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

By

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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

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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.

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

   - 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


- Caligiuri: intellectual inception of idea, oxylipins extraction and analysis, enzyme activity assay, statistical analysis, manuscript preparation
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- Ravandi: intellectual input on direction of study and manuscript editing
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- Caligiuri: intellectual inception of idea, oxylipins extraction and analysis, statistical analysis, manuscript preparation
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- Pierce: study design, intellectual input on analyses, manuscript editing


- Caligiuri: intellectual input, data/statistical analysis, figure and table creation, manuscript preparation
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- 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


• 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


• 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
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<th>Definition</th>
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</thead>
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<tr>
<td>6kPGF$_{1α}$</td>
<td>6-keto prostaglandin F$_{1α}$</td>
</tr>
<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid</td>
</tr>
<tr>
<td>ARA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AI</td>
<td>augmentation index</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BP</td>
<td>brachial blood pressure</td>
</tr>
<tr>
<td>cBP</td>
<td>Central blood pressure</td>
</tr>
<tr>
<td>cDBP</td>
<td>central diastolic blood pressure</td>
</tr>
<tr>
<td>cSBP</td>
<td>central systolic blood pressure</td>
</tr>
<tr>
<td>cXBP</td>
<td>central mean blood pressure</td>
</tr>
<tr>
<td>CVA</td>
<td>cerebrovascular accident</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DASH</td>
<td>dietary approaches to stop hypertension</td>
</tr>
<tr>
<td>DBP</td>
<td>brachial diastolic blood pressure</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexanoic acid</td>
</tr>
<tr>
<td>DGLA</td>
<td>dihomo gamma linolenic acid</td>
</tr>
<tr>
<td>DiHDP A</td>
<td>dihydroxydocosapentanoic acid</td>
</tr>
<tr>
<td>DHET</td>
<td>dihydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>DiHOME</td>
<td>dihydroxyoctadecenoic acid</td>
</tr>
<tr>
<td>END</td>
<td>enterodiol</td>
</tr>
<tr>
<td>ENL</td>
<td>enterolactone</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentanoic acid</td>
</tr>
<tr>
<td>EpOME</td>
<td>epoxyoctadecenoic acid</td>
</tr>
<tr>
<td>HDOHE</td>
<td>hydroxydocosahexanoic acid</td>
</tr>
<tr>
<td>HEPE</td>
<td>hydroxyeicosapentanoic acid</td>
</tr>
<tr>
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</tr>
<tr>
<td>HETrE</td>
<td>hydroxyeicosatrienoic acid</td>
</tr>
<tr>
<td>HHTrE</td>
<td>hydroxyheptadecatrienoic acid</td>
</tr>
<tr>
<td>HODE</td>
<td>hydroxyoctadecadienoic acid</td>
</tr>
<tr>
<td>HOTrE</td>
<td>hydroxyoctadecatrienoic acid</td>
</tr>
<tr>
<td>HYPERFlax</td>
<td>anti-HYPERtensive effects of dietary Flaxseed</td>
</tr>
<tr>
<td>LA</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
</tbody>
</table>
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 auscultatory measurement of $\geq 140$ mmHg and/or $\geq 90$ mmHg, for systolic and diastolic blood pressure, respectively. Using an oscillimetric automated device, high blood pressure is defined as $\geq 135$ mmHg and/or $\geq 85$ 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 auscultatory 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 $\geq 180/110$ mmHg. A hypertensive emergency is present when blood pressure is $\geq 180/110$ 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

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

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 auscultatory 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, antidepressants, 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 99th 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 abnormality, 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 intracranial 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


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


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>
<thead>
<tr>
<th>Dietary Intervention</th>
<th>Study Type</th>
<th>Participant Characteristics</th>
<th>Intervention</th>
<th>Duration</th>
<th>Results</th>
<th>Potential Adverse Effects</th>
<th>Level of Evidence</th>
<th>Class of Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food Derived Bioactive Peptides (7)</td>
<td>Meta-analysis of RCTs</td>
<td>n=15 trials, 826 participants Mixed normotensive and hypertensive individuals. Most were &gt;50 years old with more males than females.</td>
<td>100-400 mL of fermented milk or hydrolyzed protein powder that contained 2.6 mg – 1500 mg of bioactive peptides.</td>
<td>Average: 7.4 weeks Range: 4-12 weeks</td>
<td>SBP: -5.3 (-7.1, -3.1) DBP: -2.4 (-3.8, -1.0)</td>
<td>No observed adverse effects</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Garlic (8)</td>
<td>Meta-analysis of RCTs</td>
<td>n=11 studies, 565 patients Normotensive and hypertensive participants</td>
<td>Garlic powder, aged garlic extract, or distilled garlic oil Dose: 12.3-2400 mg /day</td>
<td>12-23 weeks</td>
<td>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</td>
<td>Garlic odour</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>DASH Diet with and without sodium reduction (9)</td>
<td>RCT - crossover</td>
<td>n= 412 average age = 48 years SBP:135 mmHg (120-159) DBP: 86 mm Hg (80-95)</td>
<td>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)</td>
<td>30 days/diet</td>
<td>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:</td>
<td>No adverse effects observed</td>
<td>A</td>
<td>I</td>
</tr>
</tbody>
</table>
Dietary Sodium Reduction (10).

| 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 | 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) | Increase in renin-angiotensin activity | A | I |

Fibre (11).

| 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)

<p>| 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 |</p>
<table>
<thead>
<tr>
<th>Diet</th>
<th>Meta-analysis of RCTs</th>
<th>N=164 adults with pre-hypertension or stage 1 hypertension</th>
<th>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</th>
<th>B</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa Powder OR Black or Green Tea (13)</td>
<td>1 trial – average baseline blood pressure was hypertensive</td>
<td>Or Flaxseed lignans (360-600 mg secoisolariciresinol diglucoside). Lignans: SBP: 0.28 (-3.5, 4.0) DBP: -1.8 (-4.3, 0.72) SBP: -4.6 (-11.9, 2.6) DBP: -4.1 (-6.8, -1.4)</td>
<td>No observed adverse effects</td>
<td>B</td>
<td>Cocoa – I Tea- III no effect</td>
</tr>
<tr>
<td>Diet rich in protein or monounsaturated fatty acids (14).</td>
<td>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</td>
<td>Cocoa: average 2 weeks Tea: average 4 weeks</td>
<td>No observed adverse effects</td>
<td>B</td>
<td>I</td>
</tr>
<tr>
<td>Soy Nuts (15).</td>
<td>Randomized Crossover Trial</td>
<td>N=60 Post-menopausal women (n=12 hypertensive)</td>
<td>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.</td>
<td>8 weeks</td>
<td>Normotensive: 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)</td>
</tr>
<tr>
<td>Mediterranean Diet (16).</td>
<td>Meta-analysis of prospective, cross-sectional, and RCTs</td>
<td>Studies that included blood pressure measurement: N=14 RCTs, 3,060 subjects</td>
<td>A diet that is rich in monounsaturated fatty acids, primarily from olive oil, frequent consumption of fruits, vegetables, whole grains, and low-fat dairy products. Less frequent</td>
<td>4 weeks – 5 years</td>
<td>SBP: -2.35 (-3.51, -1.18) DBP: -1.58 (-2.02, -1.13)</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Intervention</th>
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<tbody>
<tr>
<td>Long chain omega 3s Eicosapentanoic and Docosahexanoic acid (41)</td>
</tr>
<tr>
<td>Vitamin C (42)</td>
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<tr>
<td>Potassium (43)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Participant Characteristics</th>
<th>Intervention</th>
<th>Duration</th>
<th>Results</th>
<th>Potential Adverse Effects</th>
<th>Level of Evidence</th>
<th>Class of Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random Effects Meta-analysis of RCTs</td>
<td>n=70 RCTs with 93 data points (4489 participants)</td>
<td>Capsules (marine oil or ethyl esters) or food sources of EPA/DHA</td>
<td>Dose of EPA+DHA: 0.1 – 15.0 g/day</td>
<td>21 days – 365 days</td>
<td>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)</td>
<td>Fish aftertaste Belching Increased bleeding time with higher doses</td>
<td>A</td>
</tr>
<tr>
<td>Meta-analysis of RCTs</td>
<td>N=29 trials, 1407 participants Average age range: 22-74 years Normotensive and hypertensive participants</td>
<td>Supplement of 60-4000 mg/day Median dose: 500 mg/day</td>
<td>2-26 weeks Median: 8 weeks</td>
<td>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)</td>
<td>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.</td>
<td>A</td>
<td>IIA</td>
</tr>
<tr>
<td>Meta-analyses of RCTs</td>
<td>33 RCT trials n=2609 Participants were either normotensive or hypertensive</td>
<td>Intervention: 48-200 mmol/day Median dose: 75 mmol/day (2.9 g/day) from supplements or diet</td>
<td>4 days – 3 years, median: 5 weeks</td>
<td>Total Participants: SBP: -3.11 (-1.91, -4.31) DBP: -1.97 (-0.52, -3.42)</td>
<td>Rare cases included: flatulence, upset stomach, or diarrhea.</td>
<td>A</td>
<td>IIA</td>
</tr>
<tr>
<td>Supplement</td>
<td>Meta-analysis</td>
<td>n</td>
<td>Participants</td>
<td>Baseline</td>
<td>BP Effect</td>
<td>Side Effects</td>
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<tr>
<td>Coenzyme Q10</td>
<td>Meta-analysis of 12 trials (3 RCTs 1 open label, 8 crossover)</td>
<td>RCT: n=120 Crossover: n=18 Open-label: n=214</td>
<td>RCT: 100-120 mg/day Crossover: 100 mg/day Open-label: 30-225 mg/day</td>
<td>RCT: 8-12 weeks Crossover: 10 weeks Open-label: 1-56 weeks</td>
<td>Very rare mild side effects such as nausea, upset stomach, or diarrhea</td>
<td>B II A</td>
<td></td>
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<tr>
<td>Calcium</td>
<td>Meta-analysis including RCTs</td>
<td>n= 13 trials, 485 participants Average age = 45 years (16-86 years) SBP ≥140 DBP ≥85 Average BP: 148/91. Essential hypertension</td>
<td>Oral calcium (citrate, lactogluconate, carbonate, or oyster shell electrolyte) supplementatio n Mean dose 1.1 g/day (0.4 – 2.0 g/day)</td>
<td>8-15 weeks</td>
<td>Higher Baseline SBP &gt;145: SBP: -2.5 (-4.5, -0.6) DBP: -0.8 (-2.1, 0.4) No observed side effects below the daily tolerable upper intake level of 2.5 g/day for &gt; 1 year of age.</td>
<td>B II B</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>Meta-analysis including</td>
<td>n=12 trials, 544 participants</td>
<td>Oral magnesium (oxide, pidolate,</td>
<td>8-26 weeks</td>
<td>SBP: -1.3 (-4.0, 1.5) DBP: -2.2 (-3.4, -0.9) No observed side effects below the tolerable upper</td>
<td>B II B</td>
<td></td>
</tr>
<tr>
<td>RCTs</td>
<td>SBP ≥140 DBP ≥85 Average BP: 148/91. Essential HTN lactate, citrate, aspartate, hydrochloride) Mean dose: 17 mmol (10-40 mmol/day).</td>
<td>SBP &gt;150 mmHg: SBP: -2.22 (-9.04, 4.61) DBP: -2.93 (-6.19, 0.34)</td>
<td>intake level of 350 mg/day (14.4 mmol) for &gt; 9 years of age</td>
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</table>
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>
<thead>
<tr>
<th>Lifestyle Modification</th>
<th>Study Type</th>
<th>Participant Characteristics</th>
<th>Intervention</th>
<th>Duration</th>
<th>Results (mmHg)</th>
<th>Potential Adverse Effects</th>
<th>Level of Evidence</th>
<th>Class of Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Loss (59).</td>
<td>Meta-analysis of RCTs</td>
<td>n=25 trials, 4874 participants Mean initial body weight: 88.3 kg Mean initial BMI: 30.7 kg/m²</td>
<td>Weight loss: diet, physical activity or a combination of both</td>
<td>Mean total duration: 67 weeks</td>
<td>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</td>
<td>No observed adverse effects</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Aerobic Exercise (60).</td>
<td>Meta-analysis of RCTs</td>
<td>n=54 trials, 2419 participants</td>
<td>Supervised or unsupervised aerobic activity</td>
<td>3 weeks-2 years Median: 12 weeks</td>
<td>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)</td>
<td>Must be approved by physician</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Reduction of Alcohol Consumption (61).</td>
<td>Meta-analysis of RCTs</td>
<td>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</td>
<td>Reduction of alcohol consumption (average: 67% reduction in alcohol consumption)</td>
<td>Average: 8 weeks (1-104 weeks)</td>
<td>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)</td>
<td>No observed adverse effects</td>
<td>B</td>
<td>I</td>
</tr>
<tr>
<td>Stress Reduction (62).</td>
<td>Meta-analysis of RCTs</td>
<td>n=23 trials, 960 participants Mixed pre-hypertensive and hypertensive</td>
<td>Various stress reduction techniques including: Biofeedback, progressive muscle relaxation,</td>
<td>≥8 weeks</td>
<td>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)</td>
<td>No observed adverse effects</td>
<td>A</td>
<td>I for Transcendental Meditation Programs IIB for biofeedback, progressive</td>
</tr>
<tr>
<td>Intervention</td>
<td>Study Design</td>
<td>Sample Size</td>
<td>Intervention Details</td>
<td>Blood Pressure Change</td>
<td>Side Effects</td>
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<tr>
<td>Nasal continuous positive airway pressure (nCPAP)</td>
<td>RCT</td>
<td>N=118</td>
<td>Effective nocturnal nCPAP Control: sub-therapeutic nocturnal nCPAP</td>
<td>SBP: -2.3 (-5.0, 0.50) DBP: -1.3 (-5.4, 2.7)</td>
<td>Rhinitis, nasal congestion, claustrophobia</td>
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<tr>
<td>Resistance Training</td>
<td>Meta-analysis of RCTs</td>
<td>N=9 trials, 351 participants</td>
<td>Resistance training (static, circuit, or conventional training)</td>
<td>SBP: -5.0 (-7.6, -2.3) DBP: -2.8 (-5.0, -0.5)</td>
<td>Must be approved by a physician.</td>
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<tr>
<td>Cessation of Cigarette Smoking</td>
<td>Observational</td>
<td>N=8170</td>
<td>Followed 8170 and compared blood pressure outcomes in those who quit smoking or continued to</td>
<td>SBP: +3.8 (3.5-4.0) DBP: +2.9 (2.7-3.1)</td>
<td>Increased risk of hypertension over time</td>
<td></td>
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</tr>
</tbody>
</table>
| 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>
<thead>
<tr>
<th>Pharmaceutical</th>
<th>Study Type</th>
<th>Participant Characteristics *</th>
<th>Intervention</th>
<th>Duration</th>
<th>Results (mmHg)</th>
<th>Potential Adverse Effects</th>
<th>Level of Evidence</th>
<th>Class of Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiazides (73).</td>
<td>Meta-analysis of RCT</td>
<td>n=7138 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)</td>
<td>Standard Dose</td>
<td>24 hours</td>
<td>SBP: -8.8 (-8.3,-9.4)</td>
<td>Dizziness, impotence, nausea, muscle cramps</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>β-blockers (73).</td>
<td>Meta-analysis of RCT</td>
<td>n=7890 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)</td>
<td>Standard Dose</td>
<td>24 hours</td>
<td>SBP: -9.2 (-8.6,-9.2)</td>
<td>Feeling cold, fatigue, nausea</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Angiotensin Converting Enzyme Inhibitor (73).</td>
<td>Meta-analysis of RCT</td>
<td>n=14062 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)</td>
<td>Standard Dose</td>
<td>24 hours</td>
<td>SBP: -8.5 (-7.9,-9.0)</td>
<td>Cough</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Angiotensin II Receptor Blocker (73).</td>
<td>Meta-analysis of RCT</td>
<td>n=17940 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)</td>
<td>Standard Dose</td>
<td>24 hours</td>
<td>SBP: -10.3 (-9.9,-10.8)</td>
<td>No reported adverse effect</td>
<td>A</td>
<td>I</td>
</tr>
<tr>
<td>Calcium Channel Blocker (73).</td>
<td>Meta-analysis of RCT</td>
<td>n=11974 SBP: 154 mmHg (139-170) DBP: 97 mmHg (87-106)</td>
<td>Standard Dose</td>
<td>24 hours</td>
<td>SBP: -8.8 (-8.3,-9.2)</td>
<td>Flushing, ankle edema, dizziness</td>
<td>A</td>
<td>I</td>
</tr>
</tbody>
</table>
*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


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Author Contributions
This review was written by SPB Caligiuri and GN Pierce.

