Metabolism and Physiological Actions of Milled Flaxseed in Humans as a Function of Dose, Participant Age and Cardiovascular Disease Status

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ABSTRACT

Basic and clinical research documents the benefits of dietary milled flaxseed (MFX), a rich source of alpha-linolenic acid (ALA) and lignans, in the attenuation of risk factors key to regulating cardiovascular disease (CVD) progression. ALA has antihypertensive properties and the lignan metabolites, enterodiol (END) and enterolactone (ENL), have antioxidative potential. The effectiveness of these bioactives to reduce risk factors of CVD may be dependent upon their plasma concentrations. To study this, we first designed and validated a method using supported liquid extraction and gas chromatography/mass spectrometry to isolate and quantify enterolignans in plasma. Applying this technique, we examined MFX doses of 10-40 g/d administered to healthy, younger adults (18-49 years of age) for 4 weeks. Ten g/d was sufficient to significantly increase circulating ALA (1.5 fold) and enterolignans (5-31 fold). There was no significant dose-dependent response. In another investigation, younger (18-29 years of age) and older (45-69 years of age) healthy adults were studied to determine if age influenced enterolignan metabolism. CVD is associated with advanced age but older people may not be able to obtain lignan metabolites from dietary MFX. Following 4 weeks of MFX consumption, both age groups increased plasma total enterolignans (END + ENL) with no between-group differences. This suggested that older and younger adults metabolize MFX lignans equally. A final study assessed MFX bioactives in plasma of peripheral artery disease patients >40 years of age. Plasma enterolignans increased 10-50 fold and ALA 1-2 fold after only one month of MFX ingestion. Dietary MFX also attenuated total (11%) and LDL (15%) cholesterol in these patients after 1-6 months of administered MFX compared to placebo. The attenuation in cholesterol was due to the high fiber content of flaxseed, and not to ALA and enterolignans, despite their marked

increase in circulation. MFX did not interfere with cholesterol-lowering medications but instead decreased cholesterol levels beyond the effects of medications alone.

To conclude, dietary supplementation with MFX resulted in an increase in plasma enterolignan and ALA concentrations in healthy younger and older adults and in patients with pre-existing CVD. The cholesterol-lowering benefits of MFX were additional to cholesterol-lowering drugs and likely attributed to MFX fiber.

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I dedicate this book to my precious daughters

Jaimie and Lauryn

and to my loving husband, Wayne.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ABI	ankle brachial index
ACC	American College of Cardiology
AHA	American Heart Association
ALA	α-linolenic acid
ANOVA	analysis of variance
Apo A-I	apolipoprotein A-I
Apo B	apolipoprotein B
BMD	bone mineral density
BMI	body mass index
BP	blood pressure
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CAD	coronary artery disease
CHO	carbohydrate
CLM	cholesterol lowering medication
CRP	C-reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
DASH	**D**ietary **A**pproaches to **S**top **H**ypertension
DBP	diastolic blood pressure
DGLA	dihomo-gamma-linolenic acid
DHA	docosahexaenoic acid

DPA	docosapentaenoic acid
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acids
EI	electron ionization
END	enterodiol
ENL	enterolactone
EPA	eicosapentaenoic acid
EtOAc	ethyl acetate
FA	fatty acid
FAME	fatty acid methyl ester
FBS	fasting blood sugar
FLAX-PAD	**FLAX**seed – **P**eripheral **A**rterial **D**isease
FMD	flow-mediated dilation
FX	flaxseed
FXO	flaxseed oil
g/d	grams per day
G	group
GC/FID	gas chromatography/flame-ionization detection
GC/MS	gas chromatography/mass spectrometry
GC/MS/SIM	gas chromatography/mass spectrometry/single ion monitoring
GC/MS/μSIS	gas chromatography/mass spectrometry/micro-selected ion storage
GLA	gamma-linolenic acid
GRAS	generally recognized as safe

HC	hypercholesterolemic
HD	hemodialysis
HDL-C	high density lipoprotein cholesterol
HL	high lignan
HPLC-CEAD	high performance liquid chromatography – coulometric electrode array detection
HRT	hormone replacement therapy
IC	intermittent claudication
LA	linoleic acid
LC/MS	liquid chromatography mass spectrometry
LC/MS/MS	liquid chromatography mass spectrometry/mass spectrometry
LDL-C	low density lipoprotein - cholesterol
LDLr	low density lipoprotein receptor
LL	low lignan
LLE	liquid liquid extraction
LN	lupus nephritis
LOD	limit of detection
LOQ	limit of quantitation
MAP	mean arterial pressure
MetS	metabolic syndrome
MFX	milled flaxseed
MI	myocardial infarction
MO	mineral oil

MP	menopausal
MRI	magnetic resonance imaging
MUFA	monounsaturated fatty acid
N/A	not applicable
NC	no change
NHANES	**N**ational **H**ealth and **N**utrition **E**xamination **S**urvey
NM	not measured
NS	not significant
NZW	New Zealand white rabbit
OA	oleic acid
oxLDL-C	oxidized LDL-cholesterol
PAD	peripheral artery disease
PDFM	partially defatted flaxseed meal
PDFX	partially defatted flaxseed
PL	placebo
PM	postmenopausal
PPAR-γ	peroxisome proliferator activated receptors
PUFA	polyunsaturated fatty acid
RC	randomized crossover design
RCT	randomized controlled trial
RP	randomized parallel
RSD	relative standard deviation
SAA	serum amyloid A

SBP	systolic blood pressure
SD	Sprague Dawley
SDG	secoisolariciresinol diglucoside
SECO	secoisolariciresinol
SEM	standard error of the mean
SFA	saturated fatty acid
SHR	spontaneously hypertensive rat
SIM	single-ion monitoring
μSIS	micro-selected ion storage
SLE	supported liquid extraction
S/N	signal-to-noise
SO	safflower oil
SPE	solid phase extraction
STZ	streptozotocin
T	time
T2DM	type 2 diabetes mellitus
TC	total cholesterol
TLC	thin-layer chromatography
TG	triglyceride
TMS	trimethylsilyl
TPR	total peripheral resistance
TxA_2	thromboxane A_2
UC	uncontrolled

USDA	United States Department of Agriculture
vLDL-C	very low density lipoprotein – cholesterol
W	walnuts
WC	waist circumference
WFX	whole flaxseed
WO	walnut oil

LIST OF COPYRIGHTED MATERIALS FOR WHICH PERMISSION WAS OBTAINED

CONTRIBUTIONS OF AUTHORS

1) **Chapter V: Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults (2) (page 53).**

   I, Andrea Edel, made the following contributions to this study: I designed and validated the analytical extraction and instrumentation methods. I analyzed all data, performed statistics, prepared all figures and tables and wrote the manuscript. Other contributors include: Dr. Grant Pierce (Co-investigator, provided clinical samples for enterolignan analysis and edited the manuscript) and Dr. Michel Aliani (Principal Investigator, provided project conception, acquired financial support, edited the manuscript and shared responsibility for final content).

