 10 below.

Initial: _____

11) Subjects ingesting more than 2 servings of fish per week, taking omega-3 fatty acid supplements, and/or consuming milled flaxseed or flax oil on a regular basis (ie: ≥1 tablespoon of milled flaxseed or 1 teaspoon of flax oil per week). If the participant chooses to stop taking these supplements or foods, a washout period of 4 weeks is allowed before entry into the trial.
12) Patients having participated in an investigational drug program in the preceeding 30 days or unable or unwilling to comply with the protocol.
13) Subjects with allergies to any ingredient in the study product or control (including gluten).
14) Patients who will undergo surgery or intend to move outside Winnipeg during the trial period.

A total of 100 participants will participate in this study.

This research is being done because in a study previously performed, dietary flaxseed effectively reduced blood pressure in participants with peripheral artery disease already taking anti-hypertensive medication. This study is trying to find out if dietary flaxseed can lower blood pressure in individuals newly diagnosed with hypertension and not yet given any drugs to control the blood pressure. This will determine if flaxseed can act as an alternative to anti-hypertensive medication.

Study Procedures

The study coordinator will go through the exclusion and inclusion criteria over the telephone to perform a pre-screening for eligibility into the study. Next, an appointment will be scheduled for you to see the study co-coordinator, research nurse, and Dr. Brian Penner for

an overview of the study, to provide an opportunity for you to ask any questions, and to sign the informed consent form. Next, your blood pressure will be measured to see if you are hypertensive. The health care professional will perform a brachial blood pressure measurement just as typically done at the doctor's office (ie: a cuff will be placed around your arm, inflated, and then deflated while the research nurse reads your blood pressure). Then you will be set up with an automated blood pressure machine. You will be left to sit quietly in a room and your blood pressure will be taken on the same arm 5 times at about 1-2 minute intervals to assess if you meet the inclusion criteria: average systolic blood pressure is 135-160 or diastolic blood pressure is 85-100. If you meet this criterion, you will also see Dr. Brian Penner, the lead medical doctor at the Hypertension Clinic at Health Sciences Centre for an examination to eliminate any macrovascular target organ damage and to ensure you meet all inclusion criteria for the study. This examination includes an EKG, physical examination, and a blood and urine sample collection for analysis at the lab. This is done to exclude conditions such as diabetes, kidney disease, and heart disease.

You will be notified shortly after this first meeting to notify you if you meet all study criteria. Then we will schedule a baseline appointment where you will be asked to return within several days fasted (9-12 hour overnight fast) for blood collection and urine collection. The amount of blood collected will be approximately 30 mL (equivalent to 2 tablespoons) and the amount of urine collected is approximately 50 mL (approximately 3 tablespoons). The blood and urine will be analyzed for its content of proteins, carbohydrates, and fats to assess metabolism, health, and response to the intervention. At the same appointment, your blood pressure will be measured again as in the screening appointment. Also, height, weight, and waist circumference will be measured in order to calculate body mass index (BMI) and assess if the intervention may cause any changes in weight or waist

Initial: _____

circumference. You will also be asked of your smoking status and to fill out a 24-hour food recall with the study co-ordinator using the Multiple-Pass Method as established by the USDA and the standardized international physical activity questionnaire (short). These two surveys are used to assess your typical food intake and physical activity habits. These assessments will be repeated at month 2, 4, and 6, as detailed below in Table 1.

Each patient in both groups will participate in the study for 6 months. There will be a minimum of 5 visits for the entire study. The total approximate time required for the entire study (all visits) is approximately 7 hours.

On the same day of your assessments, at months, 2, 4, and 6, you may see Dr. Brian Penner, the lead medical doctor at the hypertension clinic at the Health Sciences Centre, for a blood pressure follow-up. Dr. Brian Penner will assess if anti-hypertensive medication is necessary. If deemed necessary, Dr. Brian Penner will prescribe anti-hypertensive medication according to the Canadian Hypertension Education Program guidelines, and record the type and dose of anti-hypertensive medication prescribed. Dr. Brian Penner, the research nurses, and study investigators will not know if you are in the flax or the control group. If, Dr. Brian Penner would like to follow-up with you sooner than 2 month intervals, you will be asked to return to his clinic for more visits as standard of care.

A summary of the visits and tests in this study are summarized in Figure 24 and Table 21 below.

Figure 24: Flow Chart of Study Overview

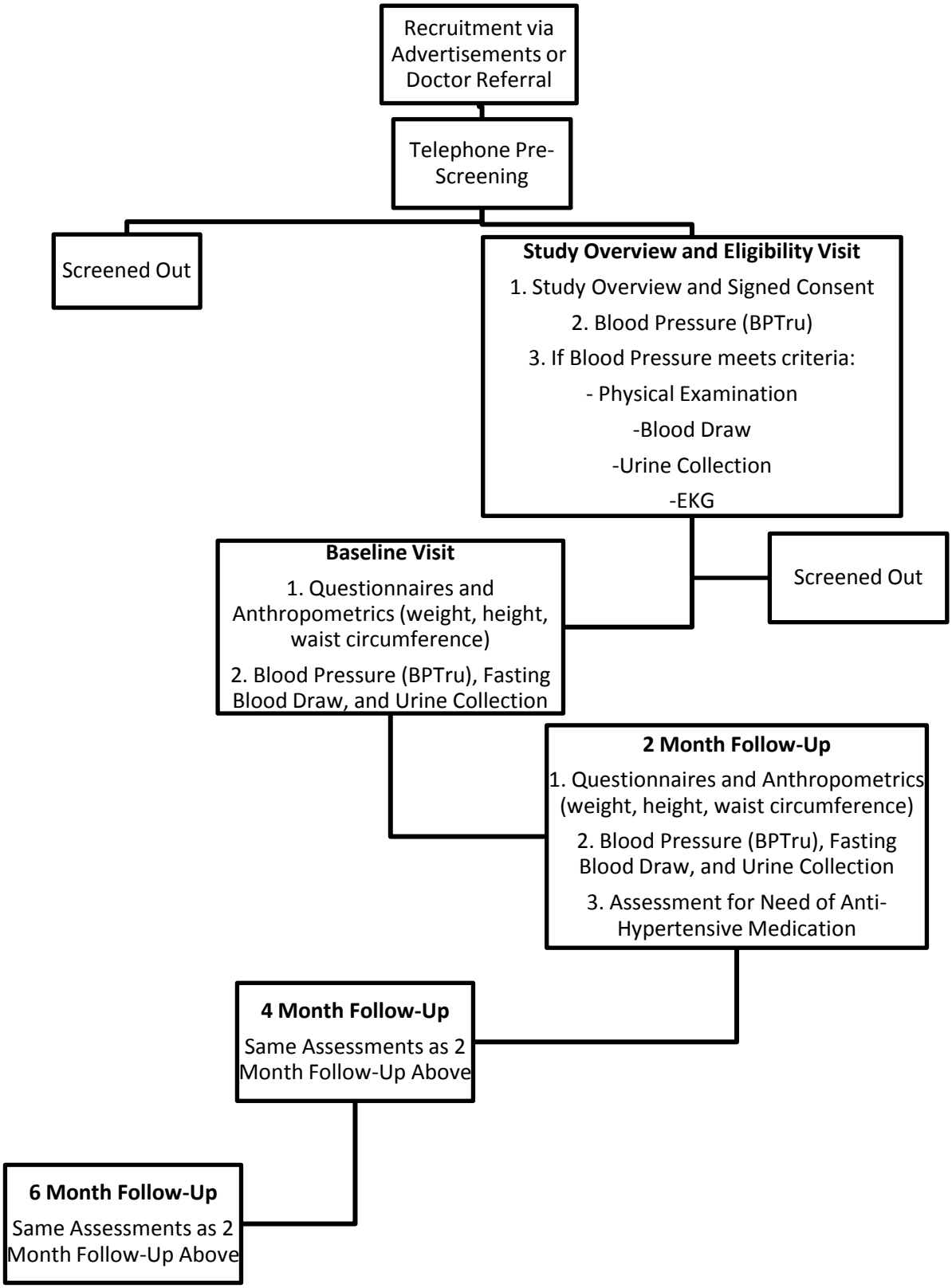


Table 23: Summary of Study Assessments and Visits

Time Point	Tests Participant Undergoes
Baseline	• Study overview • Inclusion/Exclusion Criteria will be assessed • Signed consent • Automated brachial blood pressure • If blood pressure meets criteria: examination to exclude macrovascular target organ damage (physical examination, EKG, blood and urine collection) *Performed on another day* • Automated brachial blood pressure • Fasting blood drawn • Urine collected • 24-hour food recall and physical activity record • Height, weight, waist circumference measured
2 months	• Automated brachial blood pressure • Fasting blood drawn • Urine collected • 24-hour food recall and physical activity record • Height, weight, waist circumference measured • Assessment for need of anti-hypertensive medication
4 months	• Automated brachial blood pressure • Fasting blood drawn • Urine collected Initial: _____

	• 24-hour food recall and physical activity record • Height, weight, waist circumference measured • Follow-up on anti-hypertensive medication
6 months	• Automated brachial blood pressure • Fasting blood drawn • Urine collected • 24-hour food recall and physical activity record • Height, weight, waist circumference measured • Follow-up on anti-hypertensive medication

Initial: _____

Dietary Intervention

At baseline you will be randomized (put into a group by chance, like flipping a coin) into one of two study groups (flax or control) as described below. You will have an equal, one in two, chance of being placed in either group. The trial is double-blinded, meaning that neither you nor the research staff and doctors will know whether you are in the intervention or control group; only the food delivery individual will know who belongs to which group. The trial is also controlled, meaning that there must be a group provided food products that are believed to have no therapeutic effect (made with wheat) and will serve as a comparison against the flax containing food products. You will be asked what types and flavours of food products you'd like and the Hospital staff will deliver a one month's supply at a time to your residence for the 6 months of the trial.

You will be given a variety of foods and instructed to consume one food item every day for 6 months. The intervention group will be given products that contain 30 grams of milled flaxseed and the control group will receive similar products made with wheat. There is a great variety of food items available to the participants with regard to type and flavour. A one month supply of food products will be delivered to your residence by Hospital staff. You will choose the food products and flavours that you want to eat over the month in advance by contacting the Hospital staff person. The food given to you will need to be placed in a freezer, refrigerated or stored at room temperature, depending upon the food product. A label placed on each food will instruct you on how each food should be stored. You will then have the ability to choose the food product that you will eat over each day. You will need to eat one and only one food product each day in addition to your daily meals. In some cases, you may feel that the food can replace one of your daily meals. You will be

instructed to return any unused food products from the previous month when the delivery of food products occurs each month.

You will be informed about lifestyle management of hypertension and encouraged to adopt the lifestyle changes such as increased physical activity, reducing caffeine, alcohol, and salt intake, and following the Dietary Approaches to Stop Hypertension regimen.

During the study if you start a new medication or supplement as advised by your doctor, please inform the study personnel in order to take note of the change in your records.

During the course of the study, should any new relevant findings become available that may alter your willingness to continue participating in the trial, this information will be disclosed to you as soon as possible.

In an emergency, the group to which you have been assigned will be uncovered and the information will be made available.

The researcher may decide to remove you from the study in any situation deemed to be harmful to you such as worsening health status.

You can stop participating in the study at any time.

Initial: _____

Your regular care will not be affected in any way whether you participate or not in this study. No consultation/permission is required from your doctor for you to withdraw from the study at any time.

If you agree to participate in the trial, the research study staff will send a letter to your primary care physician notifying them of your participation in the trial.

Please notify us if you are pregnant or planning to become pregnant. If you are pregnant or planning to become pregnant, you will not be permitted to enroll or continue with this study. Pregnancy tests will not be administered for the trial.

Emergency Contact

Lori Berard

Research Nurse, BN

Health Sciences Centre Hospital

Winnipeg, MB, R3A 1R9

204-789-3228

Risks and Discomforts

No serious adverse effects are expected based upon literature and prior experience in running flaxseed clinical trials. Studies carried out with flaxseed at St. Boniface Hospital for 1 year using the same dosage have resulted in no significant adverse events.

There are some minor risks associated with blood collection such as bruising, pain/discomfort in the area of blood collection, or swelling. Slight gastrointestinal

discomfort (eg: bloating, flatuelence) may arise from the fibre content that the food products offer. However, a graded increase in the fibre content will be provided during the first month to avoid any discomfort.

Your condition or symptoms may not improve or may worsen while participating in this study.

Benefits

Information from this study will be used by the study doctors. The information may or may not show effects of dietary flaxseed on blood pressure. There may or may not be direct medical benefit to you from participating in this study. We hope the information learned from this study will benefit other individuals in the management of hypertension in the future.

Confidentiality

Information gathered in this research study may be published or presented in public forums. However, your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law.

All study documents related to you will bear only your assigned patient number.

Initial: _____

For quality assurance purposes, organizations that may inspect and/or copy your research includes regulatory agencies such as the Natural Health Products Directorate of Health Canada.

The University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Review Committee may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address, or telephone number will leave St. Boniface Hospital. Once the study is complete, all confidential information will remain locked in a secure area.

The blood and urine collected during the study will be de-identified and will be labeled with an anonymous participant number. Therefore, the samples will have no personal identifying information. The samples will be stored in a locked -80°C freezer for up to 10 years at the Health Sciences Centre and St. Boniface Hospital Research Centre.

Voluntary Participation/Withdrawal from the Study

Your decision to take part in the study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your other medical care at this site. If your study doctor feels

that it is in your best interest to withdraw you from the study, your study doctor will remove you without your consent.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study about the trial feel free to contact the Study Coordinator/Student Investigator Stephanie Caligiuri at (204) 801-8593 or if you have a research-related injury or concern about the treatment, contact the study nurse at 204-789-3228.

For questions about your rights as a research participant you may contact the University of Manitoba Biomedical Research Ethics Board at (204) 789-3389.

For more information you may visit ClinicalTrials.gov which is a website that provides information about federally and privately supported clinical trials. A description of this clinical trial will be available on http://ClinicalTrials.gov. This website will not include information that can identify you. At most, the website wil include a summary of the results. You can search this website at any time.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

Initial: _____

I have read this consent form. I have had the opportunity to discuss this research study with the research staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to particpate in this research study by any statement or implied statements. Any relationship (such as employee, student, or family member) I may have with the study team has not affected by decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize that inspection of my medical records by the Food and Drug Administration, the Natural Health Product Directorate of Health Canada, The University of Manitoba Biomedical Research Ethics Board and the St. Boniface Hospital Research Centre Review Committee.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Initial: _____

Participant's Printed Name : _____

Participant's Signature _____ Date:_____

Study Co-ordinator's Printed Name: _____

Study Co-ordinator's Signature _____ Date:_____

Initial: _____

Appendix 3 – Health Canada Clinical Trial Application

Clinical Trial Application – Natural Health Product Directorate

Project Title: The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Investigators

Principal Investigator

Grant Pierce, PhD
St. Boniface Hospital Research Centre
351 Tache Avenue
Winnipeg, MB R2H 2A6
(204) 235-3414 gpierce@sbrc.ca

Qualified Investigator

Brian Penner, MD
Health Sciences Centre Hospital
707 Mcdermot Avenue - GF329 General

Centre

Winnipeg, MB, R3A 1R9
204-787-2684 bpenner@cc.umanitoba.ca

Associate Investigators

Dr. Amir Ravandi, MD PhD
351 Tache Avenue
Winnipeg, MB R2H 2A6
(204) 235-3325 aravandi@sbrc.ca

Dr. Michel Aliani, PhD
351 Tache Avenue
Winnipeg, MB R2H 2A6
(204) 474-8070 maliani@sbrc.ca

Dr. Delfin Rodriguez-Leyva
St. Boniface Hospital Research Centre
351 Tache Avenue
Winnipeg, MB R2H 2A6
(204) 235-3855 drodriguez@sbrc.ca

Student Investigator/Study Co-ordinator

Stephanie Caligiuri, M.Sc., PhD student
St. Boniface Hospital Research Centre
351 Tache Avenue
Winnipeg, MB R2H 2A6
(204) 235-3855 umcaligs@cc.umanitoba.ca

Module 1
Administrative/Clinical Information

Table of Contents

1.2 Application Information

CLINICAL TRIAL APPLICATION AND ATTESTATION FORM
Natural Health Products Directorate

HC USE ONLY		
File Number	Submission Number	Date/Time of Receipt

Please refer to the *Clinical Trial Guidance Document* for help **Please print clearly** *Denotes mandatory

Part 1: Applicant and Contact Information

A. Sponsor

Individual/Company/Institution/Organization (Full Name – No Abbreviations)* St. Boniface Hospital Research Centre			Company Code (if known)
Street / Suite / PO Box* 351 Tache Avenue			
City / Town* Winnipeg	Province / State* Manitoba	Country* Canada	Postal / ZIP Code* R2H 2A6

Contact Information of Sponsor (if Sponsor is an individual) or Senior Official (if Sponsor is a company, institution, or organization)

Name ☐ Mr. ☐ Ms. ☒ Dr.	Surname* Pierce	Given Name* Grant
Title* Director of Research/Professor	Language Preferred ☒ English ☐ French	
Telephone No.* 204-235-3206	Fax No. 204-235-0793	E-mail gpierce@sbrc.ca

B. Contact for this Application

☐ Same as A

Name ☐ Mr. ☒ Ms. ☐ Dr.	Surname* Caligiuri	Given Name* Stephanie
Title* Study Coordinator/Student Investigator	Language Preferred ☒ English ☐ French	
Company Name (Full Name – No Abbreviations)* St. Boniface Hospital Research Centre		**Address same as Sponsor** ☐
Street / Suite / PO Box* R4022 351 Tache Avenue		
City / Town* Winnipeg	Province / State* MB	Country* Canada	Postal / ZIP Code* R2H 2A6
Telephone No.* 204-235-3855	Fax No. 204-235-0793	E-mail umcaligs@cc.umanitoba.ca

C. Representative in Canada (must be completed if Sponsor is located outside Canada)

☐ Same as B ☒ Not applicable

Name ☐ Mr. ☐ Ms. ☐ Dr.	Surname*	Given Name*
Title*	Language Preferred ☐ English ☐ French	
Company Name (Full Name – No Abbreviations)*		
Street / Suite / PO Box*		
City / Town*	Province / State*	Country*	Postal / ZIP Code*
Telephone No.*	Fax No.	E-mail

Part 2: Research Ethics Board(s)

A. Research Ethics Board(s) that APPROVED the protocol*	☒ Includes member knowledgeable in complementary or alternative health care (identify member and expertise in the cover letter) *
Research Ethics Board Attestation enclosed? ☐ Yes ☒ Approval not obtained at this time (Research Ethics Board Attestation must be submitted prior to commencement of the trial)	

B. Research Ethics Board(s) that REFUSED the protocol*

Information regarding Research Ethics Board(s) that have refused to approve the protocol enclosed?
☐ Yes
☒ Not applicable
☐ Refusals not known at this time

Part 3: Clinical Trial Application Information

A. Clinical Trial Application (CTA) **† pharmaceutical, biologic, or medical device must be used according to its approved conditions of use**

Indicate the type of application (select one or more):
☐ Phase I-Bioequivalency ☐ Phase I-Healthy Subjects ☐ Phase I-Other ☐ Phase II ☒ Phase III
☐ NHP + Pharmaceutical† ☐ NHP + Biologic† ☐ NHP + Medical Device†
☐ CTA Amendment (go to part 4)

Protocol # (if known)	Protocol Title The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

B. Reference Submission

☐ Not applicable

If other submission(s) that contains the evidence to support the safety, efficacy and/or quality of the NHP to be used in the clinical trial are referenced in this application, the following information must be provided:
Submission / NHP-MF # 127296 NPN / DIN / DIN-HM # N/A

Letter of Access is enclosed? ☐ Yes ☒ Not applicable

C. Content of Application* **† must be submitted in electronic format, in addition to hard copy**
‡ if unavailable at this time, must be provided prior to commencement of the trial

Confirm, by checking the boxes below, that all application components have been provided. Include, in the cover letter, a brief rationale explaining why any required component of the application has not been provided. All required components of the application must be submitted in hard copy.