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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.

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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 - entero lactone, 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, lariiresinol 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 spontaneously 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).

![Percentage of Population with Controlled Blood Pressure at Different Time Points](image1)

![Percentage of Population with Stage 1 Uncontrolled Hypertension by Different Time Points](image2)
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

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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>
<thead>
<tr>
<th>Oxylipin</th>
<th>Oxylipin full name</th>
<th>Physiological/Pathophysiological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-HODE</td>
<td>9-hydroxyoctadecadienoic acid</td>
<td>Produced via the lipoxygenase pathway.</td>
</tr>
<tr>
<td>13-HODE</td>
<td>13-hydroxyoctadecadienoic acid</td>
<td>HODEs are present in human monocyte-oxidized LDL (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HODEs activate peroxisome proliferator –activated receptor γ (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>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)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HODEs are associated with oxidative stress (4)</td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>9,10-dihydroxydecenoic acid 12,13-dihydroxydecenoic acid</td>
<td>The DiHOMEs (leukotoxin diols) are produced by the metabolism of the EpOMEs (leukotoxins) by soluble epoxide hydrolase.</td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td></td>
<td>The DiHOMEs are associated with cytotoxicity and oxidative stress (5, 6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>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).</td>
</tr>
<tr>
<td>13-oxoODE</td>
<td>13-oxooctadecadienoic acid</td>
<td>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)</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>9,10,13-trihydroxyoctadecenoic acid</td>
<td>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)</td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>9,12,13-trihydroxyoctadecenoic acid</td>
<td></td>
</tr>
<tr>
<td><strong>Dihomo Gamma Linolenic Acid</strong></td>
<td>15-HETrE</td>
<td>15-hydroxyeicosatrienoic acid</td>
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</tbody>
</table>

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<thead>
<tr>
<th><strong>Alpha-Linolenic Acid</strong></th>
<th>9-HOTrE</th>
<th>9-hydroxyoctadecatrienoic acid</th>
<th>13-hydroxyoctadecatrienoic acid</th>
<th>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)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13-HOTrE</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Arachidonic Acid</strong></th>
<th>5,6-DiHETrE</th>
<th>5,6-dihydroxyeicosatrienoic acid</th>
<th>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)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8,9-DiHETrE</td>
<td>8,9-dihydroxyeicosatrienoic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11,12-DiHETrE</td>
<td>11,12-dihydroxyeicosatrienoic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14,15-DiHETrE</td>
<td>14,15-dihydroxyeicosatrienoic acid</td>
<td></td>
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</tbody>
</table>

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<tr>
<th><strong>HETEs</strong></th>
<th>5-HETE</th>
<th>5-hydroxyeicosatetraenoic acid</th>
<th>HETEs are produced via the lipoxygenase and epoxygenase pathways. HETEs are chemotactic for neutrophils (18, 19).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8-HETE</td>
<td>8-hydroxyeicosatetraenoic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-HETE</td>
<td>11-hydroxyeicosatetraenoic acid</td>
<td></td>
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<tr>
<td></td>
<td>12-HETE</td>
<td>12-hydroxyeicosatetraenoic acid</td>
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<tr>
<td></td>
<td>15-HETE</td>
<td>15-hydroxyeicosatetraenoic acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16-HETE</td>
<td>16-hydroxyeicosatetraenoic acid</td>
<td>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 thromboembolic stroke in New Zealand white rabbits (21).</td>
</tr>
<tr>
<td>18-HETE</td>
<td>18-hydroxyeicosatetraenoic acid</td>
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<td></td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>PGD&lt;sub&gt;2&lt;/sub&gt; is produced via the cyclooxygenase pathway and can inhibit platelet aggregation; however at higher concentrations can be pro-aggregatory. PGD&lt;sub&gt;2&lt;/sub&gt; can increase cyclic adenosine monophosphate levels. Prostaglandin D&lt;sub&gt;2&lt;/sub&gt; either has no effect on vascular tone or has constrict and dilate mesenteric arteries in dogs (22). PGD&lt;sub&gt;2&lt;/sub&gt; is implicated in the innate immune response (23). However, an analog of PGD&lt;sub&gt;2&lt;/sub&gt; inhibited polymorphonuclear neutrophil recruitment in vitro (24).</td>
<td></td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; is produced via the cyclooxygenase pathway and can induce renal vascular resistance in rats (25). PGE&lt;sub&gt;2&lt;/sub&gt; can either be pro-inflammatory and vasoconstrictive or anti-inflammatory and vasodilatative depending upon which receptor it is bound, EP&lt;sub&gt;1-4&lt;/sub&gt; (26).</td>
<td></td>
</tr>
<tr>
<td>6ketoPGF&lt;sub&gt;1α&lt;/sub&gt;</td>
<td>6keto prostaglandin F&lt;sub&gt;1α&lt;/sub&gt;</td>
<td>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).</td>
<td></td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>Prostaglandin F&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt; is produced via the cyclooxygenase pathway and induces vasoconstriction in bovine, canine, and human coronary arteries (30)</td>
<td></td>
</tr>
<tr>
<td>TXB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Thromboxane B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>TXB&lt;sub&gt;2&lt;/sub&gt; is produced via the cyclooxygenase pathway and is the stable product of the short-lived TXA&lt;sub&gt;2&lt;/sub&gt;. Therefore, TXB&lt;sub&gt;2&lt;/sub&gt; is considered the marker for TXA&lt;sub&gt;2&lt;/sub&gt;. TXA&lt;sub&gt;2&lt;/sub&gt; is an endothelial derived eicosanoid that potently induces vasoconstriction and aggregation of platelets (27).</td>
<td></td>
</tr>
</tbody>
</table>

**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 | 12-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). |
| 18-HEPE | 18-hydroxyeicosapentanoic acid |

**Docosahexanoic Acid**

| 4-HDOHE | 4-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). |
| 7-HDOHE | 7-hydroxydocosahexanoic acid |
| 10-HDOHE | 10-hydroxydocosahexanoic acid |
| 11-HDOHE | 11-hydroxydocosahexanoic acid |
| 13-HDOHE | 13-hydroxydocosahexanoic acid |
| 14-HDOHE | 14-hydroxydocosahexanoic acid |
| 16-HDOHE | 16-hydroxydocosahexanoic acid |
| 17-HDOHE | 17-hydroxydocosahexanoic acid |
| 20-HDOHE | 20-hydroxydocosahexanoic acid |
| 19,20-DiHDP | 19,20-dihydroxydocosapentanoic acid | 19,20-DiDHPA is produced from docosahexanoic acid likely via the epoxygenase pathway. 19,20-DiDHPA 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\textsuperscript{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 (DiHETE) indicates the presence of two hydroxyl groups, one at the 5\textsuperscript{th} and one at the 6\textsuperscript{th} carbon. DiHETE 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 H₂O 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\textsubscript{2} to thromboxane A\textsubscript{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\textsubscript{2} to prostaglandin I\textsubscript{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\textsubscript{2} versus normal subjects. Those with variant angina exhibit markedly elevated levels of TXB\textsubscript{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\textsubscript{2} and PGF\textsubscript{2a} (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\textsuperscript{2+}-activated K\textsuperscript{+} 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 erythrocyte 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 assess 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


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


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Abbreviations: ALA - alpha-linolenic acid, ARA - arachidonic acid, DGLA - Dihomo-\(\gamma\)-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, HET\textsubscript{r}E - hydroxyeicosatrienoic acid, HHT\textsubscript{r}E - hydroxyheptadecatrienoic acid, HODE - hydroxyoctadecadienoic acid, HOT\textsubscript{r}E - hydroxyoctadecatrienoic acid, LA - linoleic acid, oxoODE - oxooctadecadienoic acid, oxo\textsubscript{O}Tr\textsubscript{r}E - 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>
<thead>
<tr>
<th>Oxylipin</th>
<th>CID Mass Transition</th>
<th>Analyte Retention Time</th>
<th>Internal Standard</th>
<th>Detector Response Factor</th>
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**EPA Derived Oxylinps**

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**DHA Derived Oxylinps**

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<td>14-HDoHE</td>
<td>343.0-161.0</td>
<td>15.3</td>
<td>15-HETE-d_{8}</td>
<td>1.06</td>
</tr>
<tr>
<td>16-HDoHE</td>
<td>319.0-219.0</td>
<td>15.0</td>
<td>15-HETE-d_{8}</td>
<td>3.13</td>
</tr>
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<td>17-HDoHE</td>
<td>343.0-245.2</td>
<td>15.3</td>
<td>15-HETE-d_{8}</td>
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<tr>
<td>20-HDoHE</td>
<td>343.0-240.9</td>
<td>14.8</td>
<td>15-HETE-d_{8}</td>
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<td>19,20-DiHDPA</td>
<td>361.0-229.0</td>
<td>13.3</td>
<td>14,15-DHET-d_{11}</td>
<td>0.21</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Oxylipin (nM)</th>
<th>Younger Week 0</th>
<th>Younger Week 4</th>
<th>Older Week 0</th>
<th>Older Week 4</th>
</tr>
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<tbody>
<tr>
<td>LA Derived Oxylipins</td>
<td></td>
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<tr>
<td>9-HODE</td>
<td>11.6 ± 1.6</td>
<td>9.6 ± 1.1</td>
<td>9.4 ± 1.8</td>
<td>6.4 ± 0.62</td>
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<tr>
<td>13-HODE</td>
<td>9.4 ± 0.73</td>
<td>7.9 ± 0.82</td>
<td>10 ± 1.3</td>
<td>7.6 ± 0.76</td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>4.5 ± 1.1</td>
<td>2.7 ± 0.37</td>
<td>2.4 ± 0.34</td>
<td>2.2 ± 0.28</td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td>2.7 ± 0.25</td>
<td>3.4 ± 0.51</td>
<td>3.0 ± 0.44</td>
<td>2.6 ± 0.29</td>
</tr>
<tr>
<td>9-OXOoDE</td>
<td>2.9 ± 1.6</td>
<td>2.1 ± 1.1</td>
<td>4.6 ± 1.7</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>13-OXOoDE</td>
<td>5.7 ± 2.2</td>
<td>4.0 ± 1.0</td>
<td>4.8 ± 1.1</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>3.5 ± 0.33</td>
<td>2.9 ± 0.25</td>
<td>5.6 ± 0.84</td>
<td>3.2 ± 1.4</td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>3.0 ± 0.24</td>
<td>3.0 ± 0.69</td>
<td>4.5 ± 0.58</td>
<td>2.7 ± 0.31</td>
</tr>
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<td>DGLA Derived Oxylipins</td>
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<tr>
<td>15-HETrE</td>
<td>0.31 ± 0.10</td>
<td>0.31 ± 0.10</td>
<td>0.38 ± 0.14</td>
<td>0.17 ± 0.04</td>
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<tr>
<td>ALA Derived Oxylipins</td>
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<tr>
<td></td>
<td>1.2 ± 0.31</td>
<td>1.2 ± 0.13</td>
<td>0.87 ± 0.27</td>
<td>0.69 ± 0.34</td>
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<tr>
<td><strong>ARA Derived Oxylipins</strong></td>
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<tr>
<td>6kPGF&lt;sub&gt;1α&lt;/sub&gt;</td>
<td>1.1 ± 0.10</td>
<td>0.85 ± 0.13</td>
<td>1.0 ± 0.092</td>
<td>1.1 ± 0.071</td>
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<tr>
<td>12-HHTrE</td>
<td>0.59 ± 0.28</td>
<td>0.0 ± 0.0</td>
<td>0.30 ± 0.14</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>5,6-DHET</td>
<td>0.14 ± 0.032</td>
<td>0.20 ± 0.031</td>
<td>0.19 ± 0.025</td>
<td>0.17 ± 0.030</td>
</tr>
<tr>
<td>8,9-DHET</td>
<td>0.24 ± 0.052</td>
<td>0.20 ± 0.067</td>
<td>0.26 ± 0.036</td>
<td>0.24 ± 0.049</td>
</tr>
<tr>
<td>11,12-DHET</td>
<td>0.43 ± 0.045</td>
<td>0.48 ± 0.067</td>
<td>0.52 ± 0.054</td>
<td>0.42 ± 0.044</td>
</tr>
<tr>
<td>14,15-DHET</td>
<td>0.51 ± 0.042</td>
<td>0.52 ± 0.058</td>
<td>0.54 ± 0.05</td>
<td>0.42 ± 0.036</td>
</tr>
<tr>
<td>5-HETE</td>
<td>0.34 ± 0.12</td>
<td>0.37 ± 0.18</td>
<td>1.1 ± 0.23</td>
<td>0.39 ± 0.17</td>
</tr>
<tr>
<td>8-HETE</td>
<td>0.18 ± 0.10</td>
<td>0.26 ± 0.19</td>
<td>0.38 ± 0.12</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>9-HETE</td>
<td>0.27 ± 0.27</td>
<td>0.086 ± 0.086</td>
<td>0.78 ± 0.30</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td>11-HETE</td>
<td>0.69 ± 0.40</td>
<td>0.85 ± 0.48</td>
<td>0.95 ± 0.19</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td>12-HETE</td>
<td>0.77 ± 0.34</td>
<td>0.45 ± 0.12</td>
<td>1.0 ± 0.20</td>
<td>0.56 ± 0.12</td>
</tr>
<tr>
<td>15-HETE</td>
<td>0.68 ± 0.12</td>
<td>0.75 ± 0.14</td>
<td>0.91 ± 0.22</td>
<td>0.57 ± 0.15</td>
</tr>
<tr>
<td>16-HETE</td>
<td>0.18 ± 0.10</td>
<td>0.18 ± 0.09</td>
<td>0.20 ± 0.065</td>
<td>0.19 ± 0.081</td>
</tr>
<tr>
<td></td>
<td>18-HETE</td>
<td>20-HETE</td>
<td>EP Derived Oxylipins</td>
<td>DHA Derived Oxylipins</td>
</tr>
<tr>
<td>----------------</td>
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</tr>
<tr>
<td>5-HEPE</td>
<td>0.24 ± 0.14</td>
<td>0.39 ± 0.14</td>
<td>1.0 ± 0.34</td>
<td>0.56 ± 0.18</td>
</tr>
<tr>
<td>9-HEPE</td>
<td>0.047 ± 0.047</td>
<td>0.0 ± 0.0</td>
<td>0.82 ± 0.31</td>
<td>0.49 ± 0.29</td>
</tr>
<tr>
<td>12-HEPE</td>
<td>0.056 ± 0.056</td>
<td>0.16 ± 0.08</td>
<td>0.32 ± 0.12</td>
<td>0.12 ± 0.062</td>
</tr>
<tr>
<td>4-HDOHE</td>
<td>0.29 ± 0.079</td>
<td>0.25 ± 0.076</td>
<td>0.56 ± 0.18</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>7-HDOHE</td>
<td>0.051 ± 0.027</td>
<td>0.057 ± 0.029</td>
<td>0.40 ± 0.18</td>
<td>0.10 ± 0.069</td>
</tr>
<tr>
<td>10-HDOHE</td>
<td>0.28 ± 0.10</td>
<td>0.14 ± 0.06</td>
<td>0.53 ± 0.24</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>11-HDOHE</td>
<td>0.039 ± 0.026</td>
<td>0.086 ± 0.044</td>
<td>0.57 ± 0.22</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>13-HDOHE</td>
<td>0.15 ± 0.051</td>
<td>0.18 ± 0.046</td>
<td>0.49 ± 0.14</td>
<td>0.11 ± 0.063</td>
</tr>
<tr>
<td>14-HDOHE</td>
<td>0.039 ± 0.17</td>
<td>0.18 ± 0.065</td>
<td>0.57 ± 0.13</td>
<td>0.10 ± 0.056</td>
</tr>
<tr>
<td>16-HDOHE</td>
<td>0.21 ± 0.05</td>
<td>0.31 ± 0.08</td>
<td>0.44 ± 0.13</td>
<td>0.23 ± 0.050</td>
</tr>
<tr>
<td>17-HDOHE</td>
<td>0.91 ± 0.31</td>
<td>0.41 ± 0.22</td>
<td>0.96 ± 0.57</td>
<td>0.26 ± 0.19</td>
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<tr>
<td>20-HDOHE</td>
<td>0.22 ± 0.17</td>
<td>1.6 ± 0.66</td>
<td>1.0 ± 0.48</td>
<td>0.86 ± 0.38</td>
</tr>
<tr>
<td>19,20-DiHDPA</td>
<td>0.99 ± 0.12</td>
<td>1.3 ± 0.16</td>
<td>1.2 ± 0.14</td>
<td>1.0 ± 0.07</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM.