2) **Chapter VI: The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults (3) (page 87).**

   I, Andrea Edel, made the following contributions to this study: I performed all fatty acid and enterolignan extractions and analyses, and conducted all statistical analyses. I prepared all graphs and tables and interpreted all results. I also wrote 80% of the manuscript and conducted revisions. Other contributors include: Amanda F. Patenaude (Research Associate, cholesterol analysis), Melanie N. Richard (Research Assistant, platelet aggregation), Dr. Elena Dibrov (Research Associate, study facilitator), J. Alejandro Austria (Research Assistant, food distribution), Dr. Harold M. Aukema (Co-Investigator, urine thromboxane analysis), Dr. Grant N. Pierce (Principal Investigator, provided project conception and developed the research plan, acquired financial support,

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3) **Chapter VII: Age-dependency in the metabolism of flaxseed lignans by healthy adults (4) (page 131).**

   I, Andrea Edel, made the following contributions to this study: I isolated all plasma enterolignan samples and analyzed them using GC/MS. I interpreted all data, conducted all statistics, prepared all figures and tables and wrote the manuscript. Other contributors include: Dr. Grant Pierce (Principal Investigator, designed the original study by Patenaude et al (5) provided all plasma samples, edited the manuscript and shared responsibility for final content) and Dr. Michel Aliani (Co-Investigator, assisted in enterolignan extraction design and financial support, provided statistical guidance, edited the manuscript and shared responsibility for final content).

4) **Chapter VIII: Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol lowering medications alone in patients with peripheral artery disease (6) (page 167).**

   I, Andrea Edel, made the following contributions to this study: I isolated all plasma enterolignan samples and analyzed them using GC/MS. I performed all correlations with blood lipids, conducted all statistics that were done with SPSS statistical software, interpreted all data and prepared all figures and tables. I contributed towards writing 70% of the manuscript. Other contributors include: Dr. Delfin Rodriguez-Leyva (Cardiologist, analyzed and interpreted cholesterol and triglyceride data, provided

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CHAPTER I: INTRODUCTION

Epidemiological and observational studies clearly identify that the risk of developing cardiovascular disease (CVD) can be reduced through lifestyle modifications. Nutrition is one of these lifestyle modifications. The Mediterranean diet, high in the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from marine sources, has demonstrated a reduced risk of suffering myocardial infarctions or cerebrovascular events. Dietary flaxseed is receiving significant attention as a plant-based source of omega-3 fatty acids, with potentially similar cardioprotective properties.

Dietary flaxseed consumed as the whole seed may offer more than just the health benefits contributed by the omega-3 fatty acid, alpha-linolenic acid (ALA). In addition, it also contains several other bioactive ingredients that may potentially contribute to overall cardiovascular health. Flaxseed is a rich source of soluble fiber with well-documented cholesterol-lowering benefits and is the primary source of the lignan secoisolariciresinol diglucoside (SDG). SDG is metabolized by microflora in the gut to enterodiol (END) and enterolactone (ENL), which are the bioactive forms of SDG with observed estrogenic or antiestrogenic behavior and antioxidative potential. Studies involving humans ingesting whole or milled flaxseed have predominantly examined the health effects resulting from ALA, despite the presence of other potentially beneficial cardioprotective constituents, namely fiber and lignans. Further evidence is required to understand the role of enterolignans as potential bioactives in flaxseed and the possible synergism with ALA and fiber.

Nutritional bioactives, like drugs, circulate within the blood and must be accurately quantified to represent tissue-available amounts. This is a critical step prior to assessing their

biological actions. We have developed and validated an analytical method to isolate and quantify plasma enterolignans in humans. As such, we have applied this method to a number of samples from healthy and unhealthy populations with the aim of understanding flaxseed lignan metabolism in both nonclinical and clinical populations. First, in healthy subjects, we wanted to study the influence of milled flaxseed dose on circulating concentrations of flaxseed bioactives with the hypothesis that increasing doses would lead to an associated dose-dependent response in plasma bioactives. Secondly, we examined the effect of subject age on lignan metabolism. Advanced age is associated with declines in gut microflora diversity, which are the essential precursors for lignan metabolism. If enterolignans have potential to exert cardioprotective properties in elderly subjects, a population prone to CVD development, they must first be bioavailable and were, therefore, examined in healthy subjects without the influence of disease. We hypothesized that there would be an age-dependency in lignan metabolism primarily due to age-related alterations in gut microflora; however, it is difficult to predict the outcome on plasma END and ENL concentrations due to limited, although growing, evidence concerning the bacterial strains associated with enterolignan conversion (7-9). Increased age is also associated with dietary changes, one often being increased dietary fiber intake, and longer intestinal transit times. These factors would promote the conversion of END to ENL in aging populations, thereby increasing circulating concentrations of ENL in older adults compared to younger adults. Finally, we investigated lignan metabolism in unhealthy older adults with documented peripheral artery disease (PAD). These patients presented many risk factors of CVD including hypertension, hyperlipidemia, diabetes, older age and were either current or ex-smokers. We hypothesized that increased age would potentially limit lignan metabolism and, therefore, reduce

circulating concentrations of enterolignans, and the presence of CVD would augment this effect. Reductions in total- and LDL-C concentrations were expected due to the high total fiber content of the MFX diet (10), yet the influence of concurrent cholesterol lowering medications was unclear. Concentrations of circulating flaxseed bioactives were also correlated with several risk factors of CVD to assess possible relationships.