Number of diskettes / CDs: 1

☒ Cover letter	☒ Clinical Trial Application and Attestation Form ('attestation' signed by the Senior Officials)
☒ Table of contents	☒ Clinical Trial Site Information Form‡
☒ Investigator's Brochure†	☒ Research Ethics Board Attestation form for each site‡
☒ Protocol Synopsis & Evaluation Review Template†	☒ Qualified Investigator Undertaking form‡
☒ Protocol†	☐ Designated Party Authorization Form (if applicable)
☒ Informed Consent Form	☐ Animal Tissue Form(s) (if applicable)
☒ Quality Overall Summary – NHP Template†	☐ Authorization for a Third Party to Import an NHP form (if applicable)
☒ Quality Data	☐ Letters of Access (if applicable)
☐ Product Monograph (if available)	
☐ Approved Label (if available)	
☐ Information regarding refusals by other regulatory authorities or Research Ethics Boards outside Canada (if applicable)	

Part 4: Clinical Trial Application – Amendment

☒ Not applicable

A. Reference Submission*

Please provide the submission number of the approved CTA to which changes will be made.

CTA Submission #

Protocol # (if known)	Protocol Title

B. Content of Amendment*

Indicate the type of change(s) to the approved CTA (select one or more) and provide the revised documents, as well as a cover letter outlining the changes made and the reason(s) for the changes.

☐ Cover letter	☐ Source material of any of the medicinal ingredients
☐ Clinical Trial Application and Attestation Form ('attestation' signed by the Senior Officials)	☐ Specifications
☐ Dosage regimen (dose, frequency, quantity per dosage unit, potency, and/or duration of use) within established safety range	☐ Changes to or from synthetically manufactured medicinal ingredient
☐ Comparator	☐ Manufacturing information
☐ Placebo (substitution)	☐ Dosage formulation
☐ Risk information	☐ Addition or substitution of a non-medicinal ingredient not on the NHPD List of Acceptable Non-medicinal Ingredients
☐ Protocol	☐ Animal Tissue Form(s)
☐ Investigator's Brochure	☐ Other changes affecting quality (specify):
☐ Informed Consent Form	
☐ Other changes affecting safety or efficacy (specify):	

Part 5: Clinical Trial Site Information

A. Clinical Trial Site*

Clinical Trial Site Information Form enclosed for all sites?

☒ Yes ☐ No ☐ Site information not known at this time

(Clinical Trial Site Information Form must be submitted prior to commencement of the trial at each site)

B. Qualified Investigator*

Qualified Investigator Undertaking form enclosed for all sites? (There must be only one Qualified Investigator for each clinical trial site)

☒ Yes ☐ No ☐ Qualified Investigator not known at this time

(Qualified Investigator Undertaking form must be submitted prior to commencement of the trial at each site)

Part 6: Study Product Information

(copy sections 1A-C if more than one NHP is to be studied, copy sections 1A-B/2 if more than 10 ingredients are contained in the NHP/placebo)

Primary Brand Name / Product Code*	Other(s) if any
Flaxseed	

Was animal tissue used in the processing of the NHP or the placebo, although not present in the final product?
If yes, complete Animal Tissue Form. ☐ Yes ☒ No

1A: Medicinal Ingredient(s) of the NHP

Ingredient No.	A Standard or Grade	B Scientific Monograph		C* Proper Name	D Common Name	E* Quantity per dosage unit	F* Synthetic		G** Animal Tissue	
		Yes	No				Yes	No	Yes	No
1.	Milled	☐	☒	Linum usitatissimum	Flaxseed	30 g	☐	☒	☐	☒
2.		☐	☐				☐	☐	☐	☐
3.		☐	☐				☐	☐	☐	☐
4.		☐	☐				☐	☐	☐	☐
5.		☐	☐				☐	☐	☐	☐
6.		☐	☐				☐	☐	☐	☐
7.		☐	☐				☐	☐	☐	☐
8.		☐	☐				☐	☐	☐	☐
9.		☐	☐				☐	☐	☐	☐
10.		☐	☐				☐	☐	☐	☐

**if yes, complete Animal Tissue Form

Ingredient No.	H Potency (if applicable)		I* Source (if more than one enter on new line within the same cell)		J Extract (if applicable)		K Method of preparation
	Amount	Constituent	Proper Name	Material	Ratio	Quantity Dried Equivalent	
1.	30 g	Whole seed	Linum usitatissimum	N/A	N/A	N/A	Milled seed will be either sprinkled on food or baked into products
2.							
3.							
4.							
5.							
6.							
7.							
8.							
9.							

10.							

1B: Non-medicinal Ingredient(s) of the NHP					
Ingredient No.	Proper Name	Common Name *	Purpose *	Animal Tissue Used **	
				Yes	No
1.	N/A			☐	☐
2.				☐	☐
3.				☐	☐
4.				☐	☐
5.				☐	☐
6.				☐	☐
7.				☐	☐
8.				☐	☐
9.				☐	☐
10.				☐	☐

** if yes, complete Animal Tissue Form

Ingredient No.	Standard or Grade	Source (if more than one enter on new line within the same cell)	
		Proper Name	Material
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			

1C: Proposed Conditions of Use of the NHP According to Protocol

Proposed Use or Purpose *
The flaxseed will be orally ingested on a daily basis to assess the efficacy of reducing blood pressure in participants newly-diagnosed with hypertension

Dosage Form*	Duration of Use*	Sterile*	Route of Administration*
Muffins, bagels, snack bars, or packets of milled flaxseed containg 10 g of flaxseed in the 1st week, 20 g in the 2nd week, and 30 g thereafter until the end of the 6 month period.	6 months	☐ Yes ☒ No	Oral ingestion

Proposed Dose

Treatment Group*	Amount to Be Taken at One Time		Frequency*	Directions of Use*
	No. of Dosage Units* (e.g. 1, 2, 3…)	Dosage Unit* (e.g. capsule, mL, tsp)		
1	1	30 g	1/day	Oral Ingestion

Risk Information

Cautions and Warnings*
There are no known serious cautions or warnings based on previous trials with flaxseed. Some patients may experience increased bowel movements and some intestinal discomfort due to the increased fibre load. However, in order to avoid this, the first month of the trial contains a gradual increase in the amount of flaxseed provided. In our last trial, Health Canada was particularly worried about the effects of flaxseed on platelet aggregation. We found no significant effect of the flaxseed on platelet aggregation.

Contraindications*
There are no known contraindications. However, in this trial individuals with inflammatory bowel disease will be excluded due to the high fibre load of flaxseed.

Known Adverse Reactions*
No adverse reactions have been reported in previous clinical trials administering flaxseed

2: Placebo Ingredients

Ingredient No.	Standard or Grade	Scientific Monograph		Proper Name*	Common Name	Quantity per dosage unit	Synthetic*		Animal Tissue Used**	
		Yes	No				Yes	No	Yes	No
1.	Milled	☐	☒	Triticum	Wheat	30 g	☐	☒	☐	☒
2.		☐	☐				☐	☐	☐	☐
3.		☐	☐				☐	☐	☐	☐
4.		☐	☐				☐	☐	☐	☐
5.		☐	☐				☐	☐	☐	☐

6.		☐	☐				☐	☐	☐	☐
7.		☐	☐				☐	☐	☐	☐
8.		☐	☐				☐	☐	☐	☐
9.		☐	☐				☐	☐	☐	☐
10.		☐	☐				☐	☐	☐	☐

** if yes, complete Animal Tissue Form

Ingredient No.	Potency (if applicable)		Source (if more than one enter on new line within the same cell)*		Extract (if applicable)		Method of preparation
	Amount	Constituent	Proper Name	Material	Ratio	Quantity Dried Equivalent	
1.							
2.							
3.							
4.							
5.							
6.							
7.							
8.							
9.							
10.							

I, the undersigned, certify that the information and material included in this clinical trial application is accurate and complete[1].

Name of Authorized Signing Official (print) Dr. Grant Pierce	Signature	Date (yyyy / mm / dd)
Title Principal Investigator Executive Director of Research at St. Boniface Hospital Professor of Physiology and Pharmacology	Telephone No. 204-235-3206	Fax No. 204-235-0793
Name of Company to which the Authorized Signing Official Belongs St. Boniface Hospital		

[1] If the signing official is a third party acting on behalf of the Sponsor identified in Part 1, the Designated Party Authorization Form must be signed by the Sponsor and filed with the complete application.

Part 7: Clinical Trial Attestation

In regard to the clinical trial that is the subject of this application, I attest that:

a) All information contained in, or referenced by, this application is complete and accurate and is not false or misleading.

b) The clinical trial will be conducted in accordance with the protocol and the requirements as set out in Part 4 of the *Natural Health Products Regulations.* The clinical trial will be conducted according to Good Clinical Practices.

c) All changes to clinical trials will be reported to the Natural Health Products Directorate, and all reporting requirements will be met, as specified in Part 4 of the *Natural Health Products Regulations*.

d) The trial WILL NOT commence at any site until receipt of a Notice of Authorization from the Natural Health Products Directorate and until the approval of Research Ethics Board(s) is obtained.

e) Records will be maintained for a period of 25 years and will be accessible for on-site inspection by Health Canada inspectors.

Name of Senior Medical Officer or Scientific Officer in Canada (print)* Dr. Brian Penner MD.	Signature*	
Telephone No. 204-787-2864	E-mail bpenner@cc.umanitoba.ca	Date (yyyy / mm / dd)
Name of Senior Executive Officer or Department Head (print)* Dr. Grant N. Pierce	Signature*	
Telephone No. 204-235-3206	E-mail gpierce@sbrc.ca	Date (yyyy / mm / dd)

1.2.2 Information on Prior Related Applications

A CTA has been submitted for a previous clinical trial in which Grant Pierce was the principal investigator at St. Boniface Hospital Research Centre. This CTA investigated the effect of flaxseed on cardiovascular health in participants with peripheral artery disease. This past study utilized the same natural health products as proposed in the current CTA. This previous application was successfully approved by Health Canada and the appropriate ethics boards (Study identification #: 10941-011).

1.2.3

TITLE PAGE
SPONSOR NAME: Flax Canada 2015
Product: Flaxseed
Research Number:
Other Names: Linseed, *Linum usitatissimum*

INVESTIGATOR'S BROCHURE

Edition number: 1
Release Date: May 17 2013

Replaces Previous Edition Number: N/A
Date: N/A

Confidentiality Statement

Please treat this Investigator’s Brochure as a confidential document for the sole information and use of the investigator's team and the Institutional Review Board/Independent Ethics Committee.

1. TABLE OF CONTENTS OF INVESTIGATOR'S BROCHURE

2. SUMMARY

Flaxseed (*Linum usitatissimum*) is a small brown oil seed produced from a blue flowering plant primarily grown in the Canadian prairies. Flaxseed can be eaten whole or milled; in addition, the oil, fibre, and protein fractions from the seed can be extracted for consumption. Flaxseed can be sprinkled on top of oatmeal, cereal, yogurt, added into shakes, or baked into products (eg: muffins, breads, pastas). A recent investigation concluded that baking did not degrade the bioactive ingredients in flaxseed.[1]

Flaxseed is thought to have three bioactive components: (1) the omega-3 fatty acid, alpha-linolenic acid (ALA), (2) lignans, and (3) dietary fibre.[2] ALA is more bioavailable when ingested as milled flaxseed rather than as whole seed. Consuming 30 g of milled flaxseed every day for 12 weeks resulted in significantly increased plasma alpha-linolenic acid levels by 3-fold, whereas, whole flaxseed increased plasma ALA by 2-fold (not significant). [3] Milled flaxseed also results in adequate absorption of enterolignans from the lignans present in flaxseed; plasma enterolignans changed from below quantitation limit to on average 209 nM and 304 nM for enterodiol and enterolignan, respectively.[4]

Flaxseed has been granted Generally Recognized as Safe (GRAS) status by the Food and Drug Act and viewed as safe to consume for the general public.[5] Overall, toxicology and safety data in both human and animal trials have concluded flaxseed as safe to consume.[6-14]

Flaxseed has exhibited cardioprotective effects in animal models pre-disposed to cardiovascular disease by reducing atherogenicity,[15] plasma cholesterol, [16] plasma glucose,[16] plasma trans fats,[17]and blood pressure.[18]

Clinical studies have also indicated cardioprotective effects of this oilseed. A previous trial (FlaxPAD) conducted by our lab [19] observed that the consumption of 30 g of

milled flaxseed per day in baked products resulted in a significant reduction of both systolic and diastolic blood pressure in patients with peripheral artery disease (PAD). Despite the participants being on anti-hypertensive medications, a significant proportion had uncontrolled hypertension and experienced an improvement in blood pressure; a 15 and 7 mm Hg drop in systolic and diastolic blood pressure, respectively, was observed over 6 months.[20] Significant declines in blood pressure were already observed after one month of dietary intervention.

The bioactive components of flaxseed have also exhibited a capacity to lower blood pressure independent of anti-hypertensive medication. Overweight individuals provided a hypocaloric diet enriched with rapeseed oil (a source of ALA) resulted in weight loss and a reduction in blood pressure of 10 and 8.4 mm Hg in systolic and diastolic blood pressure, respectively. The reduction in diastolic but not systolic blood pressure was significantly greater than the olive oil control group.[21] Dyslipidemic individuals provided flax oil every day for 12 weeks resulted in a 3.1 and 6.3 mm Hg drop in systolic and diastolic blood pressure, respectively, which was significantly greater than the safflower oil control group.[22] In pre-hypertensive individuals, consumption of a combination of rapeseed, flax, and rice oil resulted in an approximate drop in systolic and diastolic blood pressure of 10 and 3 mm Hg, respectively, which was significantly greater than the control group.[23]

When individuals with metabolic syndrome were provided just the lignans from flaxseed, it resulted in approximately a 4 mm Hg drop in systolic blood pressure in women but not men.[24]

A meta-analysis concluded that increasing dietary fibre intake resulted in a reduction in blood pressure in hypertensive patients on average of 6 and 4 mm Hg in systolic and

diastolic blood pressure, respectively.[25]

Despite evidence illustrating the potential of flaxseed or its bioactive components as a potent cardioprotective functional food/natural health product, the efficacy of flaxseed as an effective treatment to reduce blood pressure in newly diagnosed hypertensive patients has yet to be investigated. Whether flaxseed can work as a monotherapy to reduce blood pressure or must be used in conjunction with anti-hypertensive medications needs to be determined. In addition, the ability of flaxseed to prevent or reduce the need for anti-hypertensive medication has yet to be determined. Lastly, the mechanisms of action behind the anti-hypertensive properties of flaxseed have not been identified.

The hypothesized therapeutic effect of flaxseed in the current study is the reduction of blood pressure in those newly diagnosed with hypertension and reducing the dosage or entirely preventing the need for anti-hypertensive medication. A randomized, double-blinded, and controlled clinical trial will be run in which newly-diagnosed hypertensive patients not taking anti-hypertensive medication will be recruited. Parameters such as 24-hour ambulatory blood pressure, resting brachial blood pressure, and plasma and urine metabolomics will be analyzed to assess the efficacy and mechanisms of action of dietary flaxseed in hypertension management.