Abbreviations: 6-keto prostaglandin F$_{1α}$, 6kPGF$_{1α}$; alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-γ-linolenic acid, DGLA; dihydroxydocosapentanoic acid, DiHDP; 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).
**Figure 4: Heat Map of Average Individual Fold Differences Between Age Groups and Fold Changes Between Time Points in the Plasma Oxylipin Profile**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxylipin</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>Younger</th>
<th>Older</th>
<th>4/0 weeks</th>
<th>4/0 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9-HODE</td>
<td>Older/Younger</td>
<td>Older/Younger</td>
<td>4/0 weeks</td>
<td>4/0 weeks</td>
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<td></td>
<td>13-HODE</td>
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<td></td>
<td>9,10-DiHOME</td>
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<td></td>
<td>12,13-DiHOME</td>
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<td></td>
<td>9-OXOoDE</td>
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<td>9,10,13-TriHOME</td>
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<td>5,6-DiHETrE</td>
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<td>19,20-DiHDPA</td>
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<sup>a</sup> LA: Linoleic Acid; DGLA: Docosahexaenoic Acid; ALA: Alpha-Linolenic Acid; ARA: Arachidonic Acid; EPA: Eicosapentaenoic Acid; DHA: Docosahexaenoic Acid.
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; hydroxoyoctadecadienoic 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-hydroxyoctadecatrieneic 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. β Significant age*time interaction. The data were analyzed using a 2-way general linear model.
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. βSignificant age*time interaction (p<0.05). Data were analyzed using a 2-way general linear model.
A

Plasma 11-HDOHE (nM)

Younger

Older

Week 0

Week 4

B

Plasma 5-HEPE (nM)

Younger

Older

Week 0

Week 4

C

Plasma 9-HOTrE (nM)

Younger

Older

Week 0

Week 4
**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


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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.

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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 Oxytripins via an Alpha-Linolenic Acid Induced Inhibition of Soluble Epoxide Hydrolase


Stephanie P.B. Caligiuri M.Sc, Harold M. Aukema Ph.D, Amir Ravandi M.D.,Ph.D., Randy Guzman M.D., Elena Dibrov Ph.D, Grant N. Pierce Ph.D

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

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<th>Internal Standard</th>
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<td></td>
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<td>369.1&gt;168.9</td>
<td>6.57</td>
<td>TXB₂-d₄</td>
<td>1.52</td>
</tr>
</tbody>
</table>

**EPA Derived Oxylipins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>Relative Retention</th>
<th>Adduct</th>
<th>Rel. Ret.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HEPE</td>
<td>317.0&gt;115.0</td>
<td>14.9</td>
<td>5-HETE-d₈</td>
<td>0.830</td>
</tr>
<tr>
<td>12-HEPE</td>
<td>317.0&gt;179.0</td>
<td>14.5</td>
<td>5-HETE-d₈</td>
<td>1.49</td>
</tr>
<tr>
<td>18-HEPE</td>
<td>317.0&gt;215.0</td>
<td>13.8</td>
<td>15-HETE-d₈</td>
<td>0.662</td>
</tr>
</tbody>
</table>

**DHA Derived Oxylipins**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>Relative Retention</th>
<th>Adduct</th>
<th>Rel. Ret.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HDoHE</td>
<td>343.0&gt;101.1</td>
<td>16.2</td>
<td>5-HETE-d₈</td>
<td>1.40</td>
</tr>
<tr>
<td>7-HDOOHE</td>
<td>343.0&gt;140.7</td>
<td>15.6</td>
<td>5-HETE-d₈</td>
<td>1.03</td>
</tr>
<tr>
<td>10-HDOOHE</td>
<td>343.0&gt;152.7</td>
<td>15.2</td>
<td>5-HETE-d₈</td>
<td>2.41</td>
</tr>
<tr>
<td>11-HDOOHE</td>
<td>343.0&gt;149.1</td>
<td>15.5</td>
<td>5-HETE-d₈</td>
<td>1.78</td>
</tr>
<tr>
<td>13-HDOOHE</td>
<td>343.0&gt;192.9</td>
<td>15.1</td>
<td>15-HETE-d₈</td>
<td>3.10</td>
</tr>
<tr>
<td>14-HDOOHE</td>
<td>343.0&gt;161.0</td>
<td>15.3</td>
<td>15-HETE-d₈</td>
<td>1.06</td>
</tr>
<tr>
<td>16-HDOOHE</td>
<td>319.0&gt;219.0</td>
<td>15.0</td>
<td>15-HETE-d₈</td>
<td>3.13</td>
</tr>
<tr>
<td>17-HDOOHE</td>
<td>343.0&gt;245.2</td>
<td>15.3</td>
<td>15-HETE-d₈</td>
<td>0.320</td>
</tr>
<tr>
<td>20-HDOOHE</td>
<td>343.0&gt;240.9</td>
<td>14.8</td>
<td>15-HETE-d₈</td>
<td>1.12</td>
</tr>
<tr>
<td>19,20-DiHDPA</td>
<td>361.0&gt;229.0</td>
<td>13.3</td>
<td>14.15-DHET-d₁₁</td>
<td>0.210</td>
</tr>
<tr>
<td>Maresin</td>
<td>358.9&gt;172.8</td>
<td>11.6</td>
<td>LTB₄-d₄</td>
<td>0.0499</td>
</tr>
</tbody>
</table>
Abbreviations: alpha-linolenic acid, ALA; arachidonic acid, ARA; Dihomo-\(\gamma\)-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-hydroxyoctadecatrienic 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

<table>
<thead>
<tr>
<th>Oxylipin</th>
<th>Control Baseline (n=45)</th>
<th>Control 6 months (n=38)</th>
<th>Flax Baseline (n=53)</th>
<th>Flax 6 months (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic Acid Derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-HODE</td>
<td>8.49 (6.48, 10.5)</td>
<td>7.79 (6.48, 9.09)</td>
<td>8.90 (6.62, 11.2)</td>
<td>8.27 (6.70, 9.84)</td>
</tr>
<tr>
<td>13-HODE</td>
<td>9.25 (7.38, 11.1)</td>
<td>8.90 (7.72, 10.1)</td>
<td>10.6 (8.03, 13.2)</td>
<td>8.34 (7.30, 9.38)</td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>2.97 (2.44, 3.51)</td>
<td>3.29 (2.47, 4.12)</td>
<td>4.08 (2.73, 5.42)</td>
<td>2.66 (2.32, 3.01)</td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td>2.86 (2.07, 3.65)</td>
<td>3.02 (2.50, 3.53)</td>
<td>3.55 (2.63, 4.46)</td>
<td>2.52 (2.22, 2.83)</td>
</tr>
<tr>
<td>13-OXoODE</td>
<td>6.89 (4.21, 9.57)</td>
<td>5.82 (4.61, 7.03)</td>
<td>10.1 (3.51, 16.7)</td>
<td>5.57 (4.25, 6.88)</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>3.39 (0.760, 6.03)</td>
<td>2.40 (1.94, 2.85)</td>
<td>6.28 (0.209, 12.3)</td>
<td>2.37 (1.88, 2.86)</td>
</tr>
<tr>
<td>TriHOME</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>2.04 (0.933, 3.15)</td>
<td>1.63 (1.35, 1.91)</td>
<td>3.70 (0.548, 6.86)</td>
<td>1.51 (1.17, 1.84)</td>
</tr>
<tr>
<td>Alpha-Linolenic Acid Derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-HOTrE</td>
<td>0.701 (0.488, 0.914)</td>
<td>0.727 (0.541, 0.912)</td>
<td>0.862 (0.672, 1.05)</td>
<td>1.05 (0.801, 1.30)†</td>
</tr>
<tr>
<td>Dihomo Gamma Linolenic Acid Derived</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-HETrE</td>
<td>0.461 (0.373, 1.06)</td>
<td>0.405 (0.324, 0.486)</td>
<td>0.720 (0.373, 1.06)</td>
<td>0.490 (0.207, 0.773)</td>
</tr>
<tr>
<td>Arachidonic Acid Derived</td>
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<td></td>
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<tr>
<td>5,6-DHET</td>
<td>0.253 (0.215, 0.291)</td>
<td>0.279 (0.226, 0.332)</td>
<td>0.289 (0.225, 0.353)</td>
<td>0.258 (0.170, 0.347)</td>
</tr>
<tr>
<td>8,9-DHET</td>
<td>0.161 (0.128, 0.194)</td>
<td>0.164 (0.129, 0.200)</td>
<td>0.221 (0.157, 0.284)</td>
<td>0.186 (0.128, 0.243)</td>
</tr>
<tr>
<td>11,12-DHET</td>
<td>0.657 (0.582, 0.732)</td>
<td>0.697 (0.587, 0.807)</td>
<td>0.678 (0.597, 0.759)</td>
<td>0.587 (0.505, 0.668)†</td>
</tr>
<tr>
<td>14,15-DHET</td>
<td>0.783 (0.719, 0.848)</td>
<td>0.867 (0.778, 0.956)</td>
<td>0.870 (0.785, 0.954)</td>
<td>0.756 (0.670, 0.841)†‡</td>
</tr>
<tr>
<td>5-HETE</td>
<td>1.27 (0.994, 1.55)</td>
<td>1.30 (1.03, 1.58)</td>
<td>1.58 (1.03, 2.13)</td>
<td>1.46 (0.850, 2.08)</td>
</tr>
<tr>
<td>8-HETE</td>
<td>0.609 (0.443, 0.775)</td>
<td>0.580 (0.401, 0.760)</td>
<td>0.878 (0.536, 1.22)</td>
<td>0.690 (0.337, 1.04)</td>
</tr>
<tr>
<td>11-HETE</td>
<td>0.742 (0.594, 0.891)</td>
<td>0.704 (0.546, 0.863)</td>
<td>0.897 (0.522, 0.127)</td>
<td>0.787 (0.242, 1.33)</td>
</tr>
<tr>
<td>12-HETE</td>
<td>3.63 (2.46, 4.79)</td>
<td>4.64 (2.55, 6.73)</td>
<td>4.07 (2.51, 5.62)</td>
<td>4.54 (2.88, 6.20)</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1.13 (0.942, 1.32)</td>
<td>1.15 (0.915, 1.38)</td>
<td>1.22 (0.882, 1.57)</td>
<td>1.13 (0.751, 1.52)</td>
</tr>
</tbody>
</table>
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)
‡ indicates the flax group had significantly lower levels of 14,15-DHET at 6 months versus baseline (p<0.05).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Flax</th>
<th>Control</th>
<th>Flax</th>
<th>Control</th>
<th>Flax</th>
<th>Control</th>
<th>Flax</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-HETE</td>
<td>0.116 (0.0570, 0.175)</td>
<td>0.185 (0.0638, 0.306)</td>
<td>0.153 (0.0805, 0.226)</td>
<td>0.111 (0.0426, 0.179)</td>
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<tr>
<td>18-HETE</td>
<td>0.257 (0.179, 0.335)</td>
<td>0.293 (0.214, 0.373)</td>
<td>0.379 (0.194, 0.564)</td>
<td>0.278 (0.0793, 0.477)</td>
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</tr>
<tr>
<td>PGD2</td>
<td>5.32 (4.24, 6.41)</td>
<td>4.77 (3.46, 6.07)</td>
<td>8.00 (4.04, 12.0)</td>
<td>6.32 (4.49, 8.15)</td>
<td></td>
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</tr>
<tr>
<td>PGE2</td>
<td>0.364 (0.312, 0.416)</td>
<td>0.340 (0.291, 0.390)</td>
<td>0.334 (0.290, 0.379)</td>
<td>0.357 (0.310, 0.403)</td>
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<tr>
<td>6k-PGF1 alpha</td>
<td>0.714 (0.657, 0.772)</td>
<td>0.728 (0.650, 0.806)</td>
<td>0.719 (0.642, 0.795)</td>
<td>0.773 (0.666, 0.880)</td>
<td></td>
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<tr>
<td>PGF2a</td>
<td>0.288 (0.172, 0.404)</td>
<td>0.253 (0.112, 0.394)</td>
<td>0.279 (0.135, 0.424)</td>
<td>0.174 (0.0553, 0.292)</td>
<td></td>
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</tr>
<tr>
<td>11dh-TXB2</td>
<td>124 (80.2, 168)</td>
<td>109 (73.3, 145)</td>
<td>97.5 (71.6, 124)</td>
<td>109 (79.2, 138)</td>
<td></td>
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<tr>
<td>TXB2</td>
<td>0.178 (0.0533, 0.302)</td>
<td>0.0888 (0.0262, 0.152)</td>
<td>0.0961 (0.0475, 0.145)</td>
<td>0.0813 (0.0329, 0.130)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Eicosapentanoic Acid Derived**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Flax</th>
<th>Control</th>
<th>Flax</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HEPE</td>
<td>0.553 (0.318, 0.788)</td>
<td>0.423 (0.263, 0.582)</td>
<td>0.787 (0.384, 1.19)</td>
<td>0.822 (0.299, 1.35)</td>
</tr>
<tr>
<td>12-HEPE</td>
<td>0.724 (0.435, 1.01)</td>
<td>1.08 (0.451, 1.71)</td>
<td>1.04 (0.590, 1.49)</td>
<td>1.29 (0.644, 1.94)</td>
</tr>
<tr>
<td>18-HEPE</td>
<td>0.0774 (0.00357, 0.158)</td>
<td>0.149 (0.0540, 0.245)</td>
<td>0.180 (0.0375, 0.322)</td>
<td>0.225 (0.00757, 0.442)</td>
</tr>
</tbody>
</table>