CHAPTER II: REVIEW OF LITERATURE

1. CARDIOVASCULAR DISEASE

Cardiovascular diseases (CVDs) are currently the leading cause of death worldwide (11). They accounted for ~17.5 million deaths in 2012 which is expected to rise to an estimated 23.3 million by the year 2030 (11). In the United States alone, CVDs account for over one-third of all deaths, with CVD-associated disabilities inflicting over 4 million people (12). The estimated direct and indirect cost of CVDs in the U.S. in 2010 was 444 billion dollars (13) with $109, $94 and $54 billion attributed to coronary heart disease, hypertension and stroke, respectively (14).

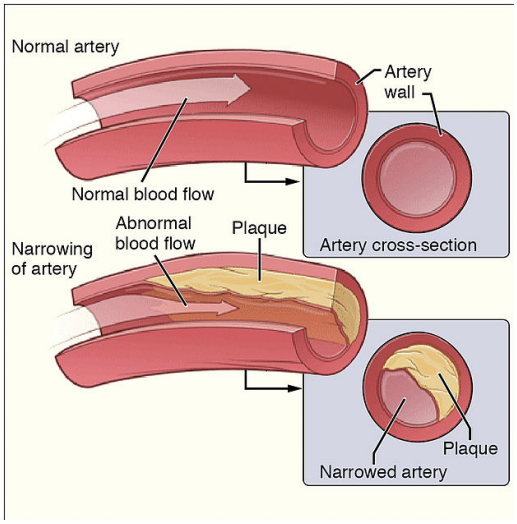
1.1 Atherosclerosis in cardiovascular disease

Atherosclerosis is a disease affecting the arterial system that can lead to CVD. Plaques form along the arterial lumen appearing as fatty streaks. As the plaque progresses, it infiltrates the vessel lumen, impeding blood flow and oxygen to vital organs (**Figure 1**). If the plaque becomes inflamed or fragile, it may burst forming a thrombus leading to vessel stenosis, or worse, an obstruction. In the heart, this could result in a myocardial infarction (MI) and in the brain, a stroke. Atherosclerosis progresses slowly over time beginning in childhood and progressing with more vigor in one's thirties. The effects of this disease typically become more lethal with advanced age, with negative outcomes surfacing as early as 50 years of age.

The onset of atherosclerosis is unclear; however, several theories exist. It is postulated that damage to the endothelial layer lining the artery becomes the site for plaque

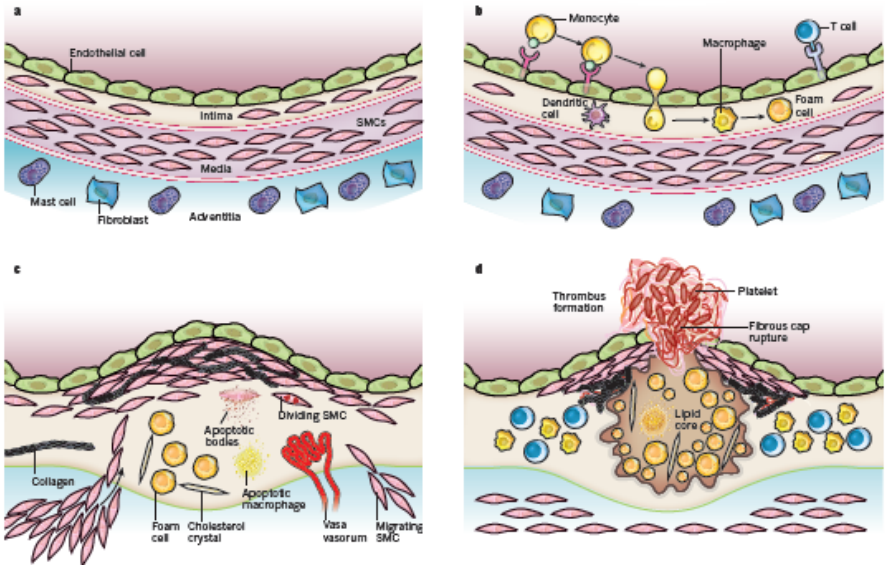
build-up. Several proposed causes of this damage include smoking, turbulent blood flow with hyperlipidemia and uncontrolled hypertension. Smoking is thought to accelerate the disease progression. At the damaged site, an influx of platelets, fats, cholesterol, cellular debris and calcium accumulate (**Figure 2**). This may result in the stimulation of other signaling pathways within the artery that lead to foam cell formation in the subendothelial space promoting the migration and proliferation of vascular smooth muscle cells. During the wound healing process a fibrous cap forms over the plaque, temporarily stabilizing it. The atherosclerotic vessel then becomes thicker and harder than the original healthy vessel, which may result in contractile dysfunction. Coronary artery disease (CAD) and peripheral artery disease (PAD) are two atherosclerotic diseases that are highly predictive of CVD.

Figure 1. A normal healthy artery and an atherosclerotic artery.



"2113ab Atherosclerosis" by OpenStax College - Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013. Licensed under CC BY 3.0 via Wikimedia Commons. The image has been cropped. http://commons.wikimedia.org/wiki/File:2113ab_Atherosclerosis.jpg#/media/File:2113ab_Atherosclerosis.jpg.

Figure 2. Stages in the development of atherosclerotic lesions.



The normal muscular artery and the cell changes that occur during disease progression to thrombosis are shown. **a**, The normal artery contains three layers. The inner layer, the tunica intima, is lined by a monolayer of endothelial cells that is in contact with blood overlying a basement membrane. In contrast to many animal species used for atherosclerosis experiments, the human intima contains resident smooth muscle cells (SMCs). The middle layer, or tunica media, contains SMCs embedded in a complex extracellular matrix. Arteries affected by obstructive atherosclerosis generally have the structure of muscular arteries. The arteries often studied in experimental atherosclerosis are elastic arteries, which have clearly demarcated laminae in the tunica media, where layers of elastin lie between strata of SMCs. The adventitia, the outer layer of arteries, contains mast cells, nerve endings and microvessels. **b**, The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes (the most numerous of the leukocytes recruited) into macrophages, and their uptake of lipid, yielding foam cells. **c**, Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die in advancing lesions, some by apoptosis. Extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque, often denoted the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **d**, Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with tissue factors in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow. Permission on May 13/2015 from Macmillan Publishers Ltd: Nature, P Libby, PM Ridker, and GK Hansson. (1), © 2011. http://www.nature.com.proxy2.lib.umanitoba.ca/nature/journal/v473/n7347/pdf/nature10146.pdf.