3. INTRODUCTION

Flaxseed (linseed, *Linum usitatissimum*) is thought to contain three bioactive components: (1) the omega-3 fatty acid, alpha-linolenic acid, (2) antioxidant lignans, primarily the lignan, secoisolariciresinol diglucoside (SDG), and (3) dietary fibre.[2] Flaxseed is considered a functional food as it provides health benefits beyond the effects of traditional

nutrients that it contains. Research is being conducted on flaxseed as a treatment for hypertension as flax is a prominent crop in the Canadian prairies and has previously shown to reduce blood pressure in patients with peripheral artery disease already taking anti-hypertensive medication.[20] Flaxseed in animal models has also reduced atherogenicity caused by a high cholesterol diet or a high trans fat diet in an animal model pre-disposed to high plasma cholesterol and cardiovascular disease (LDL receptor knockout mouse).[15, 17]

4. PHYSICAL, CHEMICAL, AND PHARMACEUTICAL PROPERTIES, AND FORMULATION

Flaxseed is a small brown seed produced from a blue flowering plant of a summer crop grown primarily in the Canadian prairies.[26] Flaxseed contains approximately 36-45% lipid, of which approximately 52% is the fatty acid alpha-linolenic acid (ALA). Flaxseed also contains plant lignans (approximately 13 mg/g flaxseed) of which secoisolariciresinol diglucoside (SDG) is the main lignan (~2653 mg/100 g).[26] Protein, [27] fibre,[26] and soluble carbohydrate also comprise significant portions of the seed at 20, 29, and 27%, respectively. Glutamate is the amino acid in the greatest abundance in flaxseed, next followed by aspartate and arginine.[27] Flaxseed soluble carbohydrate is comprised primarily of xylose, rhamnose, and galactose.[28] Please see Table 1 in the Appendices for further composition details.

In the current investigation, flaxseed will be milled to ensure increased bioavailability of the bioactive components (versus whole flaxseed) [3] and incorporated into food products for consumption by the study participants. These food products include

bagels, muffins, bars, and milled flaxseed on its own to sprinkle onto foods. Milled wheat will be used for the control to replace flaxseed. In some case, molasses and a small amount of wheat bran will be used to ensure the colour and texture of the food product is similar to that containing flaxseed. These products have been used previously.[20] Baking of the flaxseed does not degrade the bioactive ingredients.[1]

Each food product will be labelled with the proper storage conditions. The muffins and bagels will be stored in a freezer at -20°C, the milled flax or wheat in a fridge at 4°C, and the bars can be stored at room temperature. The ingredients and flavour acceptability of the bagels, muffins, and bars in the previous FlaxPAD trial have been published and are available in the appendices.[29, 30]

5. NON-CLINICAL STUDIES

5.1. Non-clinical pharmacology

1. Dupasquier et al. (2007)[15]

   Methods: The investigators added 1%, 5%, and 10% dietary flaxseed to a high cholesterol diet and provided the diet to low density lipoprotein receptor knockout mice (LDLrKO) for 24 weeks (n=15/group). The 1, 5, and 10% supplementation would be equivalent to approximately 14, 70, and 140 g per day for a standard human 1400 g diet.[31] The purpose of the trial was to assess the anti-atherogenic effects of a flaxseed supplemented diet.

   Results: The mice provided the cholesterol diet experienced a significant increase in plasma cholesterol and atherosclerotic plaque formation. The 10% supplemented flax diet resulted in a reduction of plasma cholesterol, saturated

fatty acids, the inflammatory marker mac- 3 in the aortic tissue, and atherosclerotic plaque development in the aorta and aortic sinus. The 1% and 5% flax supplemented diets were not as effective as they did not significantly attenuate aortic plaque development, but did significantly reduce plasma cholesterol and saturated fatty acids.

Relevance to Current Study: Dietary flaxseed supplementation may provide anti-atherogenic effects and should be studied in a human population pre-disposed to heart disease.

2. Pachecho et al. (2011) [16]

Methods: Thirty male Wistar rats were equally divided among a control, 25% flax supplemented, and a soybean oil/cellulose supplemented internal control group for 26 weeks. Plasma biochemical analysis was performed. The dose would be equivalent to approximately 350 g of flax added to a standard 1400 g human diet.[31] The purpose of the trial was to assess the effect of dietary flaxseed supplementation on healthy rats and any changes in plasma biochemistry that indicate a preventive effect against chronic disease.

Results: The flaxseed supplemented group experienced a significant increase in HDL-C and a reduction in LDL-C, body mass, and plasma glucose. The control groups by comparison had significantly higher levels of LDL-C, body mass, and plasma glucose, and lower levels of HDL-C.

Relevance to Current Study: Dietary flaxseed appears to have preventive characteristics potentially against chronic disease in a healthy model. The patient population in the current trial will include participants who are apparently healthy

other than having hypertension and will aim to assess if these results can be replicated in a human population.

3. Park et al. (2012) [18]

   Methods: Sprague-Dawley rats (n=8/group) were divided among a control diet, control diet plus 0.02% flax powder enriched with lignans, a high fat diet, and lastly a high fat diet plus 0.02% flax powder enriched with lignans for 12 weeks. The 0.02% flax supplemented diet would be equivalent to a 280 mg dose of flaxseed powder in a standard human 1400 g diet.[31] The purpose of the study was to examine the effects of flaxseed powder enriched with lignans on body weight, visceral fat, lipid profiles, blood pressure, and adipokines.

   Results: The high fat diet plus flaxseed significantly reduced visceral white adipose tissue in comparison the high fat diet group. Plasma triglycerides and LDL-C were the lowest in the high fat diet plus flaxseed group out of all four groups. The control diet plus flaxseed did not appear to make a significant difference in comparison to the control diet group. The high fat diet plus flaxseed reduced leptin levels in the rats to similar levels of the control diet groups. The high fat diet plus flaxseed also reduced systolic blood pressure by comparison to the high fat diet.

   Relevance to Current Study: This study showed the potential blood pressure lowering properties of flaxseed in a model pre-disposed to heart disease. This provides evidence to support the ability of flaxseed to exhibit anti-hypertensive properties in a human population.

4. Dupasquier et al. (2006) [32]

Methods: New Zealand white rabbits (n=8/group) were provided a standard chow control diet, control diet supplemented with 10% flaxseed, 0.5% supplemented cholesterol chow, or a 0.5% cholesterol plus 10% flaxseed chow. The rabbits were terminated at 6, 8, or 16 weeks. The purpose of the trial was to investigate the effect of flaxseed on atherosclerosis and vascular function.

Results: Rabbits consuming the cholesterol containing diets exhibited significantly higher plasma cholesterol levels and developed atherosclerosis than the diets without. Flax reduced atherosclerotic plaques caused by a high cholesterol diet, but this was attenuated by 16 weeks. Maximal tension induced in the aortic rings *ex vivo*, were impaired by the cholesterol diet at 16 weeks, this was not improved by flaxseed supplementation. Dietary flaxseed improved the aortic relaxation response to acetylcholine.

Relevance to Current Study: Dietary flaxseed added to a high cholesterol diet may be able to exert vasorelaxation and therefore, may be able to reduce blood pressure in hypertensive individuals. This will be examined in the current trial.

5. Francis et al. (2013) [33]

Methods: Male New Zealand rats were provided a regular diet for 12 weeks (Group I), 1% cholesterol-supplemented diet for 4 weeks followed by a regular diet for 8 weeks (Group II), fed as in Group II and for an additional 14 weeks with either a regular diet (Group III) or a 10% flaxseed supplemented diet (Group IV). The purpose of the trial was to determine if dietary flaxseed could induce or accelerate atherosclerotic plaque regression and reverse any cholesterol-induced vascular contractile abnormalities.

Results: Rabbits in Group II developed plaque growth stabilization and lower norepinephrine induced contraction and impaired acetylcholine induced relaxation. Dietary flaxseed significantly reduced atherosclerotic plaque development. Groups II and III exhibited improved contraction and vessel relaxation.

Relevance to Current Study: Dietary flaxseed and/or stopping high cholesterol intake resulted in atherosclerotic plaque regression and improved vessel responses.

6. Ander et al. (2004) [34]

Methods: Male New Zealand White Rabbits (n=14-16/group) were provided a regular standard chow, standard chow supplemented with 10% flaxseed, standard chow with 0.5% cholesterol, or standard chow with 0.5% cholesterol and 10% flaxseed for 16 weeks. The purpose of the study was to examine if dietary flaxseed supplementation possesses anti-arrhythmic effects during ischemia/reperfusion and if this effect is due to ALA or its conversion to EPA and DHA.

Results: After global ischemia, ventricular fibrillation occurred in 33, 0, 28, and 6% of rabbits from the control diet, flax diet, cholesterol diet, and cholesterol and flax diet, respectively. After reperfusion, ventricular fibrillation occurred in 28, 26, 64, and 18% of rabbits in the control diet, flax diet, cholesterol diet, and cholesterol and flax diet, respectively. The cholesterol diet increased the QT interval and the flaxseed supplemented diets reduced the QT interval and the incidence of arrhythmias. ALA applied to cardiomyocytes effectively reduced the action potential duration similarly to DHA.

Relevance to Current Study: Dietary flaxseed supplementation exhibits cardioprotection including anti-arrhythmic effects.

5.2. Pharmacokinetics and Product Metabolism in Animals

1. Dupasquier et al. (2007)[15]

   Methods: see above number 1 on page 7.

   Results: one of the bioactive ingredients of flaxseed, alpha-linolenic acid, was significantly elevated in the plasma of rats provided diets supplemented with flaxseed (1, 5, and 10%) in comparison to the control groups. The 10% flaxseed diet resulted in the highest plasma ALA levels and the lowest n-6/n-3 fatty acid plasma ratio of all groups. Plasma EPA levels were also higher in 5 and 10% flax supplemented groups in comparison to controls. DHA levels were not significantly higher in the flax supplemented groups versus the controls.

   Relevance to Current Study: One of the primary bioactive ingredients of flaxseed is significantly elevated in the plasma following oral consumption in animals. Flaxseed appears to significantly increase EPA but not DHA levels. Therefore, the benefits of flax lipids are likely due to ALA and EPA, not DHA.

2. Ander et al. (2010) [35]

   Methods: Male New Zealand rabbits were provided either a standard chow or a standard chow supplemented with 10% milled flaxseed. The purpose of

the trial was to assess the influence of dietary flaxseed on tissue fatty acid composition.

Results: The flax diet resulted in significantly higher levels of ALA in the plasma, liver, kidney, brain, heart, aorta, carotid, and gastrocnemius muscle than the control. EPA was significantly higher in the kidney, brain, heart, and gastrocnemius muscle versus control. DHA was significantly higher in the kidney, aorta, and gastrocnemius.

Relevance to Current Study: Dietary flaxseed results in significant deposition of ALA, EPA, and DHA in the tissues. Elevated levels of EPA and DHA in the tissue may not be reflected in the plasma.

3. Rickard et al. (1996) [36]

Methods: 42 female Sprague-Dawley rats were provided high fat diets with or without 2.5, 5, or 10 g of flaxseed per 100 g of diet or 1.1, 2.2, and 4.4 μmol SDG/day (equivalent to the levels in the respective flaxseed diets) for 4 weeks. The objective of the trial was to investigate SDG metabolism and excretion.

Results: A curve-linear relationship was observed with flax/SDG consumption and urinary lignan (SDG and its metabolites enterodiol and enterolactone) levels. A steep incline was observed between the 0 and 5% flax groups and the 0 and 2.2 μmol SDG groups. Higher doses did not result in significantly more urinary lignan levels.

Relevance to Current Study: Flax or its bioactive component, SDG, is endogenously metabolized to enterolactone and enterdiol and is excreted

through the urine. After a 5% flax supplemented diet or equivalent level of SDG, the urinary lignan levels plateau.

4. Rickard et al. (1998) [37]

   Methods: Female Sprague-Dawley rats with gavauged with a single dose of ^{3}H-SDG (3.7 kBq/g body weight) either immediately (acute group) or after 10 days of gavauge with 1.5 g unlabelled SDG per day (chronic group). Rats were terminated at 12, 24, 36, and 48 hours after the gavauge. The objective was to assess lignan bioactivity and disposition rate.

   Results: SDG metabolites accumulated in the kidneys, liver, uterus, gastrointestinal tract, and adipose tissue. Radioactivity for SDG was 1-16 fold greater at 12 versus 48 hours in the tissues, blood, and gastrointestinal tract. After 48 hours, 80% of the SDG metabolites were excreted through the feces and urine. SDG metabolite levels were 1-3 fold higher in the cecum, adipose, and liver in the chronic SDG exposure group compared to the acute exposure.

   Relevance to Current Study: SDG is effectively metabolized and incorporated into tissues within a short time span. After 48 hours, the majority of SDG and its metabolites are excreted. Therefore, chronic consumption of flax is required for levels of SDG and its metabolites to remain high.

5.3. Toxicology

Repeated Dose

Reproductive Toxicity

1. Orcheson et al. (1998) [12]

Methods: Female Sprague-Dawley rats (n=6/group) were provided a high-fat diet with flaxseed (2.5, 5, or 10%) or an equivalent level of flaxseed's natural lignan secoisolariciresinol diglycoside (SDG) through gavauge (0.75, 1.5, or 3.0 mg/day), or an estrogen inhibitor tamoxifen by gavauge (1 mg/kg body weight/day) for four weeks. The purpose of the trial was to assess (1) the effect of flaxseed and SDG on estrous cycling and any antiestrogenic activity, (2) if changes in estrous cycling were related to urinary lignan excretion, (3) any gross changes in organs or body weight as indicators of toxicity in the short term.

Results: 5% flaxseed supplementation resulted in a one day extended cycle in female rats. At approximately 2.5 mg/24 hours intake of SDG, an increase in the cycle length of female rats was observed ($r=0.77$, $p<0.05$); lower intakes of SDG including 0 mg/24 hours saw on average an increase in cycle length by 20% over four weeks, whereas the 2.5 mg and 3 mg per 24 hours intake of SDG saw approximately a 30% increase over 4 weeks. In addition, at approximately 1.5 mg/24 hours of SDG intake, on average the female rats experienced an increased percentage of acyclic or irregular cycles ($r=0.90$, $p<0.006$). There were no gross pathological changes or differences in the weight of the ovaries, heart, kidneys, spleen, cecum or colon, or differences in food intake or body weight.

Relevance to Current Study: Human trials investigating the influence of flaxseed on estrous cycling should be reviewed to determine safety of intake (as done below). However, the lowest dose of flax or SDG that influenced

estrous cycling was found at 1.5 mg/24 hours which according to the study by Orcheson et al. is equivalent to a 5% dietary flaxseed supplementation; for an average 1400 g diet/day [31] this is equivalent to a 70 g dose of flaxseed. The dose provided in the current trial is well below this dose at 30 g of flaxseed/day.

2. Tou et al. (1999) [38]

   Methods: Male and female Sprague-Dawley rats (5 females and 5 males per group) were provided a control diet, 5% or a 10% flaxseed supplemented diet at weaning for 29 days or continuously from gestation to postnatal day 132. The purpose of the trial was to assess the effect of dietary flaxseed on sex hormone levels and reproductive indices.

   Results: After 29 days of flaxseed consumption, there were no differences in sex hormone levels or reproductive indices compared to control. The longer duration of 5% flaxseed consumption resulted in delayed puberty onset in females, whereas 10% flax resulted in early puberty onset, higher estradiol levels, and longer estrous cycles in females. In males, the 10% flax diet resulted in higher testosterone and estradiol levels, and increased prostatic cell proliferation. At 5% flax supplementation, the opposite was observed; prostatic cell proliferation and prostatic weight were reduced.

   Relevance to Current Study: The study suggests that shorter term flaxseed supplementation is safe and that high levels of flax for the lifespan of a rat may warrant adverse effects. However, for an average human 1400 g diet/day [31] the 5% flaxseed supplemented diet is equivalent to a 70 g dose of flaxseed. The

dose provided in the current trial is well below this dose at 30 g of flaxseed/day.

Carcinogenicity

Reproductive Toxicity

1. Khan et al. (2007)[39]

   Methods: Female Sprague-Dawley dams were provided either a standard chow or a flaxseed supplemented diet (5 or 10%) and the pups of the litters were exposed to either the flax or standard diet through the milk of the dams for the first 25 days. An additional group was provided injections of estradiol. At 50 days old, the rats were injected with DMBA to induce tumorigenesis. The purpose of the study was to examine the levels of cadmium in defatted flax and kidney and liver bioaccumulation. Additionally, to assess the effect of in utero and post natal exposure of flax on tumor growth, estrogen imbalance, and biomarkers of increased risk of developing breast cancer.

   Results: Rats provided the 10% flax diet had an increased total number of tumors and higher levels of cadmium in the liver and kidneys compared to the control. The 5% flaxseed diet had lower levels of mammary tumors compared to the control, albeit not significantly. The flaxseed diet appeared to have no effect on estrogenic activities and did not increase uterine weight whereas the group given estradiol did. Importantly, the authors point out that the flax was grown in the United States and Finland which is different from the flax in Canada. A very similar study conducted on Canadian flax did not find any

adverse reactions or differences in tumorigenesis. This study is outlined below in number 2.

Relevance to Current Study: The current study will provide Canadian flax to the participants which is different from the flax provided in the study by Khan et al. In addition, the 10% flax supplementation is well above what the investigators will provide the participants in the current trial; 10% would be equivalent to 140 g/day and in the current trial to dose is 30 g/day. Also, the 5% flax diet did not result in any adverse effects or increased tumorigenesis.

2. Chen et al. (2003) [13]

Methods: Female Sprague-Dawley dams were provided a control standard chow, a 10% supplemented flaxseed, or 20.1 mg of SDG/100 g of diet (equivalent to levels of SDG in a 10% flaxseed diet) on the day of delivery. The pups were exposed to the diets through the milk from the dams. At 21 days old the pups were all provided the control standard chow and gavagued with DMBA to induce tumorigenesis for 21 more weeks. The purpose of the trial was to determine if exposure to flaxseed or SDG during the suckling period would protect the female offspring against carcinogen-induced mammary tumorigenesis later in life, as well as, examining any potential adverse effects or toxicity on the dams and offspring.

Results: Incidence, load, number, and size of tumors were significantly lower in the flaxseed and SDG supplemented groups compared to the control. There were no differences in estrous cycle length, serum prolactin, ovary or uterus

weight in the dams. In the offspring, there were no differences in the anogenital distance, puberty onset, estrous cycle length, body, ovary, or uterus mass.