**Docosahexanoic Acid Derived**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Flax</th>
<th>Control</th>
<th>Flax</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HDOHE</td>
<td>0.461 (0.263, 0.660)</td>
<td>0.460 (0.286, 0.634)</td>
<td>0.738 (0.379, 1.10)</td>
<td>0.586 (0.0173, 1.19)†</td>
</tr>
<tr>
<td>7-HDOHE</td>
<td>0.163 (0.0604, 0.265)</td>
<td>0.153 (0.0555, 0.251)</td>
<td>0.344 (0.0772, 0.611)</td>
<td>0.280 (0.0988, 0.659)</td>
</tr>
<tr>
<td>10-HDOHE</td>
<td>0.370 (0.227, 0.989)</td>
<td>0.340 (0.191, 0.490)</td>
<td>0.613 (0.236, 0.989)</td>
<td>0.526 (0.0417, 1.09)</td>
</tr>
<tr>
<td>11-HDOHE</td>
<td>0.701 (0.429, 0.974)</td>
<td>0.687 (0.463, 0.912)</td>
<td>1.05 (0.674, 1.43)</td>
<td>0.964 (0.394, 1.53)</td>
</tr>
<tr>
<td>13-HDOHE</td>
<td>0.213 (0.0560, 0.371)</td>
<td>0.175 (0.0791, 0.271)</td>
<td>0.350 (0.124, 0.575)</td>
<td>0.262 (0.0880, 0.613)</td>
</tr>
<tr>
<td>14-HDOHE</td>
<td>1.48 (0.929, 2.03)</td>
<td>2.07 (1.01, 3.14)</td>
<td>1.51 (0.900,2.12)</td>
<td>2.21 (0.979, 3.44)</td>
</tr>
<tr>
<td>16-HDOHE</td>
<td>0.275 (0.159, 0.392)</td>
<td>0.254 (0.156, 0.353)</td>
<td>0.417 (0.147, 0.687)</td>
<td>0.337 (0.00112, 0.672)</td>
</tr>
<tr>
<td>17-HDOHE</td>
<td>0.900 (0.578, 1.22)</td>
<td>0.814 (0.493, 1.14)</td>
<td>0.961 (0.381, 1.54)</td>
<td>0.816 (0.221, 1.41)</td>
</tr>
<tr>
<td>20-HDOHE</td>
<td>0.387 (0.254, 0.520)</td>
<td>0.366 (0.266, 0.466)</td>
<td>0.441 (0.191, 0.691)</td>
<td>0.310 (0.0234, 0.643)†</td>
</tr>
<tr>
<td>19,20-DiHDPA</td>
<td>1.50 (1.30, 1.69)</td>
<td>1.74 (1.53, 1.95)</td>
<td>1.54 (1.33,1.75)</td>
<td>1.45 (1.19, 1.71)†</td>
</tr>
</tbody>
</table>
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, ρ = -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).

![Graphs showing the association between flaxseed bioactives, linoleic acid, and sEH inhibition.](image)

**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

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

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-DiHETE 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

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Disclosures

The authors have no disclosures or conflicts of interest.

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Chapter II Section ii References


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 stiffness 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

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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 plasma 16-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 B2 and 5,6-dihydroxyeicosatrienoic acid (DiHETE) 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, DiHETE - 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flaxseed (n=31)</th>
<th>Control (n=31)</th>
<th>P-Value</th>
<th>P-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td>Group</td>
<td>Time</td>
<td>Group*Time</td>
</tr>
<tr>
<td><strong>Central End Systolic Pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>112 ± 10.1</td>
<td>118 ± 13.4</td>
<td>0.020</td>
<td>0.063</td>
<td>0.089</td>
</tr>
<tr>
<td>6 Months</td>
<td>110 ± 12.0</td>
<td>117 ± 14.1</td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>12 Months</td>
<td>109 ± 8.82</td>
<td>116 ± 15.4</td>
<td></td>
<td></td>
<td>0.017</td>
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<tr>
<td><strong>Central Tension Time Index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2293 ± 421</td>
<td>2499 ± 317</td>
<td>0.0094</td>
<td>0.0013</td>
<td>0.024</td>
</tr>
<tr>
<td>6 Months</td>
<td>2245 ± 410</td>
<td>2412 ± 326</td>
<td></td>
<td></td>
<td>0.065</td>
</tr>
<tr>
<td>12 Months</td>
<td>2123 ± 313</td>
<td>2380 ± 323</td>
<td></td>
<td></td>
<td>0.0050</td>
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<tr>
<td><strong>Central Diastolic Time Index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3343 ± 404</td>
<td>3410 ± 446</td>
<td>0.17</td>
<td>0.89</td>
<td>NS</td>
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<tr>
<td>6 Months</td>
<td>3279 ± 431</td>
<td>3475 ± 430</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>12 Months</td>
<td>3313 ± 417</td>
<td>3473 ± 512</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td><strong>Heart Rate</strong></td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6 Months</th>
<th>12 Months</th>
<th>6 Months</th>
<th>12 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>65.5 ± 10.0</td>
<td>71.8 ± 12.2</td>
<td>0.044</td>
<td>0.56</td>
<td>0.028</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>66.2 ± 10.9</td>
<td>69.2 ± 11.7</td>
<td></td>
<td></td>
<td>0.29</td>
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<tr>
<td><strong>12 Months</strong></td>
<td>65.7 ± 10.1</td>
<td>71.9 ± 11.9</td>
<td></td>
<td></td>
<td>0.037</td>
</tr>
<tr>
<td><strong>Peripheral Pulse Maximum dP/dT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>1079 ± 381</td>
<td>1059 ± 313</td>
<td>0.69</td>
<td>0.26</td>
<td>NS</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>1040 ± 360</td>
<td>993 ± 255</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>1047 ± 306</td>
<td>1029 ± 271</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ejection Duration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>314 ± 30.6</td>
<td>305 ± 27.3</td>
<td>0.28</td>
<td>0.00040</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>312 ± 28.6</td>
<td>308 ± 34.5</td>
<td></td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>303 ± 29.7</td>
<td>294 ± 28.9</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Central Diastolic Duration</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>625 ± 140</td>
<td>552 ± 113</td>
<td>0.035</td>
<td>0.66</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>620 ± 138</td>
<td>581 ± 114</td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>632 ± 127</td>
<td>562 ± 122</td>
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<td>0.031</td>
</tr>
<tr>
<td><strong>Central Buckberg Sub-endocardial Viability Ratio</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>151 ± 36.8</td>
<td>139 ± 22.8</td>
<td>0.16</td>
<td>0.0023</td>
<td>0.10</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th></th>
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<th>12 Months</th>
<th></th>
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<tbody>
<tr>
<td><strong>Central Pulse Period</strong></td>
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<tr>
<td>Baseline</td>
<td>939 ± 155</td>
<td>858 ± 133</td>
<td>0.038</td>
<td>0.60</td>
<td>0.029</td>
</tr>
<tr>
<td>6 Months</td>
<td>932 ± 154</td>
<td>889 ± 141</td>
<td>0.25</td>
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</tr>
<tr>
<td>12 Months</td>
<td>935 ± 145</td>
<td>856 ± 139</td>
<td>0.034</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
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</thead>
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<tr>
<td><strong>Central Ejection Duration/Period %</strong></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>34.0 ± 4.29</td>
<td>36.1 ± 3.88</td>
<td>0.073</td>
<td>0.0048</td>
<td>0.043</td>
</tr>
<tr>
<td>6 Months</td>
<td>34.1 ± 4.35</td>
<td>35.1 ± 3.52</td>
<td></td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>12 Months</td>
<td>32.8 ± 3.75</td>
<td>34.9 ± 4.42</td>
<td></td>
<td></td>
<td>0.045</td>
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</tbody>
</table>

<table>
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<th>12 Months</th>
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<td><strong>Period Ejection Duration/Period %</strong></td>
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</tr>
<tr>
<td>Baseline</td>
<td>66.0 ± 4.29</td>
<td>63.9 ± 3.88</td>
<td>0.071</td>
<td>0.0052</td>
<td>0.041</td>
</tr>
<tr>
<td>6 Months</td>
<td>65.9 ± 4.35</td>
<td>64.9 ± 3.52</td>
<td></td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>12 Months</td>
<td>67.2 ± 3.75</td>
<td>65.1 ± 4.42</td>
<td></td>
<td></td>
<td>0.044</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Flaxseed (n=31)</th>
<th>Control (n=31)</th>
<th>P-Value Group</th>
<th>P-Value Time</th>
<th>P-Value Group*Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central Augmentation/Pulse Height (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>30.8 ± 8.81</td>
<td>30.7 ± 9.90</td>
<td>0.78</td>
<td>0.00010</td>
<td>0.99</td>
</tr>
<tr>
<td>6 Months</td>
<td>28.4 ± 8.93</td>
<td>30.3 ± 9.48</td>
<td></td>
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<td>0.42</td>
</tr>
<tr>
<td>12 Months</td>
<td>26.4 ± 9.62</td>
<td>26.3 ± 9.96</td>
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<td>0.96</td>
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<tr>
<td><strong>Central Augmentation/Pulse Height Heart Rate Corrected (%)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>14.1 ± 4.17</td>
<td>17.3 ± 6.23</td>
<td>0.032</td>
<td>0.00010</td>
<td>0.030</td>
</tr>
<tr>
<td>6 Months</td>
<td>12.5 ± 5.10</td>
<td>15.1 ± 6.21</td>
<td></td>
<td></td>
<td>0.069</td>
</tr>
<tr>
<td>12 Months</td>
<td>11.2 ± 5.43</td>
<td>13.4 ± 5.75</td>
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<td></td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Augmentation Load</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.9 ± 3.99</td>
<td>15.9 ± 3.48</td>
<td>0.87</td>
<td>0.00010</td>
<td>0.78</td>
</tr>
<tr>
<td>6 Months</td>
<td>14.8 ± 4.12</td>
<td>15.4 ± 4.70</td>
<td></td>
<td></td>
<td>0.59</td>
</tr>
<tr>
<td>12 Months</td>
<td>13.3 ± 4.61</td>
<td>13.6 ± 4.67</td>
<td></td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td><strong>Augmentation Time Index</strong></td>
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<tr>
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<td>Baseline</td>
<td>6 Months</td>
<td>12 Months</td>
<td>P</td>
<td>T</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
<td>-------------</td>
<td>-------------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>136 ± 62.8</td>
<td>146 ± 52.5</td>
<td>0.51</td>
<td>0.00010</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Central Pulse Height</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>57.2 ± 14.5</td>
<td>58.8 ± 16.2</td>
<td>0.68</td>
<td>0.048</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>54.9 ± 15.0</td>
<td>56.3 ± 15.8</td>
<td></td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>53.9 ± 13.2</td>
<td>54.9 ± 14.6</td>
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<td>0.77</td>
</tr>
<tr>
<td><strong>Time of Start of Reflected Wave</strong></td>
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</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>132 ± 7.04</td>
<td>132 ± 8.80</td>
<td>0.65</td>
<td>0.0044</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>134 ± 6.76</td>
<td>133 ± 7.74</td>
<td></td>
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<td>0.63</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>135 ± 6.78</td>
<td>133.7 ± 7.74</td>
<td></td>
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<td>0.49</td>
</tr>
<tr>
<td><strong>Pulse Pressure Amplification Ratio Radial/Central</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>124 ± 10.6</td>
<td>126 ± 12.8</td>
<td>0.75</td>
<td>0.00020</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>6 Months</strong></td>
<td>128 ± 12.8</td>
<td>127 ± 14.5</td>
<td></td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td><strong>12 Months</strong></td>
<td>130 ± 13.5</td>
<td>132 ± 15.4</td>
<td></td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Central Augmented Pressure</strong></td>
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</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>17.5 ± 6.36</td>
<td>18.8 ± 8.12</td>
<td>0.39</td>
<td>0.00040</td>
<td>0.49</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th></th>
<th>Time 1</th>
<th>Time 2</th>
<th>p-value</th>
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<tbody>
<tr>
<td>6 Months</td>
<td>15.7 ± 6.43</td>
<td>18.0 ± 9.00</td>
<td>0.24</td>
</tr>
<tr>
<td>12 Months</td>
<td>14.5 ± 7.08</td>
<td>15.2 ± 8.12</td>
<td>0.71</td>
</tr>
</tbody>
</table>
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).
<table>
<thead>
<tr>
<th>Oxylipin</th>
<th>cSBP &gt;130/ &lt;130 mmHg</th>
<th>cDBP &gt;80/&lt;80 mmHg</th>
<th>cXBP &gt;100/&lt;100 mmHg</th>
</tr>
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<tbody>
<tr>
<td><strong>Linoleic Acid Oxylinins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-HODE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-HODE</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12,13-DiHOME</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>13-OxoODE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9,10,13 TriHOME</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9,12,13 TriHOME</td>
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<tr>
<td><strong>Dihomo γ-linolenic acid Oxylinins</strong></td>
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</tr>
<tr>
<td>15 HETrE</td>
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<tr>
<td><strong>Alpha-Linolenic Acid Oxylinins</strong></td>
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<tr>
<td>9-HoTrE</td>
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<tr>
<td><strong>Arachidonic Acid Oxylinins</strong></td>
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</tr>
<tr>
<td>11,12-DiHetrE</td>
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</tr>
<tr>
<td>14,15-DiHetrE</td>
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</tr>
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<td>5,6-DiHetrE</td>
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</tr>
<tr>
<td>8,9-DiHetrE</td>
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<td>5-HETE</td>
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<td>8-HETE</td>
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<td>15-HETE</td>
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<td>6-k-PGF1α</td>
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<td>7-HDoHE</td>
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<td>10-HDoHE</td>
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<td>11-HDoHE</td>
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<tr>
<td>13-HDoHE</td>
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</tbody>
</table>
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, HOTOTrE; 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) B₂, prostaglandin (PG) E₂ 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 6ketoPGF₁α (6kPGF₁α) in this population, the probability of high cBP ranged from 0 to 80%. 6kPGF₁α was the sole oxylipin identified as decreasing the odds of higher cDBP. The flax group at 6 months exhibited an increase in plasma 6kPGF₁α and a decrease in the probability of higher cDBP. In contrast, the control group exhibited a slight decrease in plasma 6kPGF₁α 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>95% Wald Confidence Interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central Systolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-HETE</td>
<td>14.1</td>
<td>(13.1, 15.0)</td>
<td>0.0169</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>26</td>
<td>(14, 39)</td>
<td>0.00280</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>33.4</td>
<td>(30.3, 36.9)</td>
<td>0.0268</td>
</tr>
<tr>
<td><strong>Central Diastolic Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-HETE</td>
<td>11.7</td>
<td>(11.0, 12.4)</td>
<td>0.0173</td>
</tr>
<tr>
<td>18-HETE</td>
<td>7.15</td>
<td>(6.78, 7.55)</td>
<td>0.0270</td>
</tr>
<tr>
<td>6kPGF$_{1α}$</td>
<td>0.0870</td>
<td>(0.0810, 0.0940)</td>
<td>0.0364</td>
</tr>
<tr>
<td><strong>Central Mean Arterial Pressure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11,12-DiHETrE</td>
<td>8.61</td>
<td>(8.14, 9.12)</td>
<td>0.0190</td>
</tr>
<tr>
<td>16-HETE</td>
<td>7.72</td>
<td>(7.24, 8.23)</td>
<td>0.0495</td>
</tr>
</tbody>
</table>
Probability of Higher Central Systolic Blood Pressure by Plasma 16-HETE Concentration
\[ \text{logit (p)} = -0.55 + (2.6 \times 16\text{-HETE}) \]