1.2 Risk factors of CVD

Atherosclerosis is associated with several risk factors that can exacerbate progression of CVD. These risk factors include smoking, diabetes, hypertension, elevated low-density lipoprotein cholesterol (LDL-C), attenuated high-density lipoprotein cholesterol (HDL-C), hyperhomocysteinemia, increased age, genetics and obesity. In both males and females, hypertension is the leading cause of CVD-related mortality, followed closely by smoking, obesity and physical inactivity which pose a similar risk, then high LDL-C and high blood glucose concentrations (15). Dietary factors that contribute to a greater incidence of CVD-related deaths include high sodium diets, low dietary omega-3 fatty acids, high dietary trans fatty acids, a diet low in fruits and vegetables, and a diet low in polyunsaturated fatty acids (PUFAs) (as a substitution for saturated fatty acids, SFAs) (16).

Despite age, ethnicity, gender or even genetics influencing the pathogenesis of CVD, there are a number of controllable risk factors that can attenuate their progression. These modifiable risk factors include the cessation of smoking, routine exercise and improved nutritional habits. Implementing these basic lifestyle changes can reduce one's incidence of developing CVD by more than 90% (17). CVD patients are commonly on a combination of lipid-lowering, anti-thrombotic, anti-hypertensive and blood glucose lowering therapies (18, 19). Non-adherence to drug regimes is a re-occurring problem leading to negative cardiac outcomes and death (20). Fortunately, management of risk factors can proceed through lifestyle modifications with effective changes resulting from their implementation (21).

1.3 Available treatments for CVD

Current treatments for CVD involve directly managing essential risk factors. This would include antihypertensive, cholesterol-lowering and anti-platelet therapies and medications to control blood glucose levels in diabetic patients. Other recommendations involve cessation of smoking, increased exercise and improved nutritional habits. Medical procedures for diagnosed stenotic vessels often include vascular bypass surgeries and endovascular procedures; however, solutions are often temporary and recurrent symptoms typically occur with many treatments often repeated. Unfortunately, atherosclerotic diseases are often under-diagnosed as the disease typically progresses asymptomatically until an acute event like an MI, stroke or intermittent claudication results. The need for implementing earlier diagnosis and awareness of atherosclerotic diseases is warranted, yet prevention must be equally emphasized. Dietary strategies for reducing the burden of CVD are just one of these solutions.

1.4 Nutrition as a therapy for reducing risk factors of CVD

Numerous observational and epidemiological studies are available that detail the cardiovascular benefits of consuming a healthy diet. Conversely, an unhealthy diet can aggravate risk factors of CVD and increase risk of mortality. Diets high in saturated fat, trans fats and overeating, which increases caloric intake, can lead to increased weight gain, obesity and even diabetes, whereas diets high in sodium or alcohol can lead to elevated blood pressure. Therefore, it is important to consider weight loss or healthy food alternatives that can reduce the incidence of developing CVD or can improve symptoms or slow the progression of the disease. Recent findings have correlated the benefits of a healthy diet and

weight loss with improvements in functional mobility and reduced risk of ischemic events (22), reductions in blood pressure (23) and type 2 diabetes (24).

Current guidelines from the American Heart Association (AHA) and American College of Cardiology (ACC) recommend diets high in fruits and vegetables, legumes, whole grains, low-fat dairy products, vegetable oils, nuts and lean meats like poultry and fish (16). Dietary recommendations suggest that individuals tailor their intake of these foods with their required caloric intakes and corresponding energy expenditure. Directions to follow dietary plans laid out by the AHA Diet (25), the Dietary Approaches to Stop Hypertension (DASH) Diet (26) and the United States Department of Agriculture Food Pattern (27) are additionally provided. However, it is the broadness of these recommendations that may limit the effective use of certain foods that contain bioactive ingredients that are critical in reducing risk factors of atherosclerosis and, therefore, the incidence of CVD.

In 2013, the AHA/ACC established several recommendations regarding nutrient intake for reducing specific risk factors of CVD (16). Dietary recommendations of n-3 PUFAs as a means of primary CVD prevention included fish meals twice per week or fish oil supplements providing approximately 500 to 1000 mg of EPA and DHA per serving. Daily salt intake should be restricted to ~6 g/d and saturated fat to about 5-6% of the total daily caloric intake. Dietary fiber intake for the purpose of lowering LDL-cholesterol and glucose levels was recommended at doses of 38 g/d for adult men and 25 g/d for women. The Institute of Medicine's Food and Nutrition Board, which is a branch of the National Academy of Science, also has recommended dietary allowances or adequate daily intakes for nutrients established by subject age and gender, yet these are not based on any disease states (28).

Bioactive compounds, which are essential or nonessential compounds present in nature that elicit health effects (29), are being investigated for their role in CVD prevention. Some of these compounds include phytoestrogens (lignans, isoflavones, resveratrol, lycopene), organosulfur compounds (allicin, diallyl sulfide, diallyl disulfide, allyl mercaptan), flavonoids (quercetin, kaempferol, catechin), plant sterols (sitostanol, stigmasterol, campesterol), soluble dietary fiber (β-glucan, pectin, psyllium), isothiocyanates (phenethyl, benzyl, sulforaphanes) and monoterpenes (d-Limonene, perillic acid) and have been reviewed (30).