Relevance to Current Study: 10% flaxseed or an equivalent amount of the flax lignan SDG during lactation, appeared to be protective against tumor development and have no adverse effects or toxicity in female rats.

Special Studies

3. Hemmings et al. 2003 [14]

Methods: Fischer 344 pregnant females were provided a 10% flax supplemented chow or control chow diet. The offspring, male and female Fischer 344 rats, were provided the same diet as their mother for 68 days (sample size: 12-25 rats or 5-7 rats per group used for analyses). The purpose of the study was to examine the effect of dietary flaxseed supplementation on behaviour, development, toxicity, and activity of liver γ-glutamyltranspeptidase (γGT).

Results: There were no significant differences between the control and flax groups for growth curves, behavioural or developmental indices, liver or kidney weight normalized to body weight, or plasma alanine transaminase, γGT, or glucose. Lastly, the activity of liver γGT was higher in the flax supplemented group compared to the control.

Relevance to Current Study: The level of flaxseed supplementation (10%) would be equivalent to 140 g/day for a standard human diet; well above our dose of 30 g/day. However, at this high dose, flaxseed did not appear to have an adverse or toxic effect. The authors note that the increased activity of γGT

may be beneficial in the upregulated recruitment of glutathione for homeostatic control against oxidative stress and that the levels of γGT are physiological rather than pathological.

6. CLINICAL STUDIES

6.1. Pharmacokinetics and Product Metabolism in Humans

1. Edel et al. (2013) [4]

   Methods: Healthy participants were provided functional foods containing 30 g of milled flaxseed per day for 4 weeks. Flaxseed contains the lignan, SDG, and it is metabolized in the intestines to enterodiol and enterolactone. These metabolites are absorbed in the plasma. Therefore, the purpose of the study was to establish a method for enterolactone and enterodiol extraction and quantitation and to observe changes from baseline to 4 weeks of flax consumption.

   Results: At baseline the individuals had below quantitation limit levels and after 4 weeks of flaxseed consumption, enterodiol and enterolactone were detected at 209 and 304 nM, respectively.

   Relevance to Current Study: One of the proposed bioactive components of flaxseed, SDG, is adequately metabolized and absorbed into the plasma of humans. Enterodiol and enterolactone can effectively serve as biomarkers for trial compliancy as SDG is not commonly in high levels in the human diet.

2. Kuijsten et al. (2005) [40]

Methods: Purified SDG (1.31 µmol/kg body weight) was ingested orally as a single dose to 12 healthy participants. Pharmacokinetics and excretion of SDG and its metabolites were assessed.

Results: enterodiol and enterolactone appeared in the plasma 8-10 hours after ingestion with maximum plasma concentrations at 14.8 and 19.7 hours, respectively. The average half-life was 4.4 and 12.6 hours for enterodiol and enterolactone, respectively. Within 3 days, up to 40% of the SDG was eliminated as enterolignans in the urine.

3. Austria et al. (2008) [3]

Methods: 30 g of milled flaxseed or 6 g of ALA in flax oil were baked into muffins and provided to participants to consume daily for 12 weeks. Since ALA is a proposed bioactive ingredient of flaxseed, muffin and plasma n-3 fatty acid levels were measured.

Results: After just 4 weeks of milled flaxseed and flax oil consumption, plasma levels of ALA increased significantly from baseline and were significantly greater than the levels in whole flaxseed.

Relevance to Current Study: The bioavailability of ALA is higher in milled flaxseed and flax oil than whole flaxseed. ALA is bioavailable from flax and is absorbed in the blood. Therefore, milled flaxseed will be provided in the current clinical trial.

4. Dahl et al. (2005) [41]

Methods: Flax fibre (9 g/day) or psyllium fibre (10.4 g/day) were provided to 26 healthy participants in capsule form for 2 weeks to investigate laxation. In addition, 11 fasting subjects were provided either flax bread or white bread to investigate glycemic response.

Results: Individuals provided flax fibre or psyillium exhibited an increase in laxation and fecal weight of 2.9 and 4.8 g fecal weight/g fibre, respectively. The 11 fasting subjects provided flax enriched bread had significantly lower blood glucose levels than those provided white bread (6.6 mmol/L versus 6.9 mmol/L, respectively)

Relevance to Current Study: The functional foods containing flax will likely increase laxation and improve glycemic response in the individuals in the current trial. Therefore, participants will be provided functional foods with graded increases in flax or wheat (control) for the first month of the trial.

6.2. Safety and Efficacy

Safety

Milled and whole flaxseed has been provided Generally Recognized as Safe (GRAS) status by the Food and Drug Act in 2009 to be used as an ingredient in foods. Therefore, flaxseed consumption has undergone extensive examination and the FDA has deemed it safe for consumption by the public. [5]

1. Bloeden et al. (2008) [11]

Methods: 62 hypercholesterolemic men and post-menopausal women were provided 40 g of milled flaxseed in baked products every day for 10 weeks and followed a low fat-low cholesterol diet. Parameters such as plasma cholesterol, oxidative stress, inflammation, insulin resistance, and safety were assessed at 0, 5, and 10 weeks.

Results: The flaxseed group exhibited a significant increase in plasma ALA and docosapentanoic acid. Flaxseed resulted in a significant decrease in plasma cholesterol at 5 weeks but not at 10 weeks. Those consuming flaxseed exhibited an improvement of the homeostatic model of insulin resistance at 10 weeks. No differences in oxidative stress or inflammation were noted between groups. There were no significant differences between groups for the number of adverse events, tolerability, or complete blood count. The most common adverse events associated with flaxseed were diarrhea, flatulence, and headache. There was one case of a bowel obstruction in the flax group that was deemed as "possibly related" to the flax containing bakery products. The men in the study exhibited a significant decrease in HDL-cholesterol at 5 and 10 weeks.

Relevance to Current Study: Milled flaxseed at a dose of 40 g/day appears to be an effective dose to increase plasma ALA levels and exhibit protection against insulin resistance. The exclusion criteria for the current study eliminates individuals with bowel obstruction or inflammatory bowel disorders to avoid potential bowel irritation or further obstruction. The amount of flax and wheat provided to the participants is gradually increased in order to allow

the participants to become accustomed to the fibre load and to avoid the potential side effects of diarrhea and flatulence.

2. Dodin et al. (2005) [10]

   Methods: 199 menopausal women were provided either 40 g of flaxseed or wheat germ every day for one year. Plasma cholesterol, bone mineral density, menopausal systems, and adverse effects were assessed.

   Results: Women consuming flaxseed exhibited a significant decrease in total cholesterol (-0.01 mmol/L) and HDL-cholesterol (-0.05 mmol/L) compared to the control. Bone mineral density or menopausal symptoms did not differ between the control and treatment group. Ten and five women dropped out of the study for the flax and wheat germ groups, respectively, due to digestive issues proposed to be due to the high fibre load. The flax was well-tolerated and no other adverse effects were observed.

   Relevance to Current Study: Long term consumption of flaxseed appears to be well-tolerated and safe in menopausal women.

3. Faintuch et al. 2007 [9]

   Methods: In a randomized double-blinded crossover study, 24 obese participants were provided 30 g of flaxseed flour or manioc flour (control) every day for 2 weeks. Parameters such as a general biochemical investigation, white blood cell count (WBC), C-reactive protein (CRP), serum amyloid A (SAA) and fibronectin were assessed.

Results: No intolerances were noted and no adverse effects on biochemical or hormonal indices were observed. No significant differences in hemoglobin, cholesterols, glucose, insulin, or leptin were observed. Flaxseed consumption resulted in a significant drop in fibronectin, SAA, CRP, and white blood cell counts.

4. Frische et al. (2007) [8]

   Methods: 16 pre-menopausal women were provided four different food formulations (baked goods with: (1) no flax or wheat bran, (2) 10 g of flax, (3) 28 g of wheat bran, or (4) 10 g of flax plus 28 g of wheat bran) every day for 2 menstrual cycles each in a cross-over design. Serum samples were collected at the mid-luteal phase of the second menstrual cycle for each treatment. Serum hormones, sex hormone binding globulin, and urinary lignan excretion were assessed.

   Results: None of the dietary treatments had significant effects on serum hormone levels or sex hormone binding globulin. Flaxseed consumption significantly increased urinary lignan levels and the addition of wheat bran did not alter urinary lignan concentrations.

   Relevance to Current Study: Animal studies showing potentially adverse effects of flax on hormones levels in female murine models were not replicated in a human study group. Therefore, flax consumption in females appears to be safe.

5. Phipps et al. (1993) [42]

Methods: 10 g of milled flaxseed per day or nothing was provided to 18 pre-menopausal women for 3 menstrual cycles each in a randomized cross-over trial. The purpose of the trial was to investigate the influence of flax consumption on menstrual cycle length and sex hormones.

Results: During flax consumption, the luteal phase of the menstrual cycle was longer (12.6 versus 11.4 days) and the ratio of progesterone to estradiol during the luteal phase was significantly higher during flax consumption versus the control phase. Three anovulatory cycles of 36 occurred during the control phase whereas none of 36 occurred during flax consumption. Flaxseed had no effect on levels of estradiol, estrone, progesterone, testosterone, prolactin, sex hormone binding globulin, or dehydroepiandrosterone.

Relevance to Current Study: Flaxseed consumption does not appear to have an adverse effect on female fertility or menstrual cycling. The data suggest that consuming flaxseed may even improve normal ovulation.

6. Shultz et al. (1991) [6]

Methods: Six healthy men were provided whole wheat bread containing flaxseed every day for 6 weeks which equated to 13.5 g of flaxseed/day. Hormone levels and serum biochemistry were assessed.

Results: Flaxseed consumption did not alter levels of total testosterone, free testosterone, sex hormone binding globulin, plasma glucose, blood urea nitrogen, creatinine, alkaline phosphatise, calcium, phosphorus, sodium, potassium, triglycerides, or total cholesterol.

Relevance to Current Study: flaxseed consumption for 6 weeks does not appear to have adverse effects in healthy males.

7. Tarpila et al. 2002 [7]

   Methods: 80 men and women (78% women) were provided control meals or meals supplemented with 1.3 g/100 g of food as milled flaxseed, 5.0 g/100g of food as flaxseed oil, and 3-4 g/100 g as inulin and wheat fibre for 4 weeks in a randomized crossover with washout period design. Fifteen of the participants continued on the supplemented diet for an additional 4 months. The objective was to assess the influence of flaxseed supplementation on plasma lipids, fatty acids, enterolactone, cadmium, and thiocyanates.

   Results: the supplemented diet resulted in higher levels of ALA, EPA, and DPA. Cadmium and thiocyanate plasma levels were not significantly different from the control and were well below the healthy reference range for smokers and non-smokers.

   Relevance to Current Trial: Flaxseed supplementation increases levels of plasma n-3 fatty acids and appears to be safe in terms of cadmium and thiocyanate toxicity.

8. Takeuchi et al. (2007) [43]

   Methods: In experiment 1, 127 subjects with high-normal blood pressure were provided bread made with either a control oil or a high ALA oil (2.6 g) for 12 weeks.

In experiment 2, 18 normotensive and 21 participants with high-normal blood pressure were provided either 42 g of control oil or a high ALA oil (7.8 g/day) for 4 weeks. The purpose of the study was to assess the effect of ALA on blood pressure in those with hypertension and to assess the safety of high ALA intakes.

Results: In experiment 1, systolic blood pressure was lower at 4, 8, and 12 weeks versus the control. Diastolic blood pressure was lower at 12 weeks compared to control. In experiment 2, no differences between control and treatment group were observed for lipid peroxide level, C-reactive protein, plasma prothrombin time, activated partial prothrombin time, plasma analysis, urinalysis, or adverse effects.

Relevance to Current Study: The ALA present in flaxseed may exert anti-hypertensive effects. The dose of 6 g in the current trial is below the 7.8 g/day dose from Takeuchi's trial which showed no adverse effects.

Efficacy

1. Rodriguez-Leyva et al. (2013) [20]

Methods: 110 participants with peripheral artery disease were provided food products (bagels, muffins, bars, pasta) with 30 g of milled flaxseed or wheat (control) every day for one year. Parameters such as inflammatory markers, blood biochemistry, and resting brachial blood pressure were assessed.

Results: Participants consuming flaxseed exhibited a significant increase in plasma ALA, EPA, and enterolignans, whereas the control group saw no

significant changes. Of the participants with hypertension, a mean decrease of 11.8 and 8.1 mm Hg in systolic and diastolic blood pressure, respectively, was observed over a year.

Relevance to Current Study: Dietary flaxseed consumption may have potent anti-hypertensive effects. The efficacy of this potential treatment option should be assessed in newly diagnosed hypertensive individuals not yet on anti-hypertensive medication. The potential mechanism of action also needs to be further explored.

2. Paschos et al. (2007) [22]

Methods: 59 dyslipidemic normotensive men were provided either 15 mL of flax oil or safflower oil per day for 12 weeks. The primary outcome was blood pressure.

Results: Systolic and diastolic blood pressure decreased by 3.1 and 6.3 mmHg, respectively, in the flax oil group. This was statistically greater than the safflower oil control group.

Relevance to Current Study: The bioactive components present in flax oil (ie: lipids and lignans) may be responsible for the beneficial anti-hypertensive action of flaxseed. However, the efficacy of flaxseed as an anti-hypertensive treatment in newly diagnosed patients nor the mechanism of action have been assessed.

3. Cornish et al. (2009) [24]

Methods: 100 men and women (> 50 years old) were provided either flaxseed lignans (543 mg/day) or control every day for 6 months while on a walking program. The objective of the trial was to assess the efficacy of flaxseed lignans on a composite score of metabolic syndrome.

Results: Men taking the flaxseed lignans experienced a significant decrease in diastolic blood pressure (-4 mmHg) at 6 months. There was a trend for a reduced metabolic syndrome in men taking the flaxseed lignan complex. Flaxseed had no effect on bone mineral density, body composition, lipoproteins, glucose, or markers of inflammation.

Relevance to Current Study: SDG may be a bioactive component in flaxseed with anti-hypertensive properties. Dietary flaxseed consumption may be more effective at reducing blood pressure in men than women.

4. Takeuchi et al. (2007) [43]

Methods: 127 subjects with high-normal blood pressure (average 136/88 mm Hg) were provided bread made with either a control oil or a high ALA oil (2.6 g of ALA) every day for 12 weeks. The purpose of the study was to assess the effect of ALA on blood pressure in those with pre-hypertension.

Results: Those consuming the high ALA oil exhibited a significant decrease in systolic blood pressure (~9 mm Hg) and diastolic blood pressure (~4 mm Hg) at 12 weeks. Significant improvements in systolic blood pressure were already observed by 4 weeks.

Relevance to Current Study: ALA may be the bioactive component in flaxseed with anti-hypertensive properties. Improvements in systolic blood pressure are already seen within 1 month.

Marketing Experience

Canadian flax is currently exported primarily to the USA, Europe, China, and Japan with 400 tonnes exported last year. Canada produced just under 600,000 tonnes of flaxseed last year. [44] Flaxseed has been granted GRAS in North America, is not banned in any countries and is consumed throughout parts of the world. Currently, consumers have access to flax in its whole form, milled, oil, or incorporated into products like breakfast cereals, crackers, and breads. However, ready to eat products (eg: muffins, bagels, bars) with therapeutic doses (30 g) have yet to be put on the market.

7. Summary of Data and Guidance for Investigator

Flaxseed is an oilseed produced primarily in the Canadian prairies and has been granted Generally Recognized as Safe (GRAS) status by the Food and Drug Act. The purpose for the current trial is to investigate the potential of dietary flaxseed consumption to reduce blood pressure, prevent or reduce the necessity for anti-hypertensive medication, and to understand the mechanisms of action of the potential anti-hypertensive properties.

Previous trials have indicated that milling the flaxseed increases the bioavailability of the bioactive constituents. Therefore, milled flaxseed will be

administered to participants in the current trial. Baking of the flaxseed does not appear to degrade the bioactive components and therefore can be baked into food products.