Probability of Higher Central Diastolic Blood Pressure by Plasma 16-HETE Concentration
\[ \text{logit (p)} = -0.85 + (2.5 \times 16\text{-HETE}) \]

Probability of Higher Mean Arterial Pressure by Plasma 16-HETE Concentration
\[ \text{logit (p)} = -0.34 + (2.0 \times 16\text{-HETE}) \]

Probability of Higher Central Systolic Blood Pressure by Plasma TXB$_2$ Concentration
\[ \text{logit (p)} = -0.59 + (3.5 \times \text{TXB}_2) \]

Probability of Higher Central Diastolic Blood Pressure by Plasma TXB$_2$ Concentration
\[ \text{logit (p)} = -1.1 + (2.0 \times \text{TXB}_2) \]

Probability of Higher Mean Arterial Pressure by Plasma TXB$_2$ Concentration
\[ \text{logit (p)} = -1.5 + (2.2 \times \text{TXB}_2) \]

Probability of Higher Central Systolic Blood Pressure by Plasma PGE$_2$ Concentration
\[ \text{logit (p)} = -2.0 + (4.8 \times \text{PGE}_2) \]

Probability of Higher Central Diastolic Blood Pressure by Plasma PGE$_2$ Concentration
\[ \text{logit (p)} = 1.2 - (2.4 \times \text{PGE}_2) \]

Probability of Higher Mean Arterial Pressure by Plasma PGE$_2$ Concentration
\[ \text{logit (p)} = -1.5 + (2.2 \times \text{PGE}_2) \]

Probability of Higher Central Systolic Blood Pressure by Plasma 18-HETE Concentration
\[ \text{logit (p)} = -1.1 + (2.0 \times 18\text{-HETE}) \]

Probability of Higher Central Diastolic Blood Pressure by Plasma 18-HETE Concentration
\[ \text{logit (p)} = -1.1 + (2.0 \times 18\text{-HETE}) \]

Probability of Higher Mean Arterial Pressure by Plasma 18-HETE Concentration
\[ \text{logit (p)} = -0.34 + (2.0 \times 18\text{-HETE}) \]

Probability of Higher Central Systolic Blood Pressure by Plasma 11,12-DIHETrE Concentration
\[ \text{logit (p)} = -1.5 + (2.2 \times 11,12\text{-DIHETrE}) \]

Probability of Higher Central Diastolic Blood Pressure by Plasma 11,12-DIHETrE Concentration
\[ \text{logit (p)} = -1.5 + (2.2 \times 11,12\text{-DIHETrE}) \]

Probability of Higher Mean Arterial Pressure by Plasma 11,12-DIHETrE Concentration
\[ \text{logit (p)} = -1.5 + (2.2 \times 11,12\text{-DIHETrE}) \]
**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$_{1\alpha}$, 6ketoPGF$_{1\alpha}$; dihydroxyeicosatrienoic acid, DiHETE; 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 antihypertensive 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 B₂, 6kPGF₁α, and 11,12-DiHETE to cBP was not unexpected. These oxylipins can regulate vascular tone (24-29). Previously, 11,12-DiHETE 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 B₂, 6kPGF₁α, and PGE₂ 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.
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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

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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 (DiHETe) 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), dihomo gamma linolenic acid (DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DiHETE), dihydroxyoctadecenoic acid (DiHOME), HETE (hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid, (HDOHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETE), hydroxyoctadecadienoic acid (HODE), hydroxyoctadecatrienoic acid (HOTE), 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 cerebrovascular accidents in patients living with PAD were assessed with regard to their relationship to conventional 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 99th 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 abnormality, 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. Twelve 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

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>No Angina (n=82)</th>
<th>SEM</th>
<th>Yes Angina (n=16)</th>
<th>SEM</th>
<th>No Myocardial Infarction (n=74)</th>
<th>SEM</th>
<th>Yes Myocardial Infarction (n=24)</th>
<th>SEM</th>
</tr>
</thead>
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<tr>
<td>C12:0</td>
<td>0.7</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>C14:0</td>
<td>24.7</td>
<td>1.7</td>
<td>23.2</td>
<td>4.6</td>
<td>25.3</td>
<td>2.0</td>
<td>22.1</td>
<td>2.2</td>
</tr>
<tr>
<td>C14:1</td>
<td>1.7</td>
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<td>1.5</td>
<td>0.3</td>
<td>2.0</td>
<td>0.7</td>
</tr>
<tr>
<td>C16:0</td>
<td>640.6</td>
<td>25.9</td>
<td>578.8</td>
<td>65.0</td>
<td>642.6</td>
<td>29.1</td>
<td>593.2</td>
<td>40.6</td>
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<tr>
<td>C16:1</td>
<td>59.9</td>
<td>4.2</td>
<td>56.8</td>
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<td>62.1</td>
<td>5.5</td>
<td>50.9</td>
<td>5.9</td>
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<tr>
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<td>170.7</td>
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<td>5.9</td>
<td>179.7</td>
<td>9.4</td>
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<td>21.3</td>
<td>637.2</td>
<td>52.0</td>
<td>703.3</td>
<td>22.4</td>
<td>655.7</td>
<td>41.7</td>
</tr>
<tr>
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<td>4.4</td>
<td>0.8</td>
<td>5.6</td>
<td>0.3</td>
<td>4.7</td>
<td>0.6</td>
</tr>
<tr>
<td>C20:1</td>
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<td>0.3</td>
<td>4.0</td>
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<td>3.6</td>
<td>0.3</td>
<td>3.2</td>
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<td>18.4</td>
<td>1.2</td>
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<td>3.8</td>
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<td>33.3</td>
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<td>6.6</td>
<td>194.3</td>
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<td>210.3</td>
<td>7.1</td>
<td>205.4</td>
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<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
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<td>0.1</td>
<td>0.2</td>
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<td>1.1</td>
<td>11.9</td>
<td>0.5</td>
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<td>0.8</td>
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<td>42.7</td>
<td>2.4</td>
<td>37.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

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 cerebrovascular accident (CVA)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>No TIA (n=82)</th>
<th>SEM</th>
<th>Yes TIA (n=16)</th>
<th>SEM</th>
<th>No CVA (n=88)</th>
<th>SEM</th>
<th>Yes CVA (n=10)</th>
<th>SEM</th>
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<tbody>
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<td>C12:0</td>
<td>0.7</td>
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<td>0.3</td>
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<td>1.7</td>
<td>27.0</td>
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</tr>
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<td>0.9</td>
<td>0.5</td>
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<td>0.3</td>
<td>1.7</td>
<td>0.9</td>
</tr>
<tr>
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<td>26.0</td>
<td>660.9</td>
<td>61.2</td>
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<tr>
<td>C16:1</td>
<td>60.9</td>
<td>5.1</td>
<td>51.6</td>
<td>7.9</td>
<td>58.8</td>
<td>4.8</td>
<td>64.7</td>
<td>11.8</td>
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<tr>
<td>C18:0</td>
<td>186.5</td>
<td>5.6</td>
<td>183.1</td>
<td>11.5</td>
<td>185.2</td>
<td>5.4</td>
<td>192.9</td>
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<tr>
<td>C18:1 Oleic</td>
<td>705.6</td>
<td>27.8</td>
<td>693.7</td>
<td>58.4</td>
<td>699.4</td>
<td>27.0</td>
<td>741.3</td>
<td>63.5</td>
</tr>
<tr>
<td>C18:1 Vaccenic</td>
<td>45.6</td>
<td>1.9</td>
<td>46.2</td>
<td>3.9</td>
<td>45.1</td>
<td>1.7</td>
<td>51.1</td>
<td>6.4</td>
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<tr>
<td>C18:2</td>
<td>684.2</td>
<td>21.2</td>
<td>730.2</td>
<td>54.0</td>
<td>688.1</td>
<td>21.1</td>
<td>722.7</td>
<td>58.7</td>
</tr>
<tr>
<td>C20:0</td>
<td>5.3</td>
<td>0.3</td>
<td>5.6</td>
<td>0.8</td>
<td>5.3</td>
<td>0.3</td>
<td>5.5</td>
<td>0.7</td>
</tr>
<tr>
<td>C18:3 DGLA</td>
<td>12.9</td>
<td>0.7</td>
<td>13.9</td>
<td>1.7</td>
<td>12.7</td>
<td>0.7</td>
<td>16.8</td>
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</tr>
<tr>
<td>C20:1</td>
<td>3.5</td>
<td>0.3</td>
<td>3.8</td>
<td>0.7</td>
<td>3.4</td>
<td>0.3</td>
<td>4.9</td>
<td>0.7</td>
</tr>
<tr>
<td>C18:3 ALA</td>
<td>18.3</td>
<td>1.3</td>
<td>22.4</td>
<td>2.8</td>
<td>18.9</td>
<td>1.3</td>
<td>19.2</td>
<td>2.5</td>
</tr>
<tr>
<td>C20:2</td>
<td>3.6</td>
<td>0.3</td>
<td>4.1</td>
<td>0.8</td>
<td>3.5</td>
<td>0.3</td>
<td>4.8</td>
<td>0.9</td>
</tr>
<tr>
<td>C22:0</td>
<td>15.4</td>
<td>0.7</td>
<td>15.0</td>
<td>2.0</td>
<td>15.4</td>
<td>0.7</td>
<td>14.8</td>
<td>2.2</td>
</tr>
<tr>
<td>C20:3</td>
<td>36.2</td>
<td>1.4</td>
<td>34.7</td>
<td>3.1</td>
<td>35.5</td>
<td>1.4</td>
<td>40.0</td>
<td>3.0</td>
</tr>
<tr>
<td>C20:4</td>
<td>204.9</td>
<td>6.6</td>
<td>230.7</td>
<td>14.2</td>
<td>205.5</td>
<td>6.1</td>
<td>240.6</td>
<td>24.1</td>
</tr>
<tr>
<td>C22:2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>C24:0</td>
<td>11.4</td>
<td>0.5</td>
<td>11.7</td>
<td>1.1</td>
<td>11.4</td>
<td>0.5</td>
<td>11.9</td>
<td>1.3</td>
</tr>
<tr>
<td>C20:5</td>
<td>20.0</td>
<td>1.2</td>
<td>24.0</td>
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<td>20.4</td>
<td>1.2</td>
<td>22.8</td>
<td>2.9</td>
</tr>
<tr>
<td>C24:1</td>
<td>23.7</td>
<td>0.8</td>
<td>24.9</td>
<td>2.7</td>
<td>23.9</td>
<td>0.8</td>
<td>24.3</td>
<td>3.5</td>
</tr>
<tr>
<td>C22:4</td>
<td>3.7</td>
<td>0.4</td>
<td>4.3</td>
<td>0.6</td>
<td>3.6</td>
<td>0.3</td>
<td>5.6</td>
<td>1.0</td>
</tr>
<tr>
<td>C22:5</td>
<td>13.2</td>
<td>0.6</td>
<td>14.2</td>
<td>1.2</td>
<td>13.1</td>
<td>0.6</td>
<td>15.7</td>
<td>2.0</td>
</tr>
<tr>
<td>C22:6</td>
<td>39.7</td>
<td>1.6</td>
<td>50.1</td>
<td>8.8</td>
<td>41.0</td>
<td>2.1</td>
<td>45.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Abbreviations: acute coronary syndrome (ACS), alpha-linolenic acid (ALA), cerebrovascular accident (CVA), dihomomo gamma linolenic acid (DGLA), transient ischemic attack (TIA), standard error of the mean (SEM)
Plasma Oxylin 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), cerebrovascular accident (CVA), dihydroxyeicosatrienoic acid (DiHETE), hydroxyeicosatetraenoic 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-hydroxyeicosatetraenoic acid (HETE) and prostaglandin (PG) E₂. 16-HETE and PGE₂ were 200% and 33% higher, respectively, in those with past or current angina versus those without (Table 19). Plasma 8,9-dihydroxyeicosatrienoic acid (DiHETE) and 18-hydroxyeicosapentaenoic acid (HEPE) significantly increased the odds of past myocardial infarctions (Table 18). 18-HEPE and 8,9-DiHETE 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)-PGF₁α resulted in an odds ratio less than 1 for the prevalence of TIAs, indicating a protective effect (Table 21). 6kPGF₁α was 17% lower in those who suffered from a TIA. This contributed to the ratio of TXB₂/6kPGF₁α significantly increasing the odds of a TIA by 3.8-fold for every 1 unit increase (Table 21). The ratio of TXB₂/6kPGF₁α was 200% greater in patients with a past TIA versus those with no TIA. Plasma 16-HETE and PGF₂α 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 PGF₂α 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Controls</th>
<th>Univariate OR (95% CI)</th>
<th>p-value</th>
<th>Multivariate OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Oxylipins Score</td>
<td>43</td>
<td>N/A</td>
<td>80 (71, 90)</td>
<td>0.022</td>
<td>80 (71, 90)</td>
<td>0.022</td>
</tr>
<tr>
<td>Selection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td></td>
<td></td>
<td>80 (71, 90)</td>
<td>0.022</td>
<td>80 (71, 90)</td>
<td>0.022</td>
</tr>
<tr>
<td>16-HETE</td>
<td></td>
<td></td>
<td>9.1 (8.5, 9.7)</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥65 years</td>
<td>65</td>
<td>33</td>
<td>1.8 (0.54, 6.2)</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Gender</td>
<td>25</td>
<td>73</td>
<td>0.63 (0.16, 2.4)</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking Status (current)</td>
<td>25</td>
<td>73</td>
<td>0.40 (0.083, 1.9)</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>74</td>
<td>24</td>
<td>2.2 (0.47, 11)</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>33</td>
<td>65</td>
<td>1.9 (0.62, 5.8)</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>78</td>
<td>20</td>
<td>3.9 (0.49, 32)</td>
<td>0.20</td>
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<td></td>
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<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta Blocker</td>
<td>33</td>
<td>65</td>
<td>3.1 (1.0, 9.3)</td>
<td>0.043</td>
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<td></td>
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<tr>
<td>Ca Channel Blocker</td>
<td>27</td>
<td>71</td>
<td>0.33 (0.07, 1.5)</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>47</td>
<td>51</td>
<td>1.6 (0.56, 4.8)</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARB</td>
<td>15</td>
<td>83</td>
<td>1.4 (0.33, 5.4)</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretic</td>
<td>39</td>
<td>39</td>
<td>0.89 (0.30, 2.7)</td>
<td>0.84</td>
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<tr>
<td>Aspirin</td>
<td>71</td>
<td>27</td>
<td>0.65 (0.21, 2.0)</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>70</td>
<td>28</td>
<td>2.5 (0.52, 12)</td>
<td>0.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), Hydroxyeicosatetraenoic acid (HETE), Prostaglandin (PG)
Table 18: Univariate and Multivariate Logistic Regression Models for the Risk Assessment of Myocardial Infarctions