1.5 Peripheral artery disease (PAD)

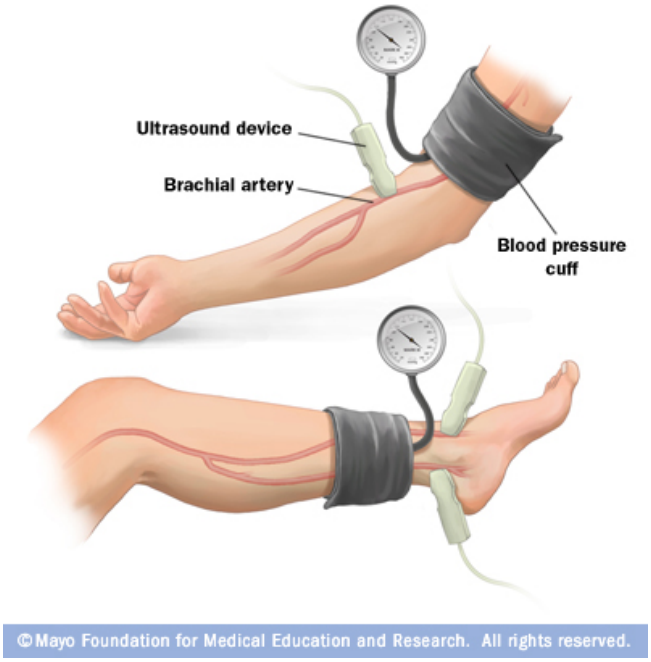
Patients with PAD exhibit many characteristics of CVD and, therefore, are at high risk for MI and stroke (19, 31). Approximately 8 million Americans suffer from PAD (32). Cigarette smoking is the primary risk factor for PAD development. Over 80% of PAD patients smoke or were prior smokers (33). Smoking increases the risk of developing lower extremity PAD by 2- to 6-fold with the risk increasing exponentially with the number of years and cigarettes smoked (34). Diabetes is another prevalent risk factor of lower extremity PAD increasing the risk by 2- to 4- fold (35). About 12-20% of PAD patients have diabetes (36). High circulating levels of total-cholesterol and LDL-cholesterol are also associated with PAD in the legs. The incidence of developing PAD rises by 5% to 10% with each 10 mg/dL increase in total-cholesterol (37). Adults greater than 60 years of age are at greatest risk for developing PAD regardless of gender; however, ethnicity may also play a role (38). Despite current therapies, PAD patients have a higher incidence of developing

CVD than CAD patients and when compared to age-matched controls, a 3- to 5-fold increase in CVD-related mortality (39).

1.5.1 Diagnosis of PAD

The diagnosis of PAD typically occurs via the Doppler technique with PAD defined as an ankle-brachial index (ABI) < 0.9 (40). Systolic blood pressures (SBPs) are measured in the brachial arteries and in the posterior tibial and dorsalis pedis arteries in the lower extremities with the patient lying supine (**Figure 3**). Both legs are recorded with the higher SBP being used in the ratio of leg to arm SBPs to calculate the ABI (40). Angiography is used to define the location of the stenotic vessel and has 98% accuracy with occlusions that are ≥ 50% (40). Other diagnostic tools include a medical questionnaire (e.g., Edinburgh Claudication questionnaire), a treadmill stress test, ultrasonography and magnetic resonance imaging (MRI). A consequence of PAD is intermittent claudication (IC). This causes pain, muscle cramping and leg hypotrophy resulting in reduced leg strength and greater functional impairment (41). Advanced PAD may result in gangrene and amputation.

Figure 3. Ankle-brachial measurement for obtaining the ankle-brachial index (ABI).



1.5.2 Nutrients capable of reducing the incidence of PAD

Several studies have examined the nutritional patterns of patients with PAD. Basic dietary profiling through the use of questionnaires noted that patients with PAD consumed diets that were deficient in folate, fiber and vitamin E (42, 43). What was being consumed, however, were high intakes of saturated fat, cholesterol and sodium (42). In two larger population-based studies using data from the United States National Health and Nutrition Examination Survey (NHANES), the odds of developing PAD were reduced (44) and higher ABIs were measured (45) when patients included more fiber, vitamins A, C, E, B_6, folate and n-3 PUFAs in their diet. Recently, a supplemented diet consisting of non-soy based legumes was found to implicate ABI (5.5% increase) in patients with PAD (46), which is a major clinical endpoint and indicator of atherogenesis. Clearly, improvements in dietary habits must be encouraged in patients with PAD in the secondary prevention of cardiovascular events (39).

2. FLAXSEED

2.1 Description and morphology of flaxseed

Flaxseed, otherwise known as linseed, is produced in over 50 countries worldwide, with Canada and China leading in global production (47). Cultivated flaxseed (*Linum usitatissimum*) consists of a tall shoot with peripheral branches each containing a periwinkle blue or white flower with five petals (**Figure 4**). In addition numerous bolls on the plant encapsulate ~10 seeds in each. Flaxseeds may be brown, a variety typically produced in Canada, or yellow as produced in the USA and known as Omega. Both of these varieties contain similar ALA omega-3 fatty acid content (48). Some genetically modified varieties of flaxseed contain up to 71% of the total fatty acids as ALA (49). Another yellow variety also exists, called Solin, which is low in ALA. Flaxseed is a small seed (3-5 mm), shaped with a pointed apex and a rounded base. The seed coat is smooth, hard and shiny and when consumed has a chewy consistency and a pleasant nutty flavor (50).

Flaxseed contains an inner embryo located at the core of the seed and is enclosed by an ovule with an inner and outer envelope (51). Approximately seven different cell types comprise a single flaxseed (52, 53). The outer portion of the ovule consists of a single layer of epidermal cells, followed by a thick layer (1 to 5 cells deep) of parenchyma cells also called ring cells. The epidermal cells provide mucilage fiber, composed of polysaccharides, polypeptides and glycoproteins (54), and the parenchyma cells may contain tannin-like compounds and/or chlorophyll. The inner portion of the ovule contains 3 cell types: a single layer of sclerenchyma fiber on the outer layer, followed by transversal cells and then pigment cells at the innermost position. An endosperm layer, containing oil and protein, tightly adheres to the seed coat and surrounds the embryo, which contains two large

cotyledons. The embryo contains ~57% (wt/wt) of the oil and is low in fiber (55). The hull is the primary location of SDG and lignans (56) and is a rich source of water-soluble mucilage fiber (57).

Figure 4. Flax flowers and brown flaxseed.

Image was purchased from istockphoto.com. Image has been cropped. Stock photo © Michael Hieber (Photo: 11023005).

2.2 Nutritional composition of flaxseed

Cultivated flaxseed is rich in fat, protein and fiber. Proximate analysis (wt/wt) of a Canadian brown flaxseed variety contains 42% fat, 27% dietary fiber, 18% protein, 7% moisture, with the remainder as minerals and vitamins (**Table 1**) (48, 58). Whole and ground

flaxseed (**Figure 5**) have similar distributions of these nutritional components; however, flax oil in its natural state is 100% fat, 53% of which is ALA, and is devoid of protein and fiber. Flaxseed is also rich in dietary lignans, the most prominent being SDG. SDG content varies with flaxseed cultivar (59), growing region and year (60). Other lignans in flaxseed include matairesinol, lariciresinol, isolariciresinol and pinoresinol, but are represented to a much lower extent (61-63).

Table 1. Proximate analysis (per 100 g) of whole and milled flaxseed, flaxseed oil and partially defatted flaxseed meal.