Overall, toxicology and safety data have deemed flaxseed as safe to consume. Some published studies have indicated that due to the high fibre load of flax, supplementation is likely to cause diarrhea and flatulence. Therefore, the dose of flax or wheat (control) will slowly be increased during the first month of the trial, ie: 10, 20, and 30 g every day for the first, second, and third week respectively. This graded increase will allow the participants to become accustomed to the fibre load. This was done successfully in the FlaxPAD trial.[20]

Appendices

Table 1: Composition of Flaxseed

Component	Percentage
Lipid [45]	36
Palmitic Acid	5.2
Stearic Acid	3.2
Oleic Acid	16.8
Linoleic Acid	15.2
Alpha-Linolenic Acid	58.8
Protein [45]	18.2
Carbohydrate [45]	27.3
Dietary Fibre	27.3
Lignans [46]	0.18
Secoisolariciresinol	92
Secoisolariciresinol-sesquilignan	3.5
Cyclolariciresinol	2.1
Lariciresinol	0.99
Hydroxysecoisolariciresinol	0.53
Pinoresinol	0.48
Metairesinol	0.29
Lariciresinol-sesquililignan	0.05
Other	0.06

References

1. Gerstenmeyer E, Reimer S, Berghofer E, Schwartz H, Sontag G: Effect of thermal heating on some lignans in flax seeds, sesame seeds and rye. *Food Chem* 138: 1847-1855, 2013

2. Bassett CM, Rodriguez-Leyva D, Pierce GN: Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. *Appl Physiol Nutr Metab* 34: 965-974, 2009

3. Austria JA, Richard MN, Chahine MN, Edel AL, Malcolmson LJ, Dupasquier CM, Pierce GN: Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. *J Am Coll Nutr* 27: 214-221, 2008

4. Edel AL, Aliani M, Pierce GN: Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults. *J Chromatogr B Analyt Technol Biomed Life Sci* 912: 24-32, 2013

5. Food and Drug Act: GRAS notice 000280: Whole and milled flaxseed.2009

6. Shultz TD, Bonorden WR, Seaman WR: Effect of short-term flaxseed consumption on lignan and sex hormone metabolism in men. *Nutr Res* 11: 1089-1100, 1991

7. Tarpila S, Aro A, Salminen I, Tarpila A, Kleemola P, Akkila J, Adlercreutz H: The effect of flaxseed supplementation in processed foods on serum fatty acids and enterolactone. *Eur J Clin Nutr* 56: 157-165, 2002

8. Frische EJ, Hutchins AM, Martini MC, Thomas W, Slavin JL: Effect of flaxseed and wheat bran on serum hormones and lignan excretion in premenopausal women. *J Am Coll Nutr* 22: 550-554, 2003

9. Faintuch J, Horie LM, Barbeiro HV, Barbeiro DF, Soriano FG, Ishida RK, Cecconello I: Systemic inflammation in morbidly obese subjects: Response to oral supplementation with alpha-linolenic acid. *Obes Surg* 17: 341-347, 2007

10. Dodin S, Lemay A, Jacques H, Legare F, Forest JC, Masse B: The effects of flaxseed dietary supplement on lipid profile, bone mineral density, and symptoms in menopausal women: A randomized, double-blind, wheat germ placebo-controlled clinical trial. *J Clin Endocrinol Metab* 90: 1390-1397, 2005

11. Bloedon LT, Balikai S, Chittams J, Cunnane SC, Berlin JA, Rader DJ, Szapary PO: Flaxseed and cardiovascular risk factors: Results from a double blind, randomized, controlled clinical trial. *J Am Coll Nutr* 27: 65-74, 2008

12. Orcheson LJ, Rickard SE, Seidl MM, Thompson LU: Flaxseed and its mammalian lignan precursor cause a lengthening or cessation of estrous cycling in rats. *Cancer Lett* 125: 69-76, 1998

13. Chen J, Tan KP, Ward WE, Thompson LU: Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced rat mammary tumorigenesis. *Exp Biol Med (Maywood)* 228: 951-958, 2003

14. Hemmings SJ, & Barker L: The effects of dietary flaxseed on the fischer 344 rat: I. development, behaviour, toxicity and the activity of liver gamma-glutamyltranspeptidase. *Cell Biochem Funct* 22: 113-121, 2004

15. Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, Yeganeh BK, Moghadasian MH, Pierce GN: Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. *Am J Physiol Heart Circ Physiol* 293: H2394-402, 2007

16. Tomaz Pacheco J, Beltrame Daleprame J, Teles Boaventura G: Impact of dietary flaxseed (linum usitatissimum) supplementation on biochemical profile in healthy rats. *Nutr Hosp* 26: 798-802, 2011

17. Bassett CM, McCullough RS, Edel AL, Patenaude A, LaVallee RK, Pierce GN: The alpha-linolenic acid content of flaxseed can prevent the atherogenic effects of dietary trans fat. *Am J Physiol Heart Circ Physiol* 301: H2220-6, 2011

18. Park JB, & Velasquez MT: Potential effects of lignan-enriched flaxseed powder on bodyweight, visceral fat, lipid profile, and blood pressure in rats. *Fitoterapia* 83: 941-946, 2012

19. Leyva DR, Zahradka P, Ramjiawan B, Guzman R, Aliani M, Pierce GN: The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with peripheral artery disease: Rationale and design of the FLAX-PAD randomized controlled trial. *Contemp Clin Trials* 32: 724-730, 2011

20. Rodriquez-Leyva D, Weighell W, Edel A, LaVallee R, Dibrov R, Pinneker R, Maddaford T, Ramjiawan B, Alian M, Guzman R, Pierce G: Potent anti-hypertensive action of dietary flaxseed in hypertensive patients. *Circulation* Submitted. 2013.

21. Baxheinrich A, Stratmann B, Lee-Barkey YH, Tschoepe D, Wahrburg U: Effects of a rapeseed oil-enriched hypoenergetic diet with a high content of alpha-linolenic acid on body weight and cardiovascular risk profile in patients with the metabolic syndrome. *Br J Nutr* 108: 682-691, 2012

22. Paschos GK, Magkos F, Panagiotakos DB, Votteas V, Zampelas A: Dietary supplementation with flaxseed oil lowers blood pressure in dyslipidaemic patients. *Eur J Clin Nutr* 61: 1201-1206, 2007

23. Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, Kasai M, Watanabe S, Aoyama T, Kondo K: Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. *J Oleo Sci* 56: 347-360, 2007

24. Cornish SM, Chilibeck PD, Paus-Jennsen L, Biem HJ, Khozani T, Senanayake V, Vatanparast H, Little JP, Whiting SJ, Pahwa P: A randomized controlled trial of the effects of flaxseed lignan complex on metabolic syndrome composite score and bone mineral in older adults. *Appl Physiol Nutr Metab* 34: 89-98, 2009

25. Whelton SP, Hyre AD, Pedersen B, Yi Y, Whelton PK, He J: Effect of dietary fiber intake on blood pressure: A meta-analysis of randomized, controlled clinical trials. *J Hypertens* 23: 475-481, 2005

26. Clifford H, Tulbek M, Xu Y: Flaxseed. *Advances in Nutrition and Food Research* 51: 1, 2006

27. Oomah BD, & Mazza G: Flaxseed proteins—a review. *Food Chem* 48: 109, 1993

28. Cui W, Mazza G, Biliaderis CG: Chemical structure, molecular size distributions and rheological properties of flaxseed gum. *J Agric Food Chem* 42: 1891, 1994

29. Aliani M, Ryland D, Pierce GN: Effect of flax addition on the flavor profile and acceptability of bagels. *J Food Sci* 77: S62-70, 2012

30. Aliani M, Ryland D, Pierce GN: Effect of flax addition on the flavor profile of muffins and snack bars. *Food Res Int* 44: 2489, 2011

31. Kendall A, Levitsky DA, Strupp BJ, Lissner L: Weight loss on a low-fat diet: Consequence of the imprecision of the control of food intake in humans. *Am J Clin Nutr* 53: 1124-1129, 1991

32. Dupasquier CM, Weber AM, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, Mitchell RW, Kroeger EA, Gilchrist JS, Moghadasian MM, Lukas A, Pierce GN: Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *Am J Physiol Heart Circ Physiol* 291: H2987-96, 2006

33. Francis AA, Deniset JF, Austria JA M, Lavallee RK, Maddaford GG, Hedley TE, Dibrov E, Pierce GN: The effects of dietary flaxseed on atherosclerotic plaque regression. *Am J Physiol Heart Circ Physiol* 2013

34. Ander BP, Weber AR, Rampersad PP, Gilchrist JS, Pierce GN, Lukas A: Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic rabbits. *J Nutr* 134: 3250-3256, 2004

35. Ander BP, Edel AL, McCullough R, Rodriguez-Leyva D, Rampersad P, Gilchrist JS, Lukas A, Pierce GN: Distribution of omega-3 fatty acids in tissues of rabbits fed a flaxseed-supplemented diet. *Metabolism* 59: 620-627, 2010

36. Rickard SE, Orcheson LJ, Seidl MM, Luyengi L, Fong HH, Thompson LU: Dose-dependent production of mammalian lignans in rats and in vitro from the purified precursor secoisolariciresinol diglycoside in flaxseed. *J Nutr* 126: 2012-2019, 1996

37. Rickard SE, & Thompson LU: Chronic exposure to secoisolariciresinol diglycoside alters lignan disposition in rats. *J Nutr* 128: 615-623, 1998

38. Tou JC, Chen J, Thompson LU: Dose, timing, and duration of flaxseed exposure affect reproductive indices and sex hormone levels in rats. *J Toxicol Environ Health A* 56: 555-570, 1999

39. Khan G, Penttinen P, Cabanes A, Foxworth A, Chezek A, Mastropole K, Yu B, Smeds A, Halttunen T, Good C, Makela S, Hilakivi-Clarke L: Maternal flaxseed diet during pregnancy or lactation increases female rat offspring's susceptibility to carcinogen-induced mammary tumorigenesis. *Reprod Toxicol* 23: 397-406, 2007

40. Kuijsten A, Arts IC, Vree TB, Hollman PC: Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside. *J Nutr* 135: 795-801, 2005

41. Dahl WJ, Lockert EA, Cammer AL, Whiting SJ: Effects of flax fiber on laxation and glycemic response in healthy volunteers. *J Med Food* 8: 508-511, 2005

42. Phipps WR, Martini MC, Lampe JW, Slavin JL, Kurzer MS: Effect of flax seed ingestion on the menstrual cycle. *J Clin Endocrinol Metab* 77: 1215-1219, 1993

43. Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, Kasai M, Watanabe S, Aoyama T, Kondo K: Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. *J Oleo Sci* 56: 347-360, 2007

44. Flax Council of Canada. Flax market snapshot. 2012. Retrieved 2013. http://www.flaxcouncil.ca/files/web/FlaxMarketSnapshot-December28th2012.pdf

45. Canadian Grain Commission. Quality of western canadian flaxseed - 2010.2011. Retrieved 2013. http://www.grainscanada.gc.ca/flax-lin/harvest-recolte/2010/hqf10-qrl10-5-eng.htm

46. Smeds AI, Eklund PC, Sjoholm RE, Willfor SM, Nishibe S, Deyama T, Holmbom BR: Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *J Agric Food Chem* 55: 1337-1346, 2007

1.2.4

PROTOCOL SYNOPSIS & EVALUATION

Date Submitted	*HPB USE ONLY*	**Submission Number**	*HPB USE ONLY*

Trial Title and Number

The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients.

Background / Rationale

Hypertension is the leading global risk for mortality as stated by the World Health Organization as high blood pressure is attributed to 13% of deaths in the world.[1] Hypertension when untreated or uncontrolled can lead to cardiac hypertrophy, arrythmias, myocardial infarctions, heart failure, stroke, and renal failure. [2]

In a landmark publication by the INTERHEART Study, it was concluded that modifiable lifestyle factors including nutrition account for 90% of the risk for myocardial infarction worldwide.[3]

Dietary flaxseed is an example of a functional food and evidence supports its ability to reduce blood pressure in patients with peripheral artery disease (PAD). In our previous clinical trial, FLAXPAD, our lab investigated the effect of dietary flaxseed on overall cardiovascular health in patients with PAD. [4, 5]

However, despite evidence to support flaxseed in reducing blood pressure in the PAD population or flaxseed's individual bioactive components in the normal normotensive population, the efficacy of flaxseed which contains ALA, dietary fibre, and enterolignans as an effective anti-hypertension treatment to reduce blood pressure in newly diagnosed hypertensive patients has yet to be investigated. In addition, the ability of flaxseed to work as a monotherapy rather than in synergism with anti-hypertensive medications also has yet to be determined. Lastly, the potential mechanism of action of blood pressure lowering by flaxseed has not been investigated.

Trial Objectives

Primary Objective:

1. To determine if dietary flaxseed consumption can reduce blood pressure in newly diagnosed hypertensive patients

Secondary Objectives:

1. To assess if dietary flaxseed consumption can reduce or prevent the necessity for anti-hypertensive medication.
2. To determine if dietary flaxseed can be effective as a monotherapy or in conjunction with anti-hypertensive medication
3. The explore mechanisms of action of flaxseed's potential anti-hypertensive properties.

Study Design

The proposed trial is a double-blinded, randomized, controlled trial to determine the efficacy of dietary flaxseed supplementation on reducing blood pressure in newly diagnosed hypertensive participants.

Study Duration

The trial will be 6 months in duration.

Number of Sites (inside and outside of Canada)

The main site where participants will go for assessment visits is at the hypertension clinic in the Health Sciences Centre Hospital. The plasma and data analysis will be done at St. Boniface Hospital Research Centre.

List of Investigators

Principal Investigator: Dr. Grant Pierce

Qualified Investigator: Dr. Brian Penner

Associate Investigators: Dr. Amir Ravandi, Dr. Michel Aliani, Dr. Delfin Rodriguez-Leyva

Study Co-ordinator/Student Investigator: Stephanie Caligiuri

Sample Size

The statistical power analysis indicated that a sample size of n = 40 subjects per group (n = 80 subjects in total) would have a power = 0.80 to detect differences between groups and across time of an effect size = 0.7 SD at an α level = 0.05. Based on the drop-out rate of 20% in the previous FLAXPAD trial, it is recommended that 50 subjects per group or n = 100 be recruited for the study. Participants will be recruited through volunteering (see Appendices, Appendix 1 and 2 for the recruitment poster and advertisements) or referrals from general practioners or Dr. Brian Penner.

Patient Population

In Manitoba, 192,181 individuals aged 12 and older in 2011 were diagnosed with high blood pressure; this equtes to 19.4% of Manitobans living with hypertension which is greater than the national prevalence of 17.6%.[6] The target population of this trial is newly diagnosed individuals; therefore, the incidence of hypertension as studied in another Canadian province, Ontario, has increased from 25.5 per 1000 adults to 32.1 per 1000 adults from 1997 to 2004[7] and this incidence rate has likely increased from 2004 to the current year.

Inclusion Criteria

1. Essential hypertension (24-hour **average** ambulatory systolic blood pressure of ≥130 OR diastolic blood pressure ≥80 OR 24-hour **awake** ambulatory systolic blood pressure ≥135 OR diastolic blood pressure ≥85[8,9])
2. Either gender
3. Untreated for hypertension
4. 18-85 years old and able to provide informed consent.
5. Females who are:
    a. highly unlikely to conceive due to surgical sterilization
    b. postmenopausal female with >2 years since last menses
    c. or non-sterilized, pre-menopausal female who agrees to: 1. Use an adequate method of contraception to prevent pregnancy (such as barrier

or hormonal); 2. Abstain from heterosexual activity for study period; or 3. Only engage in heterosexual activity with surgically sterilized male partner(s) and not planning on becoming pregnant during the study.

6. Subjects taking anti-platelet therapy must be on a stable dose for 3 months prior to the study.
7. Subjects taking lipid lowering drugs must be on a stable dose for 3 months prior to the study.
8. Subjects must have access to freezer space in their residence to hold up to one month of frozen food products associated with this study.

Exclusion Criteria

1. Patients with ischemic pain at rest in limbs, ulceration, or gangrene.
2. Clinical evidence of peripheral artery disease, previous myocardial infarction, or stroke.
3. Patient has undergone percutaneous coronary angioplasty, has had coronary bypass within the last 6 months, or has unstable angina.
4. Known secondary hypertension of any etiology.
5. Patients with confirmed and clinically significant renal or hepatic abnormalities (creatinine > 0.130 mM or creatinine clearance < 45ml/min, AST 2-3x normal, ALT > 2-3x normal) and/or electrolyte imbalance serum K+ < 3.5 or > 5.5 mM.
6. History of major bleeding.
7. Patients with bowel disease (including Crohn's disease, ulcerative colitis, celiac disease, peptic ulcer disease, irritable bowel syndrome and diverticulosis) or other diseases such as active systemic lupus erythematosus, metastatic cancer, or end stage respiratory disease.
8. Patients with clinical evidence of heart failure or an estimated life expectancy less than 2 years and with high baseline cardiac risk (post ischemic or diabetic cardiomyopathy with an ejection fraction < 40%, Canadian Cardiovascular Society Class 3 or 4 angina or need for coronary revascularization procedures).
9. Subjects that are on supplements other than those prescribed by their clinician for the entire duration of the study. See number 10 below.
10. Subjects ingesting more than 2 servings of fish per week, taking omega-3 fatty acid supplements, and/or consuming milled flaxseed or flax oil on a regular basis (ie: ≥1 tablespoon of milled flaxseed or 1 teaspoon of flax oil per week).
11. Patients having participated in an investigational drug program in the proceeding 30 days or unable or unwilling to comply with the protocol.
12. Subjects with allergies to any ingredient in the study product or control (including gluten).
13. Patients who will undergo surgery or intend to move during the trial period.

NHP Formulation

The formulation of the food products (bagels, muffins, and bars) has been previously approved in the FlaxPAD CTA (identifying #: 10941-011). The ingredients and flavour acceptability have also been published in two papers by Aliani et al. which can be found in the Appendices section, Appendix 5.

Dosage Regimen

Participants will be provided enough food items to last the duration of the month every month for the entirety of the trial. The food items include: muffins, bagels, bars, and either milled flaxseed or wheat on its own. Each food item will provide either 30 g of milled flaxseed (treatment) or milled wheat (control) and will be individually wrapped. The participants will be required to ingest only one product per day.

In the first month, the participants will be provided a graded increase in flax or wheat to allow for the participants to become accustomed to the fibre load. In the first month, 10, 20, and 30g will be provided for the first, second, and third week thereafter, respectively.

Prestudy Screening and Baseline Evaluation

The participants will meet with the study co-ordinator to go through the inclusion and exclusion criteria. If the participant meets the criteria and agrees to the informed consent, they will fill out a 24-hour food recall, physical activity questionnaire, and be set up with a 24-hour ambulatory blood pressure monitor to assess if they are hypertensive. If the participant is hypertensive they can become a participant of the trial and be provided the control or flax food products.

Treatment Visit

Participants that have met all inclusion and exclusion criteria and have provided informed consent will undergo the following baseline evaluations:

Immediately Analyzed:

1. 24-hour ambulatory blood pressure monitoring
2. Brachial blood pressure measurement

Later Analyzed:

1. 24-hour food recall and physical activity record (Appendices 3 and 4)
2. Plasma nitric oxide
3. Plasma amino acid profile
4. Plasma lipid profile (triglycercides, LDL and HDL cholesterol)
5. Plamsa glucose, liver function enzymes, kidney function markers
6. Plasma and urine metabolomics which includes proteins, lipids, and carbohydrates that are markers of metabolism and heart, blood vessel, liver, kidney, pancreatic, and intestinal health.
7. Plasma oxylipins which are metabolites of fatty acids
8. Plasma oxidized phospholipids
9. Plasma markers of hypertension such as: angiotensin II, aldosterone, angiontensin converting enzyme, and endothelin-1

Premature Withdrawal/Discontinuation Criteria

The participants are entitled to withdraw from the study at any time for any reason. However, the study will be stopped should the Data Safety and Monitoring Committee deem the study harmful to the participants. In addition, the clinical investigator may choose to withdraw a participant from the study due to medical reasons or if the participant is continually non-compliant with the study protocol.