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Controls</th>
<th>Univariate p-value</th>
<th>Multivariate p-value</th>
</tr>
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<tr>
<td></td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td></td>
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</tr>
<tr>
<td><strong>Selection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,9-DiHETrE</td>
<td>92.4 (82.0, 104)</td>
<td>92.4 (82.0, 104)</td>
<td>0.0192</td>
<td>0.0192</td>
</tr>
<tr>
<td>18-HEPE</td>
<td>4.1 (4.0, 4.3)</td>
<td></td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td><strong>Other Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥65 years</td>
<td>1.1 (0.44, 3.0)</td>
<td></td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Female Gender</td>
<td>0.71 (0.23, 2.2)</td>
<td></td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Smoking Status (current)</td>
<td>1.0 (0.35, 3.0)</td>
<td></td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>4.4 (0.96, 21)</td>
<td></td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>1.6 (0.60, 4.0)</td>
<td></td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>2.0 (0.52, 7.4)</td>
<td></td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td><strong>Medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta Blocker</td>
<td>8.8 (3.1, 25)</td>
<td></td>
<td>&lt;0.00010</td>
<td>12 (3.6, 40)</td>
</tr>
<tr>
<td>Ca Channel Blocker</td>
<td>0.44 (0.14, 1.4)</td>
<td></td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>1.6 (0.62, 3.9)</td>
<td></td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>ARB</td>
<td>0.43 (0.090, 2.0)</td>
<td></td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>Diuretic</td>
<td>1.7 (0.69, 4.4)</td>
<td></td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.62 (0.23, 1.6)</td>
<td></td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>3.1 (0.82, 11)</td>
<td></td>
<td>0.094</td>
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</tbody>
</table>

Angiotensin Converting Enzyme (ACE), Angiotensin Receptor Blocker (ARB), Calcium (Ca), (DiHETrE), hydroxyeicosapentanoic acid (HEPE), hydroxyeicosatetranoid acid (HETE), prostaglandin (PG), thromboxane (TX).
<table>
<thead>
<tr>
<th>Oxylipin</th>
<th>No Angina (n=82)</th>
<th>No ACS (n=74)</th>
<th>Yes Angina (n=16)</th>
<th>Yes ACS (n=24)</th>
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<tbody>
<tr>
<td>10-HDOHE</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.5</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>11,12-DiHETrE</td>
<td>0.6 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>11-HDOHE</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>11-HETE</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>12-HETE</td>
<td>3.8 ± 0.6</td>
<td>4.1 ± 0.9</td>
<td>4.1 ± 0.9</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td>3.4 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>3.3 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>12-HEPE</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>13-HODE</td>
<td>10.1 ± 1.0</td>
<td>9.2 ± 1.0</td>
<td>10.2 ± 1.0</td>
<td>9.3 ± 0.9</td>
</tr>
<tr>
<td>13-HDOHE</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.2 ± 0.0</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>13-OXOODE</td>
<td>9.0 ± 2.2</td>
<td>6.7 ± 0.8</td>
<td>9.3 ± 2.5</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>14,15-DiHETrE</td>
<td>0.8 ± 0.0</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>14-HDOHE</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.5</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>15-HETrE</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>16-HDOHE</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>16-HETE</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>17-HDOHE</td>
<td>0.9 ± 0.1</td>
<td>1.3 ± 0.8</td>
<td>0.7 ± 0.1</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>18-HEPE</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>18-HETE</td>
<td>0.3 ± 0.0</td>
<td>0.5 ± 0.3</td>
<td>0.3 ± 0.0</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>19,20-DiHDDA</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>20-HDOHE</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.4</td>
<td>0.3 ± 0.0</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>4-HDOHE</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>5-HETE</td>
<td>1.3 ± 0.1</td>
<td>1.9 ± 0.7</td>
<td>1.2 ± 0.1</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>5,6-DiHETrE</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>5-HEPE</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>6keto-PGF$_{1\alpha}$</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>7-HDOHE</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.4</td>
<td>0.1 ± 0.0</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>8,9-DiHETrE</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>8-HETE</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.4</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>9-HODE</td>
<td>8.8 ± 0.9</td>
<td>8.4 ± 1.2</td>
<td>8.9 ± 1.0</td>
<td>8.2 ± 0.9</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>5.5 ± 2.1</td>
<td>2.1 ± 0.3</td>
<td>5.9 ± 2.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>3.7 ± 0.4</td>
<td>3.0 ± 0.5</td>
<td>3.7 ± 0.5</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>3.2 ± 1.1</td>
<td>1.6 ± 0.3</td>
<td>3.4 ± 1.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>9-HOTrE</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Maresin</td>
<td>27.6 ± 4.4</td>
<td>20.6 ± 6.1</td>
<td>26.5 ± 4.6</td>
<td>26.3 ± 6.6</td>
</tr>
<tr>
<td>PGD$_2$</td>
<td>10.4 ± 3.7</td>
<td>4.6 ± 1.1</td>
<td>27.5 ± 17.9</td>
<td>8.6 ± 3.1</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>TXB$_2$</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Oxylin</td>
<td>No TIA (n=82)</td>
<td>SEM</td>
<td>Yes TIA (n=16)</td>
<td>SEM</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>-----</td>
<td>----------------</td>
<td>-----</td>
</tr>
<tr>
<td>10-HDOHE</td>
<td>0.5</td>
<td>0.1</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>11,12-DiHETrE</td>
<td>0.6</td>
<td>0.0</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>11-HDOHE</td>
<td>0.9</td>
<td>0.1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>11-HETE</td>
<td>0.8</td>
<td>0.1</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>12-HETE</td>
<td>3.9</td>
<td>0.6</td>
<td>3.5</td>
<td>0.8</td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td>3.3</td>
<td>0.4</td>
<td>2.7</td>
<td>0.2</td>
</tr>
<tr>
<td>12-HEPE</td>
<td>0.9</td>
<td>0.2</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>13-HODE</td>
<td>9.8</td>
<td>0.9</td>
<td>10.8</td>
<td>1.3</td>
</tr>
<tr>
<td>13-HDOHE</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>13-OXOODE</td>
<td>9.0</td>
<td>2.2</td>
<td>6.9</td>
<td>1.1</td>
</tr>
<tr>
<td>14,15-DiHETrE</td>
<td>0.8</td>
<td>0.0</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>14-HDOHE</td>
<td>1.5</td>
<td>0.2</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>15-HETrE</td>
<td>0.6</td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1.2</td>
<td>0.1</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>16-HDOHE</td>
<td>0.4</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>16-HETE</td>
<td>0.1</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>17-HDOHE</td>
<td>0.9</td>
<td>0.2</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>18-HEPE</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>18-HETE</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>19,20-DiHDPA</td>
<td>1.5</td>
<td>0.1</td>
<td>1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>20-HDOHE</td>
<td>0.4</td>
<td>0.1</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>4-HDOHE</td>
<td>0.6</td>
<td>0.1</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>5-HETE</td>
<td>1.4</td>
<td>0.2</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>5,6-DiHETrE</td>
<td>0.3</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>5-HEPE</td>
<td>0.7</td>
<td>0.1</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>6keto-PGF1α</td>
<td>0.7</td>
<td>0.0</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>7-HDOHE</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>8,9-DiHETrE</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>8-HETE</td>
<td>0.8</td>
<td>0.1</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>9-HODE</td>
<td>8.6</td>
<td>0.9</td>
<td>9.2</td>
<td>1.2</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>5.4</td>
<td>2.1</td>
<td>2.6</td>
<td>0.4</td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>3.6</td>
<td>0.4</td>
<td>3.5</td>
<td>0.6</td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>3.1</td>
<td>1.1</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>9-HETrE</td>
<td>0.8</td>
<td>0.1</td>
<td>0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Maresin</td>
<td>26.2</td>
<td>4.4</td>
<td>27.8</td>
<td>6.9</td>
</tr>
<tr>
<td>PGD2</td>
<td>26.2</td>
<td>16.1</td>
<td>5.7</td>
<td>1.0</td>
</tr>
<tr>
<td>PGE2</td>
<td>0.3</td>
<td>0.0</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>PGF2α</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>TXB2</td>
<td>0.1</td>
<td>0.0</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Abbreviations: 6 keto (6k), acute coronary syndrome (ACS), cerebrovascular accident (CVA), dihommo gamma linolenic acid (DGLA), dihydroxydocosapentanoic acid (DiHDPA), dihydroxyeicosatrienoic acid (DiHETE), dihydroxyoctadecenoic acid (DiHOME), HETE (hydroxyeicosatrienoic acid), hydroxydocosahexaenoic acid, (HDOHE), hydroxyeicosapentaenoic acid (HEPE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosatrienoic acid (HETE), hydroxyoctadecadienoic acid (HODE), hydroxyoctadecatrienoic acid (HOT), 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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Controls</th>
<th>Univariate OR (95% CI)</th>
<th>p-value</th>
<th>Multivariate OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Oxylipins Score</td>
<td>43</td>
<td>N/A</td>
<td>3.8 (3.6, 3.9)</td>
<td>0.0048</td>
<td>3.8 (3.6, 3.9)</td>
<td>0.019</td>
</tr>
<tr>
<td>TXB$<em>2$/6kPGF$</em>{1\alpha}$</td>
<td></td>
<td></td>
<td>0.066 (0.061, 0.072)</td>
<td>0.040</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6kPGF$_{1\alpha}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥65 years</td>
<td>65</td>
<td>33</td>
<td>1.8 (0.54, 6.2)</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Gender</td>
<td>25</td>
<td>73</td>
<td>0.37 (0.077, 1.7)</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking Status (current)</td>
<td>25</td>
<td>73</td>
<td>0.36 (0.076, 1.7)</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>74</td>
<td>24</td>
<td>1.3 (0.33, 5.0)</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>33</td>
<td>65</td>
<td>0.96 (0.30, 3.1)</td>
<td>0.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>78</td>
<td>20</td>
<td>0.97 (0.24, 3.8)</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta Blocker</td>
<td>33</td>
<td>65</td>
<td>3.1 (1.0, 9.3)</td>
<td>0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca Channel Blocker</td>
<td>27</td>
<td>71</td>
<td>1.7 (0.57, 5.4)</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>47</td>
<td>51</td>
<td>0.90 (0.31, 2.6)</td>
<td>0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARB</td>
<td>15</td>
<td>83</td>
<td>1.3 (0.33, 5.4)</td>
<td>0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diuretic</td>
<td>39</td>
<td>39</td>
<td>0.64 (0.20, 2.0)</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>71</td>
<td>27</td>
<td>0.47 (0.16, 1.4)</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>70</td>
<td>28</td>
<td>0.95 (0.62, 1.5)</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases</th>
<th>Controls</th>
<th>Univariate p-value</th>
<th>Multivariate p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Oxylipins Score Selection</td>
<td>43</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-HETE</td>
<td>55 (51, 60)</td>
<td>0.0016</td>
<td>55 (51, 60)</td>
<td>0.0016</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>3.3 (3.2, 3.5)</td>
<td>0.047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other Characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age $\geq$65 years</td>
<td>65</td>
<td>33</td>
<td>0.33 (0.86, 1.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>Female Gender</td>
<td>25</td>
<td>73</td>
<td>2.1 (0.55, 8.3)</td>
<td>0.28</td>
</tr>
<tr>
<td>Smoking Status (current)</td>
<td>25</td>
<td>73</td>
<td>0.81 (0.16, 4.2)</td>
<td>0.80</td>
</tr>
<tr>
<td>Hypertension</td>
<td>74</td>
<td>24</td>
<td>1.1 (0.21, 5.7)</td>
<td>0.91</td>
</tr>
<tr>
<td>Diabetes</td>
<td>33</td>
<td>65</td>
<td>0.97 (0.23, 4.1)</td>
<td>0.96</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>78</td>
<td>20</td>
<td>0.84 (0.16, 4.4)</td>
<td>0.83</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta Blocker</td>
<td>33</td>
<td>65</td>
<td>1.4 (0.36, 5.2)</td>
<td>0.66</td>
</tr>
<tr>
<td>Ca Channel Blocker</td>
<td>27</td>
<td>71</td>
<td>0.63 (0.13, 3.2)</td>
<td>0.58</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>47</td>
<td>51</td>
<td>3.1 (0.75, 13)</td>
<td>0.12</td>
</tr>
<tr>
<td>ARB</td>
<td>15</td>
<td>83</td>
<td>&lt;0.001 (&lt;0.001, &gt;999)</td>
<td>0.96</td>
</tr>
<tr>
<td>Diuretic</td>
<td>39</td>
<td>39</td>
<td>4.1 (0.99, 17)</td>
<td>0.052</td>
</tr>
<tr>
<td>Aspirin</td>
<td>71</td>
<td>27</td>
<td>0.38 (0.10, 1.4)</td>
<td>0.15</td>
</tr>
<tr>
<td>Statin</td>
<td>70</td>
<td>28</td>
<td>1.1 (0.29, 4.4)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

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, DiHETE – 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-DiHETE (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-DiHETE, 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 DiHETEs 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-DiHETE 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 cerebrovascular 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 particularly 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-DiHETE 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-DiHETE 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 difficult to ascertain if they are a cause or a consequence of the event. Because of their physiological role (i.e., 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.