Flaxseed Form	Total Fat (g)	C18:3 Undifferentiated (g)	Protein (g)	CHO (g)	Dietary Fiber (g)	SDG (mg)	Energy (kcal)
WFX[1]	42.2	22.8	18.3	28.9	27.3	82 – 2600[3]	534
MFX[1]	42.2	22.8	18.3	28.8	27.3	82 – 2600[3]	534
FXO[1]	100.0	(ALA) 53.4	0.1	0.0	0.0	0	884
PDFM[2]	11.1	(ALA) 6.0	38.9	38.9	33.3	2500	389

[1] Proximate analysis data was obtained from the United States Department of Agriculture, Agricultural Research Service, National Nutrient Database for flaxseed (58).
[2] Proximate analysis data for PDFM was provided by Omega Nutrition, Canada.
[3] SDG content was obtained from (64).
Abbreviations: ALA, alpha-linolenic acid (C18:3); CHO, carbohydrate (includes dietary fiber, starches and sugars); FXO, flaxseed oil; MFX, milled flaxseed; PDFM, partially defatted flaxseed meal; SDG, secoisolariciresinol diglucoside; WFX, whole flaxseed.

Figure 5. Whole and ground brown flaxseed.

Image purchased from istockphoto.com. Stock photo © eliane (Photo: 3439583).

2.3 Phytochemical composition of flaxseed and their related health benefits

Several hundred species of the genus *Linum* exist, yet, most studies involving flaxseed phytochemicals focus on the species *Linum usitatissimum* as it is the most produced variety. Information regarding the phytonutrient composition of flaxseed can be obtained readily from the United States Department of Agriculture (58) (**Table 2**). The phytochemical composition of flaxseed for the purpose of this discussion will be restricted to the omega-3 fatty acid ALA, the lignan SDG and fiber.

Table 2. Nutrient composition of 100 g of flaxseed. [a]

Nutrient Composition	Amounts	Nutrient Composition	Amounts
Vitamins		C18:1 undifferentiated	7.359 g
Vitamin C	0.6 mg	C20:1	0.067 g
Thiamin	1.644 mg	C22:1 undifferentiated	0.013 g
Riboflavin	0.161 mg	C24:1 c	0.064 g
Niacin	3.08 mg	Total PUFAs	28.73 g
Pantothenic acid	0.985 mg	C18:2 undifferentiated	5.903 g
Vitamin B_6	0.473 mg	C18:3 undifferentiated	22.813 g
Total folate	87 µg	C20:2 n-6 c,c	0.007 g
Total choline	78.7 mg	C20:5 n-3 (EPA)	0 g
Betaine	3.1 mg	C22:5 n-3 (DPA)	0 g
Lutein + zeaxanthin	651 µg	C22:6 n-3 (DHA)	0 g
Vitamin E	0.31 mg	Cholesterol	0 mg
γ-Tocophernol,	19.95 mg	Stigmasterol	11 mg
δ-Tocophernol,	0.35 mg	Campesterol	45 mg
Vitamin K	4.3 µg	β-sitosterol	90 mg
Minerals		**Amino acids**	
Calcium	255 mg	Tryptophan	0.297 g
Iron	5.73 mg	Threonine	0.766 g
Magnesium	392 mg	Isoleucine	0.896 g
Phosphorus	642 mg	Leucine	1.235 g
Potassium	813 mg	Lysine	0.862 g
Sodium	30 mg	Methionine	0.37 g
Zinc	4.34 mg	Cystine	0.34 g
Copper	1.22 mg	Phenylalanine	0.957 g
Manganese	2.482 mg	Tyrosine	0.493 g
Selenium	25.4 mg	Valine	1.072 g
Lipids		Arginine	1.925 g
Total SFAs	3.663 g	Histidine	0.472 g
C14:0	0.008 g	Alanine	0.925 g
C15:0	0.005 g	Aspartic acid	2.046 g
C16:0	2.165 g	Glutamic acid	4.039 g
C17:0	0.018 g	Glycine	1.248 g
C18:0	1.33 g	Proline	0.806 g
C20:0	0.052 g	Serine	0.97 g
C22:0	0.052 g	Hydroxyproline	0.175 g
C24:0	0.031 g	**Other**	
Total MUFAs	7.527 g	Glycitein	0.1 mg
C16:1 undifferentiated	0.024 g	Total isoflavones	0.1 mg

[a] Adapted from the USDA nutrient database for standard reference (nutrient data for: 12220, Seeds, flaxseed) (58). Nutrients that were measured but were below the limit of detection include: fructose, lactose, maltose, galactose, folic acid, vitamin B_{12}, vitamin A, retinol, β-carotene, α-carotene, β-cryptoxanthin, vitamin A (IU), lycopene, vitamin E (added), β-tocopherol, vitamin D (D_2 +D_3), C4:0. C6:0, C8:0, C10:0, C12:0, C18:4, C20:4

undifferentiated, ethyl alcohol, caffeine, theobromine, daidzein, genistein, biochanin A, formononetin and coumestrol.
Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid, MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

2.3.1 Fatty Acids

Flaxseed is one of the richest sources of the omega-3 fatty acid ALA (C18:3n-3) (**Figure 6A**) contributing to 54-57% of the overall fat composition. PUFAs represent the largest group of fatty acids making up 68% of the overall distribution. Linoleic acid (LA; C18:2n-6) (**Figure 6B**) is the next most prevalent PUFA at 14%. Monounsaturated fatty acids (MUFAs) represent 18% of the distribution with oleic acid (C18:1) being most prevalent. A number of SFAs are also present in flaxseed with palmitic acid (C16:0) and stearic acid (C18:0) being the largest at 5% and 3%, respectively.

ALA can be metabolized via enzymatic reactions to the longer chain PUFAs eicosapentaenoic acid (EPA; C20:5n-3), docosapentaenoic acid (DPA; C22:5n-3) and docosahexaenoic acid (DHA; C22:6n-3) (65) (**Figure 7**), the same PUFAs present in fish oil that exhibit cardioprotective properties (66). Metabolic conversion of ALA in humans is relatively inefficient with only 5-8% converted to EPA and 0.5-4% for DHA (67, 68). However, the n-6 fatty acid LA competes with these same desaturase and elongase enzymes since LA can also be elongated to produce longer chain PUFAs. A consequence of this competition is that one of the primary metabolites formed during the rate limiting step with delta-5 desaturase is arachidonic acid (AA, C20:4n-6). Eicosanoids produced from AA have pro-inflammatory action and are from the 2- and 4- series, such as thromboxane A_2 (TxA_2), which implicate platelet aggregation and vasoconstriction (66). However, eicosanoids produced from the parallel competing carbon-20 omega-3 fatty acid EPA (C20:5n-3) are

from the 3- and 5- series which are less pro-inflammatory. It is suggested that elevated amounts of intracellular ALA compete with LA at the rate-limiting step favorably producing more of the 3- and 5-series eicosanoids, leading to a less inflammatory environment.