Rescue Medication

As this is not a drug trial, rescue medication is not warranted. If deemed necessary by Dr. Brian Penner, the participants will be started on anti-hypertensive medication as standard of care by following the Canadian Hypertension Education Panel Guidelines.[10]

Washout Period

No washout period is necessary if the participants meet the inclusion and exclusion criteria.

Concomitant Medication

Participants will be asked to not consume any flax containing products other than those provided in the trial. Other herbal or nutritional supplements will also be restricted during the study. Participants who meet all the inclusion criteria and do not have any exclusion criteria will take all their routine medications as well as any additional medications prescribed throughout the study.

Variables to be Assessed

Time Point	Tests Patient Undergoes
Baseline	• Brachial blood pressure • 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Randomization into control or flax group
2 months	• Brachial blood pressure • 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Assessment for need of anti-hypertensive medication
3 months	Brachial blood pressure Follow-up on anti-hypertensive medication
4 months	Brachial blood pressure Follow-up on anti-hypertensive medication
5 months	Brachial blood pressure Follow-up on anti-hypertensive medication
6 months	• Brachial blood pressure • 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Follow-up on anti-hypertensive medication

The 24-hour food recall is based on the Multiple Pass Method developed by the Food Surveys Research Group at the United States Department of Agriculture.[11] The multiple pass steps are outlined in Appendix 3. The standardized international physical activity questionnaire (short) is an internationally recognized survey for assessment of regular

physical activity and will also be provided to the participants (https://sites.google.com/site/theipaq/) and can be found in Appendix 4.

The blood collected at baseline, month two, and month six will be assessed for:

1. Nitric oxide
2. Amino acid profile
3. Plasma lipid profile (triglycercides, LDL and HDL cholesterol)
4. Glucose, liver function enzymes, kidney function markers
5. Plasma metabolomics which includes proteins, lipids, and carbohydrates that are markers of metabolism and heart, blood vessel, liver, kidney, pancreatic, and intestinal health.
6. Plasma oxylipins which are metabolites of fatty acids
7. Plasma oxidized phospholipids
8. Plasma markers of hypertension such as: angiotensin II, aldosterone, angiontensin converting enzyme, and endothelin-1

The urine will be assessed for metabolomics as outlined in #5 above.

Efficacy Analysis

Based on the objectives of the trial as listed on page 1 and 2 of this document, measured parameters will be assessed to determine the efficacy of dietary flaxseed supplementation using statical methods as detailed below. Final data tabulation, reduction, and processing will only be carried out at the end of the study.

Safety Analysis

No adverse effects are expected based upon published literature and experience with prior trials with dietary flaxseed supplementation. Studies with flaxseed carried out at St. Boniface Hospital Research Centre for the timespan of one year with a dose of 30 g/day have reported no significant adverse events.[5] The food products provided to the participants will be prepared by companies with whom follow Good Manufacturing Practice. The food products will be prepared, frozen, and delivered to the participants monthly.

Due to the ability of flaxseed to increase stool bulk and frequency of defecation, patients with a history of bowel obstruction, irritable bowel syndrome, or diverticular disease will be excluded. When large amounts of dietary fibre are consumed, gastro-intestinal discomfort may occur. Results from our lab have indicated that the gastro-intestinal discomfort and flatulence disappear within a few weeks once the participants have become accustomed to the fibre load.

However, adverse events, as identified by the WHO scale, will be followed up, if medically indicated, with relevevant laboratory investigations under the direction of the study medical monitor. Research staff will record final outcome and resolution date of the event wherever possible.

All serious adverse events (representing a significant health hazard to the participant) will be reviewed by the medical monitor within 24 hours of becoming aware of the events. The monitor will notify the sponsors and the Ethics Review Board within 7-14 days of the event. The University of Manitoba has a Research Ethics Boards that reviews all clinical trials for safety and ethical considerations. St. Boniface Hospital also has a similar process to protect research partipants. This trial must pass both these review processess before it can proceed.

Applications to both of these boards will be filed and reviewed concurrently with the CTA. Final approvals will be forwarded to the NHPD of Health Canada.

Statistical Analysis

Comparisons between the control and treatment groups will be assessed using independent t-tests or the Mann-Whitney U test for Gaussian and non-Gaussian data, respectively, with a Bonferroni correction to prevent the inflation of Type I error. Differences between groups for variables assessed across time will be analyzed using a repeated measures multiple analysis of variance (MANOVA) in which group (treatment versus control) is the between subject factor and time (baseline, 2 months, and 6 months) is the within subject factor. Multiple regression will be utilized to assess the influence of dietary flaxseed supplementation on the outcome variables by controlling for age, sex, diet, physical activity, and body mass index (BMI). The statistical analyses may also include dichotomization of the study population by sex, BMI classification, and age. Correlation analyses will be employed to understand potential relationships between clinical findings and metabolic analyses.

Current Problems/Concerns

There are no current problems or concerns to date.

Appendices

Appendix 1 Recruitment Poster

Hôpital St-Boniface Hospital
RECHERCHE • RESEARCH

Health Sciences Centre
Winnipeg

St. Boniface Hospital Research and the Health Sciences Centre are looking for participants for a flaxseed study.

Have you been recently diagnosed with high blood pressure and <u>not</u> taking any blood pressure lowering medications?

If you've answered yes, you may be qualified for a study that involves flaxseed food products which may reduce blood pressure, cardiovascular events, and other heart related complications.
For more information, please call the study co-ordinator at (xxx) xxx-xxxx

---

Please note:

<u>Exclusion Criteria</u> includes but not limited to: individuals with peripheral artery disease, bowel disease, systemic lupus, pregnant/breastfeeding women, individuals who already consume flax or fish more than 2 times/week.

Appendix 2

Radio or Television Advertisements

***Radio and Television Advertisments may be a shorter summary of the below information.**

Previous trials conducted at St. Boniface Hospital Research Centre have shown protective effects of consuming milled flaxseed against heart disease in animal models. More specifically reducing fatty plaques in blood vessels, reducing blood cholesterol levels, reducing prevalence of arrythmias, and promoting proper blood vessel relaxation and contraction.

The World Health Organization has stated that high blood pressure is the leading cause of global mortality and is attributed to 13% of deaths. Uncontrolled high blood pressure increases the risk for stroke, heart attacks, and kidney disease. Therefore, the ability to find effective treatments to reduce blood pressure may result in saving a lot of lives.

Recently, a clinical trial run by Dr. Grant Pierce and his lab at St. Boniface Hospital Research Centre found that consuming 30 g of milled flaxseed in baked products significantly reduced blood pressure in patients with peripheral artery disease already taking anti-hypertensive medication.

However, from this trial we have more questions to answer such as (1) can milled flaxseed effectively reduce blood pressure in those newly diagnosed with high blood pressure not taking any anti-hypertesnive medication? (2) Can flaxseed reduce or prevent the need for anti-hypertensive medication? (3) Can flaxseed work on its own or best with anti-hypertensive medication? (4) How does flaxseed work to reduce blood pressure?

In order to answer these questions and provide better care for individuals with high blood pressure, we are looking to recruit participants newly diagnosed with high blood pressure not taking any anti-hypertensive medication. The trial will be 6 months in duration in which the participants will be provided baked products with flaxseed to consume every day. Assessments such as 24 hour blood pressure and blood chemistry analysis will be used to assess the effect of flaxseed on blood pressure and vascular health.

If you are interested in participating in the current trial please contact the study nurse at (xxx) xxx-xxxx

Appendix 3

24-hour food recall Mulitple Pass Method as established by the USDA and published by Raper et al. J Food Comp Anal. 2004;17:545. The below table is a modification of the table provided in Raper et al. (2004)[11]

Step	**Pass**	**Purpose**
1	Quick List	Collect list of foods and beverages consumed the day prior
2	Forgotten Foods List	Collect foods and beverages that may have been forgotten by the participant. Probe for items such as beverages, snacks, and additives (sugar and salt).
3	Time and Occasion	Ensure time and occasion for all items has been recorded
4	Detail and Review	Ensure that quantity is accurate. Ask if brand names and types (eg: low sodium, fat free, sugar free) have been recorded when possible
5	Final Review	Ask if any other items could be added and double check quantities

24-hour Food Recall Record

Participant's First and Last Name: _____ Date: _____

Please record below to the best accuracy as possible all of the foods and beverages you have consumed in the last 24 hours. Please refer to the serving size handout to aid in estimating quantity of food/beverage consumed. If you are uncertain about particular items or quantities please feel free to ask the study co-ordinator.

Example:

Time of Day	***Quantity of Food/Beverage (eg: cups, tablespoons, grams, servings)***	***Food Item (please list brand name if known)***
8:00 AM	*1.5 cups* *2 tablespoons* *2 tablespoons* *2 slices* *4 tablespoons*	*Coffee (Folgers)* *White sugar* *Half and half cream* *100% whole wheat Dempsters bread* *Skippy Crunchy Peanut Butter*

Time of Day	**Quantity of Food/Beverage (eg: cups, tablespoons, grams)**	**Food Item (please list brand name if known)**

Continued on next page

Question: Has your diet changed as a result of joining this study? If yes, how so?

1 Serving Looks Like . . .

Grain Products

1 cup of cereal flakes = fist

1 pancake = compact disc 

½ cup of cooked rice, pasta, or potato = ½ baseball 

1 slice of bread = cassette tape

1 piece of cornbread = bar of soap 

1 Serving Looks Like . . .

Vegetables and Fruit

1 cup of salad greens = baseball 

1 baked potato = fist

1 med. fruit = baseball

½ cup of fresh fruit = ½ baseball 

¼ cup of raisins = large egg

1 Serving Looks Like . . .

Dairy and Cheese

1½ oz. cheese = 4 stacked dice or 2 cheese slices

½ cup of ice cream = ½ baseball 

Fats

1 tsp. margarine or spreads = 1 dice

1 Serving Looks Like . . .

Meat and Alternatives

3 oz. meat, fish, and poultry = deck of cards 

3 oz. grilled/baked fish = checkbook 

2 Tbsp. peanut butter = ping pong ball

Appendix 4

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ **days per week**

☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**

_____ **minutes per day**

☐ Don't know/Not sure

Appendix 5

Aliani et al. Effect of flax addition on the flavor profile of muffins and snack bars. Food Res Int. 2011;44:2489-96.

Aliani et al. Effect of flax addition on the flavor profile and acceptability of bagels. J Food Sci. 2012; 77:S62-S70.

PATIENT CONSENT FORM EVALUATION	
Requirement	**Evaluation Status**
Full Disclosure of Risk	G Acceptable G Requires Revision
Clarity of Language	G Acceptable G Requires Revision
Description of Procedure	G Acceptable G Requires Revision
Confidentiality for Patient	G Acceptable G Requires Revision
Lack of Bias	G Acceptable G Requires Revision
Placebo and/or Comparator Disclosure	G Acceptable G Requires Revision

INVESTIGATORY BROCHURE EVALUATION			
Requirement	Version Dated:		**Evaluation Status**
Accuracy of Information			G Acceptable G Requires Revision
Rationale for Investigation			G Acceptable G Requires Revision
Completeness			G Acceptable G Requires Revision
Numerical Data			G Acceptable G Requires Revision
Tabulation of Actual Results			G Acceptable G Requires Revision
Side Effects			G Acceptable G Requires Revision
Summary of ADR: Deaths, Serious, Other			G Acceptable G Requires Revision
Information on Patient Exposure, Duration of Study, Location of Study, NHP Dosage			G Acceptable G Requires Revision
Dosage Formulation			G Acceptable G Requires Revision

References

1. World Health Organization: Global health risks 2013: 70, 2009

2. Mayo Clinic: High blood pressure (hypertension).20132011

3. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L, INTERHEART Study Investigators: Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet* 364: 937-952, 2004

4. Leyva DR, Zahradka P, Ramjiawan B, Guzman R, Aliani M, Pierce GN: The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with peripheral artery disease: Rationale and design of the FLAX-PAD randomized controlled trial. *Contemp Clin Trials* 32: 724-730, 2011

5. Rodriquez-Leyva D, Weighell W, Edel A, LaVallee R, Dibrov R, Pinneker R, Maddaford T, Ramjiawan B, Alian M, Guzman R, Pierce G: Potent anti-hypertensive action of dietary flaxseed in hypertensive patients. *Circulation* Submitted. 2013.

6. High blood pressure, by sex, and by province and territory.2012

7. Tu K, Chen Z, Lipscombe LL, Canadian Hypertension Education Program Outcomes Research Taskforce: Prevalence and incidence of hypertension from 1995 to 2005: A population-based study. *Cmaj* 178: 1429-1435, 2008

8. Staessen JA, Fagard RH, Lijnen PJ, Thijs L, Van Hoof R, Amery AK: Mean and range of the ambulatory pressure in normotensive subjects from a meta-analysis of 23 studies. *Am J Cardiol* 67: 723-727, 1991

9. O'Brien E, Asmar R, Beilin L, Imai Y, Mancia G, Mengden T, Myers M, Padfield P, Palatini P, Parati G, Pickering T, Redon J, Staessen J, Stergiou G, Verdecchia P, European

Society of Hypertension Working Group on Blood Pressure Monitoring: Practice guidelines of the european society of hypertension for clinic, ambulatory and self blood pressure measurement. *J Hypertens* 23: 697-701, 2005

10. Hypertension Canada: The 2013 CHEP recommendations: 2013. Retrieved 2013. **http://www.hypertension.ca/chep-recommendations**

11. Raper N, Perloff B, Ingwersen L, Steinfeldt L, Jaswinder A: An overview of USDA's dietary intake data system. *J Food Comp Anal* 17: 545, 2004

1.2.5

Protocol

Trial Title: The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Investigators

Principal Investigator	Grant Pierce, PhD St. Boniface Hospital Research Centre 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3414 gpierce@sbrc.ca
Qualified Investigator Centre	Brian Penner, MD Health Sciences Centre Hospital 707 Mcdermot Avenue - GF329 General Winnipeg, MB, R3A 1R9 204-787-2684 bpenner@cc.umanitoba.ca
Associate Investigators	Dr. Amir Ravandi, MD PhD 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3325 aravandi@sbrc.ca Dr. Michel Aliani, PhD 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 474-8070 maliani@sbrc.ca Dr. Delfin Rodriguez-Leyva St. Boniface Hospital Research Centre 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3855 drodriguez@sbrc.ca and delfin@hvil.hlg.sld.cu
Student Investigator/Study Co-ordinator	Stephanie Caligiuri, M.Sc., PhD student St. Boniface Hospital Research Centre 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3855 umcaligs@cc.umanitoba.ca

PROTOCOL TABLE OF CONTENTS

Trial Title: The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Executive Summary

Hypertension is the leading global risk for mortality and can lead to, arrythmias, myocardial infarction, stroke, renal failure, and cardiac failure. Nutritional intervention with functional foods is a very effective method in reducing the risk of heart disease which allows individuals to take control of their own health. Flaxseed and its bioactive components have displayed cardioprotection and the current trial aims to assess if consuming functional foods with dietary flaxseed can reduce blood pressure and prevent the necessity of anti-hypertensive medication in newly diagnosed hypertensive individuals. In addition, the mechanisms of action of dietary flaxseed will be explored.

In order to explore these objectives, a randomized, double-blinded, controlled clinical trial will be carried out. Various food products including bagels, muffins, snack bars, and milled flaxseed (or wheat for control) will be provided to the participants to consume every day for 6 months. Assessments such as 24-hour ambulatory blood pressure monitoring, resting brachial blood pressure, need for anti-hypertensive medication, plasma and urine metabolomics will be assessed.

Details of the study rationale and design are outlined below.

Background/Rationale

Hypertension is the leading global risk for mortality as stated by the World Health Organization as high blood pressure is attributed to 13% of deaths in the world.[1] Hypertension when untreated or uncontrolled can lead to cardiac hypertrophy, arrythmias, myocardial infarctions, heart failure, stroke, and renal failure. [2] Despite the increasing knowledge on the severity of hypertension, the proportion of Canadians who live with high blood pressure (≥140/90 mm Hg) is increasing every year; the percentage of Canadians aged ≥12 years with hypertension has grown steadily from 16% to 17.4% since 2007; this equates to more than five million individuals.[3] For those aged 65 years and older, the prevalence is even greater; 49.5% live with hypertension.[3]

Hypertension not only places a burden on the individual but also on the healthcare system; in 1998, 26.4 million visits were made by Canadians to doctors for cardiovascular disease; one half of those cases were for hypertension management.[4] It is therefore evident that hypertension places a significant burden on the lives of Canadians and approaches to reduce blood pressure are of intrinsic importance.

In a landmark publication by the INTERHEART Study, it was concluded that modifiable lifestyle factors including nutrition account for 90% of the risk for myocardial infarction worldwide.[5] According to the Institute of Food Technologists Functional Food Survey, 30% and 36% of individuals stated they always or sometimes purchase grocery products labelled to help with health conditions, respectively. [6] The population has a desire to adjust modifiable risk factors such as diet in order to improve their health.