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Chapter II Section iv References


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


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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 \( \geq 25 \) 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.
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 than 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

Recruitment via Advertisement or Doctor Referral

Telephone Pre-Screening

Study Overview and Eligibility Visit
1. Study Overview and Signed Consent
2. Blood Pressure Measurement and Physical Examination

Baseline Visit
1. Questionnaires and Anthropometrics
2. Blood Pressure Measurement
3. Fasting Blood Draw and Urine Collection

2 Month Follow-Up
1. Questionnaires and Anthropometrics
2. Blood Pressure Measurement
3. Fasting Blood Draw and Urine Collection
4. Assessment for Need of Anti-hypertensive Medication

Screened Out

Screened Out

4 Month Follow-Up
Same Assessments as 2 Month Follow-Up Above

6 Month Follow-Up
Same Assessments as 2 Month Follow-up Above
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 assessments, 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 molasses, 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 ability 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 BPTtru 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 stethoscope 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.

**Anthropometrics 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 warehouse. 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 easily 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


http://guidelines.hypertension.ca/


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 prevalence 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 prevalence 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.

![Pie chart showing proportion of pre-screened individuals by recruitment method]

**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 awareness 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.](image-url)
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 viewed 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


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 awareness 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 accessibility. 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 monitoring 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 \( \geq 140/90 \) 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


https://clinicaltrials.gov/ct2/resources/trends#RegisteredStudiesOverTime


Chapter IV - Appendices

Appendix 1 – Research Ethics Board Approval for the HyperFlax Clinical Trial

Appendix 2 – Informed Consent Document for the HyperFlax Clinical Trial
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

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You are being asked to participate in a clinical 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.
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 than 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 preceding 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
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

Recruitment via Advertisements or Doctor Referral

Telephone Pre-Screening

Screened Out

Study Overview and Eligibility Visit
1. Study Overview and Signed Consent
2. Blood Pressure (BPTru)
3. If Blood Pressure meets criteria:
   - Physical Examination
   - Blood Draw
   - Urine Collection
   - EKG

Baseline Visit
1. Questionnaires and Anthropometrics (weight, height, waist circumference)
2. Blood Pressure (BPTru), Fasting Blood Draw, and Urine Collection

Screened Out

2 Month Follow-Up
1. Questionnaires and Anthropometrics (weight, height, waist circumference)
2. Blood Pressure (BPTru), Fasting Blood Draw, and Urine Collection
3. Assessment for Need of Anti-Hypertensive Medication

4 Month Follow-Up
Same Assessments as 2 Month Follow-Up Above

6 Month Follow-Up
Same Assessments as 2 Month Follow-Up Above

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<th>Tests Participant Undergoes</th>
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<tr>
<td>Baseline</td>
<td>• Study overview&lt;br&gt;• Inclusion/Exclusion Criteria will be assessed&lt;br&gt;• Signed consent&lt;br&gt;• Automated brachial blood pressure&lt;br&gt;• If blood pressure meets criteria: examination to exclude macrovascular target organ damage (physical examination, EKG, blood and urine collection)&lt;br&gt;<strong>Performed on another day</strong>&lt;br&gt;• Automated brachial blood pressure&lt;br&gt;• Fasting blood drawn&lt;br&gt;• Urine collected&lt;br&gt;• 24-hour food recall and physical activity record&lt;br&gt;• Height, weight, waist circumference measured</td>
</tr>
<tr>
<td>2 months</td>
<td>• Automated brachial blood pressure&lt;br&gt;• Fasting blood drawn&lt;br&gt;• Urine collected&lt;br&gt;• 24-hour food recall and physical activity record&lt;br&gt;• Height, weight, waist circumference measured&lt;br&gt;• Assessment for need of anti-hypertensive medication</td>
</tr>
</tbody>
</table>
| 4 months   | • Automated brachial blood pressure<br>• Fasting blood drawn<br>• 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 |
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

Initial: _____
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.
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

Initial: _____
discomfort (eg: bloating, flatulence) 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.
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 will 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 participate 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
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<td>3</td>
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### 1.2 Application Information

**CLINICAL TRIAL APPLICATION AND ATTESTATION FORM**

*Natural Health Products Directorate*

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<tr>
<td>File Number</td>
<td>Submission Number</td>
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**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)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mr.</th>
<th>Ms.</th>
<th>Dr.</th>
<th>Surname* Pierce</th>
<th>Language Preferred</th>
<th>Given Name* Grant</th>
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<tbody>
<tr>
<td>Title*</td>
<td>Director of Research/Professor</td>
<td>Language Preferred</td>
<td>English</td>
<td>French</td>
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</tr>
<tr>
<td>Telephone No.*</td>
<td>204-235-3206</td>
<td>Fax No.</td>
<td>204-235-0793</td>
<td>E-mail</td>
<td><a href="mailto:gpierce@sbrc.ca">gpierce@sbrc.ca</a></td>
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#### B. Contact for this Application

- Same as A

<table>
<thead>
<tr>
<th>Name</th>
<th>Mr.</th>
<th>Ms.</th>
<th>Dr.</th>
<th>Surname* Caligiuri</th>
<th>Language Preferred</th>
<th>Given Name* Stephanie</th>
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<tr>
<td>Title*</td>
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<td>Company Name (Full Name – No Abbreviations)*: St. Boniface Hospital Research Centre</td>
<td>Address same as Sponsor</td>
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<tr>
<td>Fax No.</td>
<td>204-235-0793</td>
</tr>
<tr>
<td>E-mail</td>
<td><a href="mailto:umcaligs@cc.umanitoba.ca">umcaligs@cc.umanitoba.ca</a></td>
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#### C. Representative in Canada (must be completed if Sponsor is located outside Canada)

- Same as B

<table>
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<th>Surname*</th>
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### Part 2: Research Ethics Board(s)
### A. Research Ethics Board(s) that APPROVED the protocol*

- 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
- ☑ Table of contents
- ☑ Investigator’s Brochure†
- ☑ Protocol Synopsis & Evaluation Review Template†
- ☑ Protocol†
- ☑ Informed Consent Form
- ☑ Quality Overall Summary – NHP Template†
- ☒ Quality Data
- ☐ Product Monograph (if available)
- ☐ Approved Label (if available)
- ☐ Information regarding refusals by other regulatory authorities or Research Ethics Boards outside Canada (if applicable)

- ☑ Clinical Trial Application and Attestation Form (*attestation* signed by the Senior Officials)
- ☑ Clinical Trial Site Information Form‡
- ☑ Research Ethics Board Attestation form for each site‡
- ☑ Qualified Investigator Undertaking form‡
- ☐ Designated Party Authorization Form (if applicable)
- ☐ Animal Tissue Form(s) (if applicable)
- ☐ Authorization for a Third Party to Import an NHP form (if applicable)
- ☐ Letters of Access (if applicable)
### Part 4: Clinical Trial Application – Amendment

- **A. Reference Submission***
  
  Please provide the submission number of the approved CTA to which changes will be made.
  
  **CTA Submission #**

<table>
<thead>
<tr>
<th>Protocol # (if known)</th>
<th>Protocol Title</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **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.

<table>
<thead>
<tr>
<th>Change Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover letter</td>
<td></td>
</tr>
<tr>
<td>Clinical Trial Application and Attestation Form</td>
<td>(‘attestation’ signed by the Senior Officials)</td>
</tr>
<tr>
<td>Dosage regimen (dose, frequency, quantity per dosage unit, potency, and/or duration of use) within established safety range</td>
<td></td>
</tr>
<tr>
<td>Comparator</td>
<td></td>
</tr>
<tr>
<td>Placebo (substitution)</td>
<td></td>
</tr>
<tr>
<td>Risk information</td>
<td></td>
</tr>
<tr>
<td>Protocol</td>
<td></td>
</tr>
<tr>
<td>Investigator’s Brochure</td>
<td></td>
</tr>
<tr>
<td>Informed Consent Form</td>
<td></td>
</tr>
<tr>
<td>Other changes affecting safety or efficacy (specify):</td>
<td></td>
</tr>
<tr>
<td>Source material of any of the medicinal ingredients</td>
<td></td>
</tr>
<tr>
<td>Specifications</td>
<td></td>
</tr>
<tr>
<td>Changes to or from synthetically manufactured medicinal ingredient</td>
<td></td>
</tr>
<tr>
<td>Manufacturing information</td>
<td></td>
</tr>
<tr>
<td>Dosage formulation</td>
<td></td>
</tr>
<tr>
<td>Addition or substitution of a non-medicinal ingredient not on the NHPD List of Acceptable Non-medicinal Ingredients</td>
<td></td>
</tr>
<tr>
<td>Animal Tissue Form(s)</td>
<td></td>
</tr>
<tr>
<td>Other changes affecting quality (specify):</td>
<td></td>
</tr>
</tbody>
</table>

### 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)

<table>
<thead>
<tr>
<th>Primary Brand Name / Product Code*</th>
<th>Other(s) if any</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaxseed</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>A Standard or Grade</th>
<th>B Scientific Monograph</th>
<th>C* Proper Name</th>
<th>D Common Name</th>
<th>E* Quantity per dosage unit</th>
<th>F* Synthetic</th>
<th>G** Animal Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Milled</td>
<td>☐</td>
<td>☒</td>
<td>Linum usitatissimum</td>
<td>Flaxseed</td>
<td>30 g</td>
<td>☐</td>
</tr>
<tr>
<td>2.</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
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<td>3.</td>
<td>☐</td>
<td>☐</td>
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<td>4.</td>
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<td>9.</td>
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<td>10.</td>
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<td></td>
</tr>
</tbody>
</table>

**if yes, complete Animal Tissue Form

#### H: Potency (if applicable)

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>Amount</th>
<th>Constituent</th>
<th>Proper Name</th>
<th>Material</th>
<th>Ratio</th>
<th>Quantity Dried Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30 g</td>
<td>Whole seed</td>
<td>Linum usitatissimum</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Milled seed will be either sprinkled on food or baked into products

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>I* Source (if more than one enter on new line within the same cell)</th>
<th>J Extract (if applicable)</th>
<th>K Method of preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td>1.</td>
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<td>2.</td>
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<tr>
<td>9.</td>
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</tr>
</tbody>
</table>
### 1B: Non-medicinal Ingredient(s) of the NHP

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>Proper Name</th>
<th>Common Name *</th>
<th>Purpose *</th>
<th>Animal Tissue Used **</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
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<tr>
<td>10</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

**if yes, complete Animal Tissue Form**

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>Standard or Grade</th>
<th>Source (if more than one enter on new line within the same cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proper Name</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>10</td>
<td></td>
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</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Dosage Form*</th>
<th>Duration of Use*</th>
<th>Sterile*</th>
<th>Route of Administration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muffins, bagels, snack bars, or packets of milled flaxseed containing 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.</td>
<td>6 months</td>
<td>☑ Yes ☒ No</td>
<td>Oral ingestion</td>
</tr>
</tbody>
</table>

Proposed Dose

<table>
<thead>
<tr>
<th>Treatment Group*</th>
<th>Amount to Be Taken at One Time</th>
<th>Frequency*</th>
<th>Directions of Use*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Dosage Units*</td>
<td>Dosage Unit*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e.g. 1, 2, 3…)</td>
<td>(e.g. capsule, mL, tsp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>30 g</td>
<td>1/day</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>Standard or Grade</th>
<th>Scientific Monograph</th>
<th>Proper Name*</th>
<th>Common Name</th>
<th>Quantity per dosage unit</th>
<th>Synthetic*</th>
<th>Animal Tissue Used**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Milled</td>
<td>☐</td>
<td>☒</td>
<td>Triticum</td>
<td>Wheat</td>
<td>30 g</td>
<td>☑</td>
<td>☒</td>
</tr>
<tr>
<td>2.</td>
<td>☐</td>
<td>☐</td>
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<td>5.</td>
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<td></td>
</tr>
</tbody>
</table>
** if yes, complete Animal Tissue Form

<table>
<thead>
<tr>
<th>Ingredient No.</th>
<th>Potency (if applicable)</th>
<th>Source (if more than one enter on new line within the same cell)*</th>
<th>Extract (if applicable)</th>
<th>Method of preparation</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

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10.

I, the undersigned, certify that the information and material included in this clinical trial application is accurate and complete.

Name of Authorized Signing Official (print)  Signature  Date (yyyy / mm / dd)
Dr. Grant Pierce

Title  Telephone No.  Fax No.
Principal Investigator  204-235-3206  204-235-0793

Executive Director of Research at St. Boniface Hospital
Professor of Physiology and Pharmacology

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.

<table>
<thead>
<tr>
<th>Name of Senior Medical Officer or Scientific Officer in Canada (print)*</th>
<th>Signature*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Brian Penner MD.</td>
<td></td>
</tr>
<tr>
<td>Telephone No. 204-787-2864</td>
<td>E-mail <a href="mailto:bpenner@cc.umanitoba.ca">bpenner@cc.umanitoba.ca</a></td>
</tr>
<tr>
<td>Name of Senior Executive Officer or Department Head (print)*</td>
<td>Signature*</td>
</tr>
<tr>
<td>Dr. Grant N. Pierce</td>
<td></td>
</tr>
<tr>
<td>Telephone No. 204-235-3206</td>
<td>E-mail <a href="mailto:gpierce@sbrc.ca">gpierce@sbrc.ca</a></td>
</tr>
</tbody>
</table>
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).
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

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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 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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, \textit{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.\textsuperscript{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. 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).  

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. 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). Protein, fibre, 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. Flaxseed soluble carbohydrate is comprised primarily of xylose, rhamnose, and galactose. 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) 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. 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. 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 \textit{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) \cite{Francis2013}

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)\textsuperscript{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)\textsuperscript{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 enterodiol 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 gavaged with a single dose of $^{3}$H-SDG (3.7 kBq/g body weight) either immediately (acute group) or after 10 days of gavage with 1.5 g unlabelled SDG per day (chronic group). Rats were terminated at 12, 24, 36, and 48 hours after the gavage. 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
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 gavage (0.75, 1.5, or 3.0 mg/day), or an estrogen inhibitor tamoxifen by gavage (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 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)

*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 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)\textsuperscript{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)\textsuperscript{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 gavaged 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

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 $\gamma$-glutamyltranspeptidase ($\gamma$GT).

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, $\gamma$GT, or glucose. Lastly, the activity of liver $\gamma$GT 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 $\gamma$GT
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)

   *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)
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 psyllium 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.  

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.