The ability for n-3 fatty acids to reduce blood pressure has been clearly documented in the INTERMAP Study (69). Several studies in humans have demonstrated the ability of ALA or flaxseed oil (FXO) to reduce diastolic blood pressure (DBP) (70), systolic blood pressure (SBP) (71) or both DBP and SBP (72-74). ALA has also demonstrated anti-inflammatory properties as observed by an attenuation of cytokines in cultured peripheral blood mononuclear cells (75). Other recent findings on the potential benefits of dietary ALA include attenuations in triglycerides (5, 76) and C-reactive protein (CRP) levels (77), with no effect on glycemic control (78, 79), on adiponectin levels (80) or plasma lipoprotein levels (81).

Figure 6. Molecular structures of the two most prevalent polyunsaturated fatty acids in flaxseed: (A) alpha-linolenic acid (ALA) and (B) linoleic acid (LA).

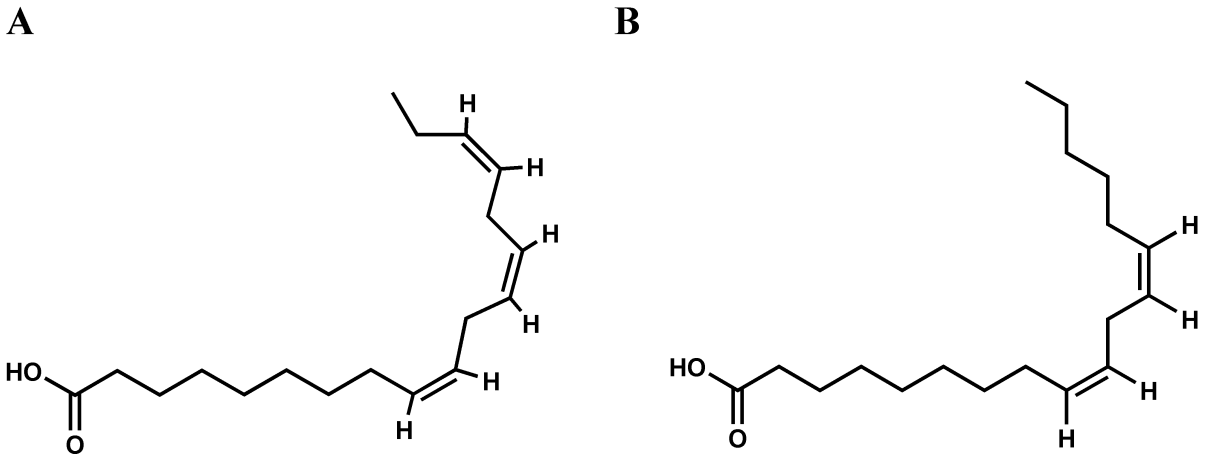
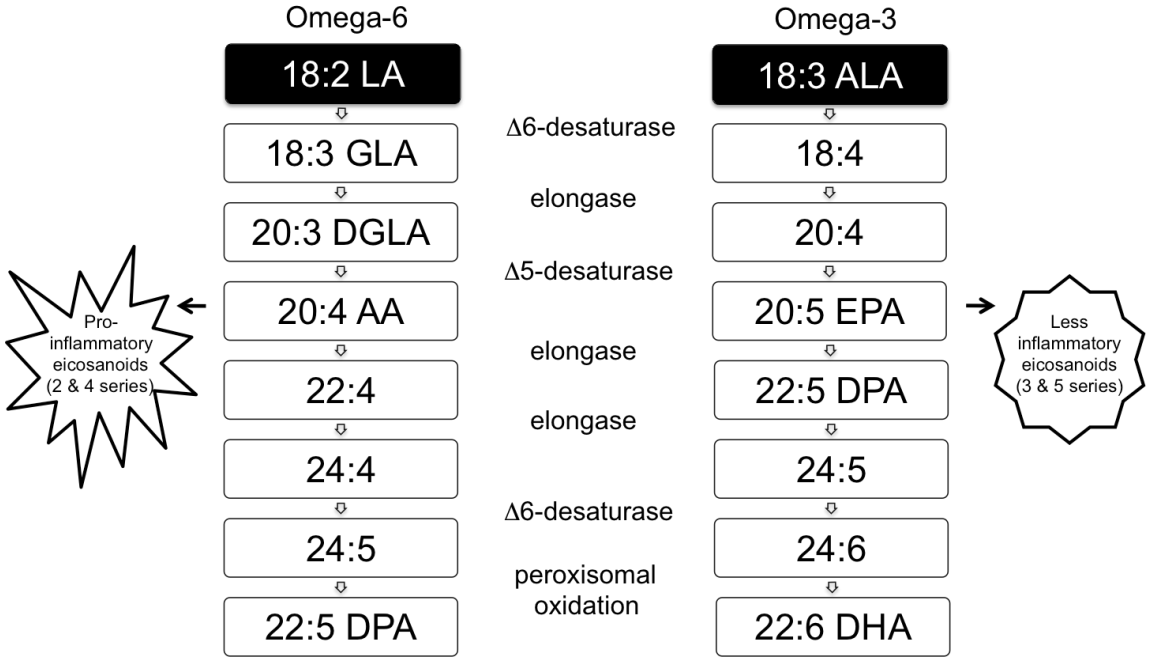


Figure 7. Competitive metabolism of linoleic acid (LA, C18:2n-6) and alpha-linolenic acid (ALA, C18:3n-3) to longer chain polyunsaturated fatty acids.



Abbreviations: AA, arachadonic acid; ALA, alpha-linolenic acid; DGLA, dihomo-gamma-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid. Figure adapted from Arterburn et al (65).