Dietary flaxseed is an example of a functional food and evidence supports its ability to reduce blood pressure in patients with peripheral artery disease (PAD). In our previous clinical trial, FLAXPAD, our lab investigated the effect of dietary flaxseed on overall cardiovascular health in patients with PAD. [7, 8] Flaxseed is thought to contain three bioactive components: the omega 3 fatty acid alpha-linolenic acid (ALA), the potent antioxidant enterolignan, and dietary fibre. [9] Significant reductions in both systolic and diastolic blood pressure were observed in the group of patients administered milled flaxseed. Despite the participants being on anti-hypertensive medications, a significant proportion had uncontrolled hypertension and experienced an improvement in blood pressure; an 11.8 and 8.1 mm Hg drop in systolic and diastolic blood pressure, respectively, was observed over a year. However, significant declines in blood pressure were already observed after one month of dietary intervention.

Similar findings have been reported in dietary interventions containing ALA; overweight individuals provided a hypocaloric diet enriched with rapeseed oil resulted in weight loss and a reduction in blood pressure of 10 and 8.4 mm Hg in systolic and diastolic blood pressure, respectively. The reduction in diastolic but not systolic blood pressure was significantly greater than the olive oil control group. [10] Dyslipidemic individuals provided flax oil every day for 12 weeks resulted in a 3.1 and 6.3 mm Hg drop in systolic and diastolic blood pressure, respectively, which was significantly greater than the safflower oil control group. [11] In pre-hypertensive individuals, consumption of a combination of rapeseed, flax, and rice oil resulted in an approximate drop in systolic and diastolic blood pressure of 10 and 3 mm Hg, respectively, which was significantly greater than the control group. [12]

However, despite evidence to support dietary ALA enrichment in the reduction of blood pressure, the efficacy of flaxseed which contains ALA, dietary fibre, and enterolignans as an effective anti-hypertension treatment to reduce blood pressure in newly diagnosed hypertensive patients has yet to be investigated. In addition, the ability of flaxseed to work as a monotherapy rather than in synergism with anti-hypertensive medications also has yet to be determined. Lastly, the potential mechanism of action of blood pressure lowering by flaxseed has not been investigated.

Trial Objectives

The primary purpose of the current investigation is to determine the efficacy of dietary flaxseed supplementation on reducing blood pressure in newly diagnosed hypertensive individuals over a period of six months. The primary endpoint is therefore blood pressure. Additional objectives include investigating the ability of dietary flaxseed to prevent or reduce the need of for anti-hypertensive medication, whether flaxseed is more effective as a monotherapy or in conjuction with anti-hypertenstive medication, and lastly, the potential mechanism of action of flaxseed's blood pressure lowering effect. The additional endpoints include:

1. Nutritional intake and physical activity (Appendices 3 and 4)
2. Plasma nitric oxide
3. Plasma amino acid profile
4. Plasma lipid profile (triglycercides, LDL and HDL cholesterol)
5. Plamsa glucose, liver function enzymes, kidney function markers

6. Plasma and urine metabolomics which includes proteins, lipids, and carbohydrates that are markers of metabolism and heart, blood vessel, liver, kidney, pancreatic, and intestinal health.
7. Plasma oxylipins which are metabolites of fatty acids
8. Plasma oxidized phospholipids
9. Plasma markers of hypertension such as: angiotensin II, aldosterone, angiontensin converting enzyme, and endothelin-1

Study Design

The proposed trial is a double-blinded, randomized, controlled trial to determine the efficacy of dietary flaxseed supplementation on reducing blood pressure in newly diagnosed hypertensive participants. The participants will be recruited either from volunteers after seeing advertisements for recruitment (see Appendix 1 and 2) or referred by a general practitioner. The participants will be referred to the hypertension clinic run by Dr. Brian Penner at the Health Sciences Hospital. If the participant meets all inclusion and exclusion criteria and signs the consent form, they will be asked to fill out a 24-hour food recall with the study co-ordinator using the Multiple-Pass Method as established by the USDA and the standardized international physical activity questionnaire (short) (https://sites.google.com/site/theipaq/) (See Appendices 3 and 4). They will also be set up with a 24-hour ambulatory blood pressure monitor in addition to undergoing a brachial blood pressure measurement to determine if the participant is hypertensive.

According to the Canadian Hypertension Education Pannel, if the participant has a **mean** 24-hour systolic blood pressure ≥130 OR diastolic ≥80 mm Hg or **awake** systolic blood pressure ≥135 OR diastolic ≥85 from the ambulatory blood pressure measurement they will be classified as hypertensive.[14, 15] If the participant has been diagnosed with

hypertension, they will officially enter the trial and return for baseline fasting blood collection (9-12 hour fast), urine collection, height, weight, and waist circumference measurements. The participants at this point will also be randomized into either the control or flax group. Both the participant, the study co-ordinator, qualified investigator (Dr. Brian Penner) and additional investigators will be blinded to the group. The participants will be provided either wheat (control) or flax (treatment) containing food products such as muffins, bagels, snack bars, and milled seeds to consume once a day for six months. A variety of flavours will be provided to assure the participants do not tire of the food products. In the first two months of the trial the participants will not be prescribed anti-hypertensive medication, as this is the lifestyle intervention period. A meta-analysis published in 2008 concluded the administration of a control to a patient with hypertension for 4-8 weeks provided no objective reason against safety.[16] The participants will be encouraged not to consume the food products within two hours of taking any medication.

At the two month mark, the participants will return to the hypertension clinic. At this point, the same baseline tests will be repeated. After assessment of the 24 hour ambulatory blood pressure, if Dr. Brian Penner deems that it is necessary to start anti-hypertensive medication according to the Canadian Hypertension Education Panel guidelines he will provide standard of care and take record of the prescriptions. After the 2 month follow-up visit, the patients will come back to the hypertension clinic for a follow-up visit with Dr. Brian Penner every month where their brachial blood pressure will be measured and medication routine assessed. The participants will continue to consume the control or flax containing food products every day throughout the entirety of the study. At the six month point, the patients will return to the hypertension clinic where the same tests from the baseline and 2 month period will be repeated.

Table Summary of Study Design

Time Point	Tests Patient Undergoes
Baseline	• Brachial blood pressure • 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* --- • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Randomization into control or flax group
2 months	• Brachial blood pressure • 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* --- • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Assessment for need of anti-hypertensive medication
3 months	Brachial blood pressure Follow-up on anti-hypertensive medication
4 months	Brachial blood pressure Follow-up on anti-hypertensive medication
5 months	Brachial blood pressure Follow-up on anti-hypertensive medication
6 months	• Brachial blood pressure

	• 24-hour ambulatory blood pressure monitoring • 24-hour food recall and physical activity record *Performed on the following day* --- • Fasting blood drawn • Urine collected • Height, weight, waist circumference measured • Follow-up on anti-hypertensive medication

Study Duration

The proposed study is expected to be four and a half years in total duration. The screening and enrollment of the 100 subjects and completion of study is anticipated to take approximately two years. Sample analysis, data entry, and writing will take approximately two and a half years thereafter.

Number of Sites (inside and outside Canada)

The study will be conducted at two sites: Health Sciences Centre Hospital (patient assessment) and St. Boniface Hospital Research Centre (sample analysis).

List of Investigators

Principal Investigator

Grant Pierce, PhD

St. Boniface Hospital Research Centre

351 Tache Avenue

Winnipeg, MB R2H 2A6

(204) 235-3414 gpierce@sbrc.ca

Qualified Investigator	Brian Penner, MD Health Sciences Centre Hospital 707 Mcdermot Avenue - GF329 General Centre Winnipeg, MB, R3A 1R9 204-787-2684 bpenner@cc.umanitoba.ca
Associate Investigators	Dr. Amir Ravandi, MD PhD 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3325 aravandi@sbrc.ca
	Dr. Michel Aliani, PhD 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 474-8070 maliani@sbrc.ca
	Dr. Delfin Rodriguez-Leyva St. Boniface Hospital Research Centre 351 Tache Avenue Winnipeg, MB R2H 2A6 (204) 235-3855 drodriguez@sbrc.ca and delfin@hvil.hlg.sld.cu

Student Investigator/Study Co-ordinator Stephanie Caligiuri, M.Sc., PhD student

St. Boniface Hospital Research Centre

351 Tache Avenue

Winnipeg, MB R2H 2A6

(204) 235-3855 umcaligs@cc.umanitoba.ca

Sample Size

A statistical power analysis was calculated for the repeated measures ANOVA design comparing two independent groups of hypertensive patients at baseline, 2 and 6 months. The primary outcome measures were systolic and diastolic blood pressure. The statistical power analysis indicated that a sample size of n = 40 subjects per group (n = 80 subjects in total) would have a power = 0.80 to detect differences between groups and across time of an effect size = 0.7 SD at an α level = 0.05. Based on the drop-out rate of 20% in the previous FLAXPAD trial, it is recommended that 50 subjects per group or n = 100 be recruited for the study.

Patient Population

5,095,240 Canadians aged 20 or older have stated they have been diagnosed with high blood pressure in 2011.[3] In Manitoba, 192,181 individuals aged 12 and older in 2011 were diagnosed with high blood pressure; this equtes to 19.4% of Manitobans living with hypertension which is greater than the national prevalence of 17.6%.[17] The target population of this trial is newly diagnosed individuals; therefore, the incidence of hypertension as studied in another Canadian province, Ontario, has increased from 25.5 per 1000 adults to

32.1 per 1000 adults from 1997 to 2004. [18] and this incidence rate has likely increased from 2004 to the current year.

Inclusion Criteria

10. Essential hypertension (24-hour **average** ambulatory systolic blood pressure of ≥130 OR diastolic blood pressure ≥80 OR 24-hour **awake** ambulatory systolic blood pressure ≥135 OR diastolic blood pressure ≥85 [14, 15])
11. Either gender
12. Untreated for hypertension
13. 18-85 years old and able to provide informed consent.
14. Females who are:
    a. highly unlikely to conceive due to surgical sterilization
    b. postmenopausal female with >2 years since last menses
    c. or non-sterilized, pre-menopausal female who agrees to: 1. Use an adequate method of contraception to prevent pregnancy (such as a barrier or hormonal); 2. Abstain from heterosexual activity for study period; or 3. Only engage in heterosexual activity with surgically sterilized male partner(s) and not planning on becoming pregnant during the study.
15. Subjects taking anti-platelet therapy must be on a stable dose for 3 months prior to the study.
16. Subjects taking lipid lowering drugs must be on a stable dose for 3 months prior to the study.
17. Subjects must have access to freezer space in their residence to hold up to one month of frozen food products associated with this study.

Exclusion Criteria

14. Patients with ischemic pain at rest in limbs, ulceration, or gangrene.
15. Clinical evidence of peripheral artery disease, previous myocardial infarction, or stroke.
16. Patient has undergone percutaneous coronary angioplasty, has had coronary bypass within the last 6 months, or has unstable angina.
17. Known secondary hypertension of any etiology.
18. Patients with confirmed and clinically significant renal or hepatic abnormalities (creatinine > 0.130 mM or creatinine clearance < 45ml/min, AST 2-3x normal, ALT > 2-3x normal) and/or electrolyte imbalance serum K+ < 3.5 or > 5.5 mM.
19. History of major bleeding.
20. Patients with bowel disease (including Crohn's disease, celiac disease, colitis, peptic ulcer disease, irritable bowel syndrome and diverticulosis) or other diseases such as active systemic lupus erythematosus, metastatic cancer, or end stage respiratory disease.
21. Patients with clinical evidence of heart failure or an estimated life expectancy less than 2 years and with high baseline cardiac risk (post ischemic or diabetic cardiomyopathy with an ejection fraction < 40%, Canadian Cardiovascular Society Class 3 or 4 angina or need for coronary revascularization procedures).
22. Subjects that are on supplements other that those prescribed by their clinician for the entire duration of the study. Please see point 10 below.

23. Subjects ingesting more than 2 servings of fish per week, taking omega-3 fatty acid supplements, and/or consuming milled flaxseed or flax oil on a regular basis (ie: ≥1 tablespoon of milled flaxseed or 1 teaspoon of flax oil per week).
24. Patients having participated in an investigational drug program in the proceeding 30 days or unable or unwilling to comply with the protocol.
25. Subjects with allergies to any ingredient in the study product or control (including gluten).
26. Patients who will undergo surgery or intend to move outside Winnipeg during the trial period.

NHP Formulation

The formulation of the food products (bagels, muffins, and bars) has been previously approved in the FlaxPAD CTA (identifying #: 10941-011). The ingredients and flavour acceptability have also been published in two papers by Aliani et al. which can be found in the Appendices section, Appendix 5.

Dosage Regimen

Participants will be provided enough food items to last the duration of the month every month for the entirety of the trial. The food items include: muffins, bagels, bars, and either milled flaxseed or wheat on its own. Each food item will provide either 30 g of milled flaxseed (treatment) or milled wheat (control) and will be individually wrapped. The participants will be requied to ingest only one product per day.

In the first month of the trial, the participants will be provided a gradual increase of flaxseed or wheat in order to allow the participants to become accustomed to the rise in dietary fibre. The food items will provide 10, 20, and 30 g of milled flaxseed or wheat in week one, two, and three, respectively. The gradual increase in flaxseed and wheat will aid in participant comfort and subject compliance as previously shown in the FlaxPAD trial.

Prestudy Screening and Baseline Evaluation

Participants that have met all inclusion and exclusion criteria and have provided informed consent will undergo the following baseline evaluations:

Immediately Analyzed:

1. 24-hour ambulatory blood pressure monitoring
2. Brachial blood pressure measurement

Later Analyzed:

1. 24-hour food recall and physical activity record
2. Height, weight, waist circumference
3. Plasma nitric oxide
4. Plasma amino acids
5. Plasma lipid profile (eg: triglycercides, LDL and HDL cholesterol)
6. Blood glucose, liver function enzymes, kidney function markers
7. Plasma and urine metabolomics which includes proteins, lipids, and carbohydrates that are markers of metabolism and heart, blood vessel, liver, kidney, pancreatic, and intestinal health.
8. Plasma oxylipins which are metabolites of fatty acids
9. Plasma oxidized phospholipids

10. Plasma markers of hypertension such as: angiotensin II, aldosterone, angiontensin converting enzyme, and endothelin-1

Treatment Visit

After the baseline evaluation, the participants will return for seven follow-up visits (two visits at both the two and six month period, and one visit each at the three, four, and five month time periods). At the two and six month periods, the participants will fill out the 24-hour food recall and physical activity record with the study co-ordinator and undergo brachial blood pressure measurement and be set up with the 24-hour ambulatory blood pressure monitor in the morning. The following morning, the participant will return to have the 24-hour ambulatory blood pressure monitor removed, as well, as for fasting blood and urine collection, height, weight, and waist circumference assessment. At the three, four, and five month time periods, the participants will see Dr. Brian Penner for brachial blood pressure measurement and assessment of medication intake if any.

The blood collected at baseline, month two, and month six will be assessed for:

1. Nitric oxide
2. Amino acid profile
3. Plasma lipid profile (eg: triglycercides, LDL and HDL cholesterol)
4. Glucose, liver function enzymes, kidney function markers
5. Plasma metabolomics which includes proteins, lipids, and carbohydrates that are markers of metabolism and heart, blood vessel, liver, kidney, pancreatic, and intestinal health.
6. Plasma oxylipins which are metabolites of fatty acids

7. Plasma oxidized phospholipids
8. Plasma markers of hypertension such as: angiotensin II, aldosterone, angiontensin converting enzyme, and endothelin-1

The urine will be assessed for metabolomics as outlined in #5 above.

Premature Withdrawal/Discontinuation Criteria

The participants are entitled to withdraw from the study at any time for any reason. However, the study will be stopped should the Data Safety and Monitoring Committee deem the study harmful to the participants. In addition, the clinical investigator may choose to withdraw a participant from the study due to medical reasons or if the participant is continually non-compliant with the study protocol.

Rescue Medication

As this is not a drug trial, rescue medication is not warranted. If deemed necessary by Dr. Brian Penner, the participants will be started on anti-hypertensive medication as standard of care by following the Canadian Hypertension Education Panel Guidelines [19].

Washout Period

No washout period is necessary if the participants meet the inclusion and exclusion criteria.

Concomitant Medication

Participants will be asked to not consume any flax containing products other than those provided in the trial. Other herbal or nutritional supplements will also be restricted during the study. Participants who meet all the inclusion criteria and do not have any exclusion

criteria will take all their routine medications as well as any additional medications prescribed throughout the study.

Variables to be Assessed

Please refer to the Summary Table of Study Design, as well as, the Prestudy Screening and Baseline Evaluation of this document.

Efficacy Analysis

Based on the objectives of the trial objectives, measured parameters will be assessed to determine the efficacy of dietary flaxseed supplementation using statical methods as detailed below. Final data tabulation, reduction, and processing will only be carried out at the end of the study.

Safety Analysis

No adverse effects are expected based upon published literature and experience with prior trials with dietary flaxseed supplementation. Studies with flaxseed carried out at St. Boniface Hospital Research Centre for the timespan of one year with a dose of 30 g/day have reported no significant adverse events. [8] The food products provided to the participants will be prepared by companies with whom follow Good Manufacturing Practice. The food products will be prepared, frozen, and delivered to the participants monthly.

Due to the ability of flaxseed to increase stool bulk and frequency of defecation, patients with a history of bowel obstruction, irritable bowel syndrome, or diverticular disease will be excluded. When large amounts of dietary fibre are consumed, gastro-intestinal discomfort

may occur. Results from our lab have indicated that the gastro-intestinal discomfort and flatulence disappear within a few weeks once the participants have become accustomed to the fibre load.

However, adverse events, as identified by the WHO scale, will be followed up, if medically indicated, with relevevant laboratory investigations under the direction of the study medical monitor. Research staff will record final outcome and resolution date of the event wherever possible.

All serious adverse events (representing a significant health hazard to the participant) will be reviewed by the medical monitor within 24 hours of becoming aware of the events. The monitor will notify the sponsors and the Ethics Review Board within 7-14 days of the event. The University of Manitoba has a Research Ethics Boards that reviews all clinical trials for safety and ethical considerations. St. Boniface Hospital also has a similar process to protect research partipants. This trial must pass both these review processess before it can proceed. Applications to both of these boards will be filed and reviewed concurrently with the CTA. Final approvals will be forwarded to the NHPD of Health Canada.