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

*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)

*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. 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.\textsuperscript{20}
## Appendices

Table 1: Composition of Flaxseed

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid $^{45}$</td>
<td>36</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>5.2</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>3.2</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>16.8</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>15.2</td>
</tr>
<tr>
<td>Alpha-Linolenic Acid</td>
<td>58.8</td>
</tr>
<tr>
<td>Protein $^{45}$</td>
<td>18.2</td>
</tr>
<tr>
<td>Carbohydrate $^{45}$</td>
<td>27.3</td>
</tr>
<tr>
<td>Dietary Fibre</td>
<td>27.3</td>
</tr>
<tr>
<td>Lignans $^{46}$</td>
<td>0.18</td>
</tr>
<tr>
<td>Secoisolaricresinol</td>
<td>92</td>
</tr>
<tr>
<td>Secoisolaricresinol-sesquilignan</td>
<td>3.5</td>
</tr>
<tr>
<td>Cyclolaricresinol</td>
<td>2.1</td>
</tr>
<tr>
<td>Lariciresinol</td>
<td>0.99</td>
</tr>
<tr>
<td>Hydroxysecoisolaricresinol</td>
<td>0.53</td>
</tr>
<tr>
<td>Pinoresinol</td>
<td>0.48</td>
</tr>
<tr>
<td>Metaresinol</td>
<td>0.29</td>
</tr>
<tr>
<td>Lariciresinol-sesquilignan</td>
<td>0.05</td>
</tr>
<tr>
<td>Other</td>
<td>0.06</td>
</tr>
</tbody>
</table>

References
References


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


   


1.2.4

PROTOCOL SYNOPSIS & EVALUATION

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. Hypertension when untreated or uncontrolled can lead to cardiac hypertrophy, arrhythmias, myocardial infarctions, heart failure, stroke, and renal failure.

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.

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.

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 practitioners or Dr. Brian Penner.

**Patient Population**
In Manitoba, 192,181 individuals aged 12 and older in 2011 were diagnosed with high blood pressure; this equates to 19.4% of Manitobans living with hypertension which is greater than the national prevalence of 17.6%. 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 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 \( \geq 130 \) OR diastolic blood pressure \( \geq 80 \) OR 24-hour awake ambulatory systolic blood pressure \( \geq 135 \) OR diastolic blood pressure \( \geq 85 \))
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.
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 (triglycerides, LDL and HDL cholesterol)
5. Plasma 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, angiotensin 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**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Tests Patient Undergoes</th>
</tr>
</thead>
</table>
| 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. 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 (triglycerides, 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, angiotensin 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. 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 relevant 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 participants. This trial must pass both these review processes 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.
Appendix 1 Recruitment Poster

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 not 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:

Exclusion Criteria 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 Advertisements 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 arrhythmias, 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-hypertensive 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 Multiple 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)\textsuperscript{11}

<table>
<thead>
<tr>
<th>Step</th>
<th>Pass</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quick List</td>
<td>Collect list of foods and beverages consumed the day prior</td>
</tr>
<tr>
<td>2</td>
<td>Forgotten Foods List</td>
<td>Collect foods and beverages that may have been forgotten by the participant. Probe for items such as beverages, snacks, and additives (sugar and salt).</td>
</tr>
<tr>
<td>3</td>
<td>Time and Occasion</td>
<td>Ensure time and occasion for all items has been recorded</td>
</tr>
<tr>
<td>4</td>
<td>Detail and Review</td>
<td>Ensure that quantity is accurate. Ask if brand names and types (eg: low sodium, fat free, sugar free) have been recorded when possible</td>
</tr>
<tr>
<td>5</td>
<td>Final Review</td>
<td>Ask if any other items could be added and double check quantities</td>
</tr>
</tbody>
</table>
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:

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>Quantity of Food/Beverage (eg: cups, tablespoons, grams, servings)</th>
<th>Food Item (please list brand name if known)</th>
</tr>
</thead>
</table>
| 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 |

Continued on next page
Question: Has your diet changed as a result of joining this study? If yes, how so?
<table>
<thead>
<tr>
<th>1 Serving Looks Like...</th>
<th>1 Serving Looks Like...</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GRAIN PRODUCTS</strong></td>
<td><strong>VEGETABLES AND FRUIT</strong></td>
</tr>
<tr>
<td>1 cup of cereal flakes = fist</td>
<td>1 cup of salad greens = baseball</td>
</tr>
<tr>
<td>1 pancake = compact disc</td>
<td>1 baked potato = fist</td>
</tr>
<tr>
<td>½ cup of cooked rice, pasta, or potato = ½ baseball</td>
<td>1 med. fruit = baseball</td>
</tr>
<tr>
<td>1 slice of bread = cassette tape</td>
<td>½ cup of fresh fruit = ½ baseball</td>
</tr>
<tr>
<td>1 piece of cornbread = bar of soap</td>
<td>¼ cup of raisins = large egg</td>
</tr>
<tr>
<td>1 Serving Looks Like...</td>
<td>1 Serving Looks Like...</td>
</tr>
<tr>
<td><strong>DAIRY AND CHEESE</strong></td>
<td><strong>MEAT AND ALTERNATIVES</strong></td>
</tr>
<tr>
<td>1½ oz. cheese = 4 stacked dice or 2 cheese slices</td>
<td>3 oz. meat, fish, and poultry = deck of cards</td>
</tr>
<tr>
<td>½ cup of ice cream = ½ baseball</td>
<td>3 oz. grilled/baked fish = checkbook</td>
</tr>
<tr>
<td><strong>FATS</strong></td>
<td></td>
</tr>
<tr>
<td>1 tsp. margarine or spreads = 1 dice</td>
<td>2 Tbsp. peanut butter = ping pong ball</td>
</tr>
</tbody>
</table>
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
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
### PATIENT CONSENT FORM EVALUATION

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Evaluation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Disclosure of Risk</td>
<td>G Acceptable</td>
</tr>
<tr>
<td></td>
<td>G Requires Revision</td>
</tr>
<tr>
<td>Clarity of Language</td>
<td>G Acceptable</td>
</tr>
<tr>
<td></td>
<td>G Requires Revision</td>
</tr>
<tr>
<td>Description of Procedure</td>
<td>G Acceptable</td>
</tr>
<tr>
<td></td>
<td>G Requires Revision</td>
</tr>
<tr>
<td>Confidentiality for Patient</td>
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### INVESTIGATORY BROCHURE EVALUATION

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References


2. Mayo Clinic: High blood pressure (hypertension).20132011


6. High blood pressure, by sex, and by province and territory.2012


[http://www.hypertension.ca/chep-recommendations](http://www.hypertension.ca/chep-recommendations)

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**

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**Student Investigator/Study Co-ordinator**

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**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, arrhythmias, 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. Flaxseed is thought to contain three bioactive components: the omega 3 fatty acid alpha-linolenic acid (ALA), the potent antioxidant enterolignan, and dietary fibre. 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. 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. 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.
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 conjunction with anti-hypertensive 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 (triglycerides, LDL and HDL cholesterol)
5. Plasma 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, angiotensin 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/](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 \(\geq 130\) OR diastolic \(\geq 80\) mm Hg or **awake** systolic blood pressure \(\geq 135\) OR diastolic \(\geq 85\) 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. 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**

<table>
<thead>
<tr>
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<th>Tests Patient Undergoes</th>
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| 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**

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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. In Manitoba, 192,181 individuals aged 12 and older in 2011 were diagnosed with high blood pressure; this equates to 19.4% of Manitobans living with hypertension which is greater than the national prevalence of 17.6%. 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.\textsuperscript{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 \( \geq 130 \) OR diastolic blood pressure \( \geq 80 \) OR 24-hour awake ambulatory systolic blood pressure \( \geq 135 \) OR diastolic blood pressure \( \geq 85 \))\textsuperscript{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.


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 than 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 required 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 (e.g., triglycerides, 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, angiotensin 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: triglycerides, 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, angiotensin 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.

**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 statistical 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. 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 relevant 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 participants. This trial must pass both these review processes 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 clinical and metabolic measurements.

Current Problems/Concerns

There are no problems or concerns to date.
References


   http://www.mayoclinic.com/health/high-blood-pressure/HI00062

   http://www.statcan.gc.ca.proxy2.lib.umanitoba.ca/tables-tableaux/sum-som/01/cst01/health03a-eng.htm


**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

**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

**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
  Winnipeg

  Province / State
  MB

  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)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Telephone No.</th>
<th>Fax No.</th>
<th>Language Preferred</th>
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<tr>
<td>Dr. Grant Pierce</td>
<td>204-235-3414</td>
<td>204-235-0793</td>
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<tr>
<td>Director of Research/Professor</td>
<td><a href="mailto:gpierce@sbrc.ca">gpierce@sbrc.ca</a></td>
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**C. Contact for this Clinical Trial**

<table>
<thead>
<tr>
<th>Contact Name</th>
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<tr>
<td>Ms. Stephanie Caligiuri</td>
<td><a href="mailto:umcaligs@cc.umanitoba.ca">umcaligs@cc.umanitoba.ca</a></td>
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Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

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351 Tache Avenue

City / Town
Winnipeg

Province / State
Manitoba

Country
Canada

Postal / ZIP Code
R2H 2A6
**Part 3: Clinical Trial Site Information**

### A. Clinical Trial Site

<table>
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<tr>
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<td>Street / Suite / PO Box</td>
<td>GA219 700 William Ave</td>
</tr>
<tr>
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</tr>
<tr>
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### 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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Dr. Brian Penner</th>
<th>Title</th>
<th>Doctor/Professor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Street / Suite / PO Box</td>
<td>707 McDermot Avenue - GF329 General Centre</td>
<td>City / Town</td>
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<tr>
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<tr>
<td>E-mail</td>
<td><a href="mailto:bpenner@cc.umanitoba.ca">bpenner@cc.umanitoba.ca</a></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telephone No.</td>
<td>204-787-2684</td>
<td></td>
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<td>Fax No.</td>
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### 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.

<table>
<thead>
<tr>
<th>Name of Contact Person</th>
<th>Mrs. Shelly Rempel-Rossum</th>
<th>Date of Approval</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Street / Suite / PO Box</td>
<td>800 Waterfront Avenue - GF329 General Centre</td>
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<td>Name of Contact Person</td>
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<td>Telephone No.</td>
<td>204-789-3389</td>
</tr>
<tr>
<td>Phone No.</td>
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</tr>
<tr>
<td>Title</td>
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<td>Title</td>
<td>REB Coordinator</td>
</tr>
<tr>
<td>E-mail</td>
<td>E-mail</td>
<td>E-mail</td>
<td><a href="mailto:mross@cc.umanitoba.ca">mross@cc.umanitoba.ca</a></td>
</tr>
</tbody>
</table>

---

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:
- [x] 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

<table>
<thead>
<tr>
<th>City / Town</th>
<th>Province / State</th>
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<tbody>
<tr>
<td>Winnipeg</td>
<td>MB</td>
<td>Canada</td>
<td>R2H 2A6</td>
</tr>
</tbody>
</table>

**Contact Information of Sponsor (if Sponsor is an individual) or Senior Official (if Sponsor is a company, institution or organization)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Telephone No.</th>
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<th>Language Preferred</th>
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</thead>
<tbody>
<tr>
<td>Dr. Grant Pierce</td>
<td>204-235-3414</td>
<td>204-235-0793</td>
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<tbody>
<tr>
<td>Director of Research/Professor</td>
<td><a href="mailto:gpierce@sbrc.ca">gpierce@sbrc.ca</a></td>
</tr>
</tbody>
</table>

#### C. Contact for this Clinical Trial

<table>
<thead>
<tr>
<th>Contact Name</th>
<th>E-mail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ms. Stephanie Caligiuri</td>
<td><a href="mailto:umcaligs@cc.umanitoba.ca">umcaligs@cc.umanitoba.ca</a></td>
</tr>
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</table>

**Company Name (Full Name – No Abbreviations)**
St. Boniface Hospital Research Centre

<table>
<thead>
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<th>Street / Suite / PO Box</th>
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<tbody>
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<td>351 Tache Avenue</td>
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### Part 3: Clinical Trial Site Information

#### A. Clinical Trial Site

<table>
<thead>
<tr>
<th>Name of Site (Full Name – No Abbreviations)</th>
<th>St. Boniface Hospital Research Centre</th>
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<td>Street / Suite / PO Box</td>
<td>351 Tache Avenue</td>
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<tr>
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Commencement Date of Clinical Trial or Clinical Trial Amendment: 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.

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Street / Suite / PO Box:
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<td>Manitoba</td>
<td>R3A 1R9</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Name of Research Ethics Board</th>
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<tr>
<td>University of Manitoba Faculty of Medicine, Research Ethics Board</td>
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Street / Suite / PO Box:

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Name of Contact Person:
Mrs. Shelly Rempel-Rossum

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<td>☑️ English</td>
</tr>
</tbody>
</table>

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:

- [x] 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):
  - [x] 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: Winnipeg
- Province / State: Manitoba
- Country: Canada
- Postal / ZIP Code: R2H 2A6

#### C. Contact for this Clinical Trial

- Contact Name: Ms. Stephanie Caligiuri
- E-mail: umcaligs@cc.umanitoba.ca

- Company Name (Full Name – No Abbreviations):
  - St. Boniface Hospital Research Centre

- Street / Suite / PO Box:
  - 351 Tache Avenue

- City / Town: Winnipeg
- Province / State: Manitoba
- Country: Canada
- Postal / ZIP Code: R2H 2A6

- Telephone No.: 204-235-3855
- Fax No.: 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 | Province | Postal Code |
| Winnipeg | Manitoba | R3A 1R9 |

#### B. Qualified Investigator

| Name | Title | Language Preferred |
| Dr. Brian Penner | Doctor/Professor | ☑ English ☐ French |

| Street / Suite / PO Box | 707 Mcdermot Avenue - GF329 General Centre |
| City / Town | Province | Postal Code |
| Winnipeg | Manitoba | R3A 1R9 |

| E-mail | Telephone No. | Fax No. |
| bpenner@cc.umanitoba.ca | 204-787-2684 | 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.

<table>
<thead>
<tr>
<th>Signature of Qualified Investigator</th>
<th>Date</th>
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<tbody>
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</table>
**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

**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

**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
Winnipeg

Province / State
Manitoba

Country
Canada

Postal / ZIP Code
R2H 2A6

**C. Contact for this Clinical Trial**

Contact Name
Ms. Stephanie Caligiuri

E-mail
umcaligs@cc.umanitoba.ca

Company Name (Full Name – No Abbreviations)
St. Boniface Hospital Research Centre

Street / Suite / PO Box
351 Tache Avenue

City / Town
Winnipeg

Province / State
Manitoba

Country
Canada

Postal / ZIP Code
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Telephone No.
204-235-3855

Fax No.
204-235-0793
### Part 3: Clinical Trial Site Information

#### A. Clinical Trial Site

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<tr>
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<tr>
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#### C. Research Ethics Board Approval

<table>
<thead>
<tr>
<th>Name of Research Ethics Board</th>
<th>St Boniface Hospital Research Review Committee</th>
</tr>
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<tbody>
<tr>
<td>Street / Suite / PO Box</td>
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<tr>
<th>Name of Research Ethics Board Chair</th>
<th>Dr Bram Ramjiawan</th>
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<table>
<thead>
<tr>
<th>Title</th>
<th>Director, Research Innovation and Regulatory Affairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-mail</td>
<td><a href="mailto:bramjiawan@sbrc.ca">bramjiawan@sbrc.ca</a></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Name, Title and Signature of Research Ethics Board Representative</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name: Dr Bram Ramjiawan</td>
<td>Title: Director, Research Innovation and Regulatory Affairs</td>
</tr>
</tbody>
</table>
Module 2

Common Technical Document – Not Applicable
Module 3
Quality Data—Not Applicable