Statistical Analysis

Comparisons between the control and treatment groups will be assessed using independent t-tests or the Mann-Whitney U test for Gaussian and non-Gaussian data, respectively, with a Bonferroni correction to prevent the inflation of Type I error. Differences between groups for variables assessed across time will be analyzed using a repeated measures multiple analysis of variance (MANOVA) in which group (treatment versus control) is the between

subject factor and time (baseline, 2 months, and 6 months) is the within subject factor. Multiple regression will be utilized to assess the influence of dietary flaxseed supplementation on the outcome variables by controlling for age, sex, diet, physical activity, and body mass index (BMI). The statistical analyses may also include dichotomization of the study population by sex, BMI classification, and age. Correlation analyses will also be employed to assess associations between clinicaal and metabolic measurements.

Current Problems/Concerns

There are no problems or concerns to date.

References

1. World Health Organization: Global health risks 2013: 70, 2009. Retrieved 2013. http://www.who.int.proxy2.lib.umanitoba.ca/healthinfo/global_burden_disease/GlobalHealthRisks_report_full.pdf
2. Mayo Clinic: High blood pressure (hypertension). 2011. Retrieved 2013. http://www.mayoclinic.com/health/high-blood-pressure/HI00062
3. Government of Canada - Statistics Canada: High blood pressure, by age group and sex. 2012. Retrieved 2013. http://www.statcan.gc.ca.proxy2.lib.umanitoba.ca/tables-tableaux/sum-som/l01/cst01/health03a-eng.htm
4. Heart and Stroke Foundation Canada: *The changing face of heart disease and stroke in Canada*. Ottawa, Canada, 1999.
5. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L, INTERHEART Study Investigators: Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): Case-control study. *Lancet* 364: 937-952, 2004
6. Sloan E: Top 10 functional food trends. 2012. Retrieved 2013. http://www.ift.org/food-technology/past-issues/2012/april/features/top-10-functional-food-trends.aspx?page=viewall
7. Leyva DR, Zahradka P, Ramjiawan B, Guzman R, Aliani M, Pierce GN: The effect of dietary flaxseed on improving symptoms of cardiovascular disease in patients with peripheral artery disease: Rationale and design of the FLAX-PAD randomized controlled trial. *Contemp Clin Trials* 32: 724-730, 2011
8. Rodriquez-Leyva D, Weighell W, Edel A, LaVallee R, Dibrov R, Pinneker R, Maddaford T, Ramjiawan B, Alian M, Guzman R, Pierce G: Potent anti-hypertensive action of dietary flaxseed in hypertensive patients. *Circulation* Submitted. 2013.
9. Bassett CM, Rodriguez-Leyva D, Pierce GN: Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. *Appl Physiol Nutr Metab* 34: 965-974, 2009
10. Baxheinrich A, Stratmann B, Lee-Barkey YH, Tschoepe D, Wahrburg U: Effects of a rapeseed oil-enriched hypoenergetic diet with a high content of alpha-linolenic acid on body weight and cardiovascular risk profile in patients with the metabolic syndrome. *Br J Nutr* 108: 682-691, 2012
11. Paschos GK, Magkos F, Panagiotakos DB, Votteas V, Zampelas A: Dietary supplementation with flaxseed oil lowers blood pressure in dyslipidaemic patients. *Eur J Clin Nutr* 61: 1201-1206, 2007
12. Takeuchi H, Sakurai C, Noda R, Sekine S, Murano Y, Wanaka K, Kasai M, Watanabe S, Aoyama T, Kondo K: Antihypertensive effect and safety of dietary alpha-linolenic acid in subjects with high-normal blood pressure and mild hypertension. *J Oleo Sci* 56: 347-360, 2007
13. Kolar AS, Patterson RE, White E, Neuhouser ML, Frank LL, Standley J, Potter JD, Kristal AR: A practical method for collecting 3-day food records in a large cohort. *Epidemiology* 16: 579-583, 2005
14. Staessen JA, Fagard RH, Lijnen PJ, Thijs L, Van Hoof R, Amery AK: Mean and range of the ambulatory pressure in normotensive subjects from a meta-analysis of 23 studies. *Am J Cardiol* 67: 723-727, 1991
15. O'Brien E, Asmar R, Beilin L, Imai Y, Mancia G, Mengden T, Myers M, Padfield P, Palatini P, Parati G, Pickering T, Redon J, Staessen J, Stergiou G, Verdecchia P,

European Society of Hypertension Working Group on Blood Pressure Monitoring: Practice guidelines of the european society of hypertension for clinic, ambulatory and self blood pressure measurement. *J Hypertens* 23: 697-701, 2005

16. DeFelice A, Willard J, Lawrence J, Hung J, Gordon MA, Karkowsky A, Targum S, Throckmorton DC, Girton J, Stertz B, Glasser SP, Lipicky RJ: The risks associated with short-term placebo-controlled antihypertensive clinical trials: A descriptive meta-analysis. *J Hum Hypertens* 22: 659-668, 2008
17. High blood pressure, by sex, and by province and territory. 2012. Retrieved 2013. http://www.statcan.gc.ca.proxy2.lib.umanitoba.ca/tables-tableaux/sum-som/l01/cst01/health70a-eng.htm
18. Tu K, Chen Z, Lipscombe LL, Canadian Hypertension Education Program Outcomes Research Taskforce: Prevalence and incidence of hypertension from 1995 to 2005: A population-based study. *Cmaj* 178: 1429-1435, 2008
19. Hypertension Canada: The 2013 CHEP recommendations. 2013. Retrieved 2013. http://www.hypertension.ca/chep-recommendations

1.2.7

CLINICAL TRIAL SITE INFORMATION FORM

Natural Health Products Directorate

A separate form for each clinical trial site must be completed by the Sponsor and filed with Health Canada.

All fields must be completed prior to submitting this form to Health Canada.

Part 1: Clinical Trial Protocol Information

Please check one of the following:

☒ **Clinical Trial Application (CTA)**

☐ **Clinical Trial Application Amendment (CTA-A)**

☐ **Clinical Trial Notification**

Protocol Title The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Protocol # (if known)

Part 2: Natural Health Product (NHP) / Sponsor Information

A. NHP Information

Brand Name / Product Code: N/A

Medicinal Ingredient(s):

☒ See Clinical Trial Application and Attestation Form

Submission Number (if known):

B. Sponsor of Clinical Trial

Name of Sponsor (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	MB	Canada	R2H 2A6

Contact Information of Sponsor (if Sponsor is an individual) or Senior Official (if Sponsor is a company, institution or organization)

Name	Telephone No.	Fax No.	Language Preferred
Dr. Grant Pierce	204-235-3414	204-235-0793	☒ English ☐ French
Title: Director of Research/Professor	E-mail: gpierce@sbrc.ca		

C. Contact for this Clinical Trial

Contact Name	E-mail
Ms. Stephanie Caligiuri	umcaligs@cc.umanitoba.ca

Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

Telephone No. 204-235-3855	Fax No. 204-235-0793	Language Preferred ☒ English ☐ French

Part 3: Clinical Trial Site Information

A. Clinical Trial Site

Name of Site (Full Name – No Abbreviations) Health Sciences Centre Hospital Hypertension Clinic		
Street / Suite / PO Box GA219 700 William Ave		
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9
Commencement Date of Clinical Trial or Clinical Trial Amendment[1] not yet determined		

B. Qualified Investigator

A Qualified Investigator Undertaking must be completed by the qualified investigator responsible for the conduct of the clinical trial at the site specified above. The completed undertaking must be retained by the clinical trial sponsor for a period of 25 years.

Name Dr. Brian Penner	Title Doctor/Professor	Language Preferred ☒ English ☐ French
Street / Suite / PO Box 707 Mcdermot Avenue - GF329 General Centre		
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9

E-mail bpenner@cc.umanitoba.ca	Telephone No. 204-787-2684	Fax No. N/A

C. Research Ethics Board Approval

A Research Ethics Board Attestation must be completed by the Research Ethics Board that reviewed and approved the protocol and informed consent form for this clinical trial at the site specified above. The completed attestation must be retained by the clinical trial sponsor for a period of 25 years.

Name of Research Ethics Board The University of Manitoba Biomedical Research Ethics Board	Date of Approval pending
Street / Suite / PO Box	
City / Town Winnipeg	Province MB	Postal Code R3E0W3

Name of Contact Person Mrs. Shelly Rempel-Rossum	Telephone No. 204-789-3389	Fax No. 204-789-3414	Language Preferred ☒ English ☐ French
Title REB Coordinator		E-mail mross@cc.umanitoba.ca	

[1] Date of commencement of the trial: For the purposes of the Clinical Trial Site Information Form this is defined as the date when the clinical trial is ready to enroll patients in the clinical trial. (Before a start date can be determined, both Health Canada and Research Ethics Boards approval must be obtained).

CLINICAL TRIAL SITE INFORMATION FORM

Natural Health Products Directorate

A separate form for each clinical trial site must be completed by the Sponsor and filed with Health Canada.

All fields must be completed prior to submitting this form to Health Canada.

Part 1: Clinical Trial Protocol Information

Please check one of the following:

☒ **Clinical Trial Application (CTA)**

☐ **Clinical Trial Application Amendment (CTA-A)**

☐ **Clinical Trial Notification**

Protocol Title The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Protocol # (if known)

Part 2: Natural Health Product (NHP) / Sponsor Information

A. NHP Information

Brand Name / Product Code: N/A

Medicinal Ingredient(s):

☒ See Clinical Trial Application and Attestation Form

Submission Number (if known):

B. Sponsor of Clinical Trial

Name of Sponsor (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	MB	Canada	R2H 2A6

Contact Information of Sponsor (if Sponsor is an individual) or Senior Official (if Sponsor is a company, institution or organization)

Name	Telephone No.	Fax No.	Language Preferred
Dr. Grant Pierce	204-235-3414	204-235-0793	☒ English ☐ French

Title	E-mail
Director of Research/Professor	gpierce@sbrc.ca

C. Contact for this Clinical Trial

Contact Name	E-mail
Ms. Stephanie Caligiuri	umcaligs@cc.umanitoba.ca

Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

Telephone No.	Fax No.	Language Preferred
204-235-3855	204-235-0793	☒ English ☐ French

Part 3: Clinical Trial Site Information

A. Clinical Trial Site

Name of Site (Full Name – No Abbreviations) St. Boniface Hospital Research Centre		
Street / Suite / PO Box 351 Tache Avenue		
City / Town Winnipeg	Province Manitoba	Postal Code R2H 2A6
Commencement Date of Clinical Trial or Clinical Trial Amendment[2] not yet determined		

B. Qualified Investigator

A Qualified Investigator Undertaking must be completed by the qualified investigator responsible for the conduct of the clinical trial at the site specified above. The completed undertaking must be retained by the clinical trial sponsor for a period of 25 years.

Name Dr. Brian Penner	Title Doctor/Professor	Language Preferred ☒ English ☐ French
Street / Suite / PO Box 707 Mcdermot Avenue - GF329 General Centre		
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9

E-mail bpenner@cc.umanitoba.ca	Telephone No. 204-787-2684	Fax No. N/A

C. Research Ethics Board Approval

A Research Ethics Board Attestation must be completed by the Research Ethics Board that reviewed and approved the protocol and informed consent form for this clinical trial at the site specified above. The completed attestation must be retained by the clinical trial sponsor for a period of 25 years.

Name of Research Ethics Board University of Manitoba Faculty of Medicine, Research Ethics Board		Date of Approval pending	
Street / Suite / PO Box			
City / Town Winnipeg	Province MB	Postal Code R3E0W3	
Name of Contact Person Mrs. Shelly Rempel-Rossum	Telephone No. 204-789-3389	Fax No. 204-789-3414	Language Preferred ☒ English ☐ French
Title REB Coordinator		E-mail mross@cc.umanitoba.ca	

[2] Date of commencement of the trial: For the purposes of the Clinical Trial Site Information Form this is defined as the date when the clinical trial is ready to enroll patients in the clinical trial. (Before a start date can be determined, both Health Canada and Research Ethics Boards approval must be obtained).

1.2.8

Canadian Research Ethics Boards Refusals

No Research Ethics Boards in Canada have refused to approve of the CT protocol.

1.2.9

Foreign Refusals

No Research Ethics Boards outside of Canada or regulatory authorities have refused approval of the CT protocol.

1.2.10

Letters of Access

No letters of access are required to access proprietary information.

1.2.11

QUALIFIED INVESTIGATOR UNDERTAKING
Natural Health Products Directorate

An undertaking must be completed by the qualified investigator responsible for the conduct of the clinical trial at the site specified below. The completed undertaking must be retained by the clinical trial sponsor for a period of 25 years.

Part 1: Clinical Trial Protocol Information

Please check one of the following:

☒ **Clinical Trial Application (CTA)**

☐ **Clinical Trial Application Amendment (CTA-A)**

☐ **Clinical Trial Notification**

Protocol Title The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Protocol # (if known)

Part 2: Natural Health Product (NHP) / Sponsor Information

A. NHP Information

Brand Name / Product Code: N/A

Medicinal Ingredient(s):

☒ See Clinical Trial Application and Attestation Form

Submission Number (if known):

B. Sponsor of Clinical Trial

Name of Sponsor (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

C. Contact for this Clinical Trial

Contact Name	E-mail
Ms. Stephanie Caligiuri	umcaligs@cc.umanitoba.ca

Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

Telephone No.	Fax No.
204-235-3855	204-235-0793

Part 3: Qualified Investigator Information			
A. Clinical Trial Site			
Name of Site (Full Name – No Abbreviations) Health Sciences Centre Hospital Hypertension Clinic			
Street / Suite / PO Box GA219 700 William Ave			
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9	
B. Qualified Investigator			
Name Dr. Brian Penner	Title Doctor/Professor	Language Preferred ☒ English ☐ French	
Street / Suite / PO Box 707 Mcdermot Avenue - GF329 General Centre			
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9	
E-mail bpenner@cc.umanitoba.ca	Telephone No. 204-787-2684	Fax No. N/A	

In respect of the identified clinical trial, I certify, as the qualified investigator for this site that:

1. I am a physician or dentist and a member in good standing of a professional medical or dental association as defined in Part 4 of the *Natural Health Products Regulations*;

2. I will supervise the medical care and medical decisions respecting this clinical trial at this site;

3. I will conduct this clinical trial in accordance with Good Clinical Practices; and

4. I will immediately on discontinuance of the clinical trial by the sponsor, in its entirety or at a clinical trial site, inform both the clinical trial subjects and the Research Ethics Board for this site of the discontinuance, provide them with the reasons for the discontinuance, and advise them in writing of any potential risks to the health of clinical trial subjects or other persons.

Signature of Qualified Investigator	Date					
	YYYY		M		D	

RESEARCH ETHICS BOARD ATTESTATION
Natural Health Products Directorate

An attestation must be completed by the Research Ethics Board that reviewed and approved the clinical trial protocol and informed consent form for this clinical trial at the site below. The completed attestation must be retained by the clinical trial sponsor for a period of 25 years.

Part 1: Clinical Trial Protocol Information

Please check one of the following:

☒ **Clinical Trial Application (CTA)**

☐ **Clinical Trial Application Amendment (CTA-A)**

Protocol Title The efficacy of dietary flaxseed for the reduction of blood pressure in newly diagnosed hypertensive patients

Protocol # (if known)

Part 2: Natural Health Product (NHP) / Sponsor Information

A. NHP Information

Brand Name / Product Code: Flaxseed

Medicinal Ingredient(s):

☒ See Clinical Trial Application and Attestation Form

Submission Number (if known):

B. Sponsor of Clinical Trial

Name of Sponsor (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

C. Contact for this Clinical Trial

Contact Name	E-mail
Ms. Stephanie Caligiuri	umcaligs@cc.umanitoba.ca

Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town	Province / State	Country	Postal / ZIP Code
Winnipeg	Manitoba	Canada	R2H 2A6

Telephone No.	Fax No.
204-235-3855	204-235-0793

Part 3: Clinical Trial Site Information

A. Clinical Trial Site

Name of Site (Full Name – No Abbreviations) Health Sciences Centre Hospital Hypertension Clinic		
Street / Suite / PO Box GA219 700 William Ave		
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9

B. Qualified Investigator

Name Dr. Brian Penner	Title Doctor/Professor	Language Preferred ☒ English ☐ French
Street / Suite / PO Box 707 Mcdermot Avenue - GF329 General Centre		
City / Town Winnipeg	Province Manitoba	Postal Code R3A 1R9

E-mail bpenner@cc.umanitoba.ca	Telephone No. 204-787-2684	Fax No. N/A

Attach separate sheets (same format) for each Clinical Trial Site.

Number of pages attached:

C. Research Ethics Board Approval

☒ Includes member knowledgeable in complementary or alternative health care (identify member and expertise in the cover letter)

Name of Research Ethics Board St Boniface Hospital Research Review Committee		Date of Approval Under review	
Street / Suite / PO Box 409 Tache Ave			
City / Town Winnipeg	Province MB	Postal Code R2H 2A6	
Name of Research Ethics Board Chair Dr Bram Ramjiawan	Telephone No. 204-235-3206	Fax No. 204-235-0793	Language Preferred ☒ English ☐ French
Title Director, Research Innovation and Regulatory Affairs	E-mail bramjiawan@sbrc.ca		

In respect of the identified clinical trial, I certify, as representative of this Research Ethics Board that:

1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the *Natural Health Products Regulations*;
2. This Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices; and
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Board have been documented in writing.

Name, Title and Signature of Research Ethics Board Representative		Date		
Name: Dr Bram Ramjiawan	Title: Director, Research Innovation and Regulatory Affairs	YYYY	M	D

Signature:						

Module 2

Common Technical Document – Not Applicable

Module 3
Quality Data– Not Applicable