2.3.2 Lignans

Lignans in flaxseed are characterized as phenolic acids and are located as an ester-linked complex in the seed coat (82). SDG (**Figure 8**) is the most prevalent lignan in flaxseed (61) and forms the structural backbone of this complex, known as the lignan macromolecule. This backbone consists of 5 SDG molecules linked by 4 hydroxy-methyl-glutaric acid molecules and has a molecular weight of ~4000 Da (83). Other known phenolic compounds bound within the lignan macromolecule are hydroxycinnamic acids (*p*-coumaric acid, ferulic acid, sinapic acid, caffeic acid present as free or glucosidic conjugates) and

flavonoids (herbacetin diglucoside and kaempferol diglucoside) (84). Flaxseed contains 1099 mg SDG/100 g flaxseed (62). The next highest food source of SDG is asparagus and is 180 fold-less than flaxseed (85). Secoisolariciresinol (SECO), the aglycone of SDG, comprises 375 to 485 mg/100 g of flaxseed (61, 62). Matairesinol (0.2 mg/100 g), pinoresinol (0.7 to 24.8 mg/100 g), lariciresinol (2.8 to 3.3 mg/100 g) and isolariciresinol (10.2 mg/100 g) are also lignans found in flaxseed (**Figure 8**), yet their presence is minimal compared to SECO (61, 62).

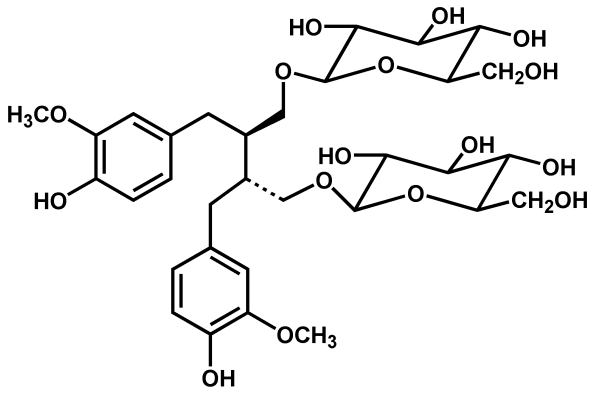
SDG consumed orally is not bioavailable in plasma or urine; however, the products of SDG metabolism, END and ENL (**Figure 8**) can be absorbed and therefore circulate systemically (86). SDG is first metabolized by microflora in the intestines to END, which may then be further metabolized to ENL (**Figure 9**). Other lignans in flaxseed can also be metabolized to END and/or ENL, but their overall influence on enterolignan content is much less than the contribution by SDG. The involvement of gut microflora and bacteria in the conversion of lignans was first demonstrated in rats as urinary concentrations of enterolignans were negligible in both germ-free and antibiotic treated animals, but not in control animals (87, 88). SDG hydrolysis is not suspected to occur in the oral cavity or upper gastrointestinal tract as artificial conditions simulating stomach and intestinal juice, at physiological temperature, did not hydrolyse the glycosidic bond (8). Lignan metabolites undergo enterohepatic circulation *in vivo* as they become conjugated in the liver following intestinal absorption and are then excreted in bile or urine and reabsorbed and repackaged as β-glucuronide or sulfate conjugates (89, 90) (**Figure 10**). Initially, it was concluded that enterolignans could only be conjugated in the liver; however, it is now understood that phase II metabolism already takes place in colonic epithelial cells (91) as glucuronic acid

conjugates of enterolignans (END and ENL) were measured in blood samples taken from the portal vein of rats (87). It was during this same study that the enterohepatic circulation of lignans was discovered as inserted bile fistula significantly mitigated enterolignans in the urine of female rats (87). Rat plasma, urine and bile all contained END and ENL primarily as glucuronide conjugates, with small amounts present as mono- and disulfates conjugates (87). Human urine contained all conjugated forms; however, the monoglucuronide was the primary conjugate with 73-95% as END and 86-98% as ENL (89, 92). No unconjugated or di-conjugated forms of either enterolignan were detected in urine. Similar to urine, 80% of the conjugates of END and ENL in human plasma were glucuronides (93).

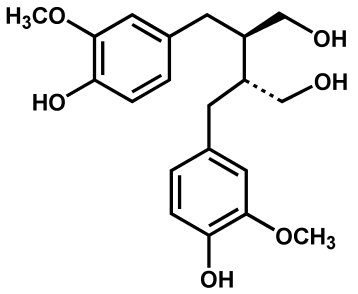
The health benefits of consuming plant lignans, like SDG, are suggested to be due to their estrogenic and antioxidative behavior. Enterolignans closely resemble the active form of estrogen, which is 17β-estradiol, and therefore can weakly bind to the estrogen receptor exerting estrogenic or anti-estrogenic effects (94). In addition, the antioxidative properties of enterolignans have been demonstrated *in vitro* (95, 96) and *in vivo* (97). These may have implications in CVD prevention as oxidative damage is suggested to play a role in disease progression (98). The cholesterol lowering effects of flaxseed lignan are uncertain and warrant further investigation. Attenuations in total- and LDL-cholesterol have been observed only in hypercholesterolemic patients at higher lignan doses of 600 mg/d (99) or in the LDL-C/HDL-C ratio (100). Other studies involving healthy adults have observed no effect (101, 102). Ox-LDL, a risk factor of CVD, was also reduced by a high-lignan diet (99). Other studies have demonstrated improved glycemic control (103), decreased blood glucose levels (104), reduced CRP (105, 106) and a mild attenuation in DBP (107) with a lignan-

supplemented diet. Beneflax (543 mg/d of flaxseed lignan in tablet form contains 32.9% SDG) does not pose a risk for hypotension or hypoglycemia in healthy older adults (108).

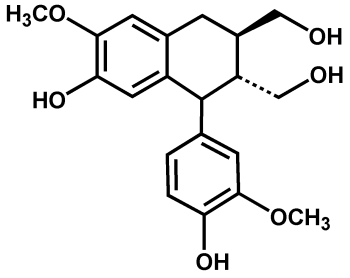
Figure 8. Chemical structures of the major plant lignans in flaxseed and the mammalian lignans, enterodiol and enterolactone.



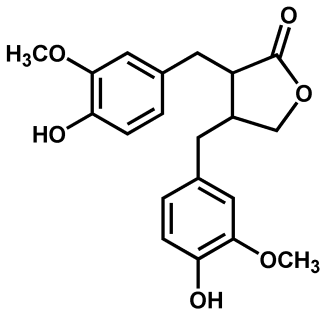
Secoisolariciresinol diglucoside



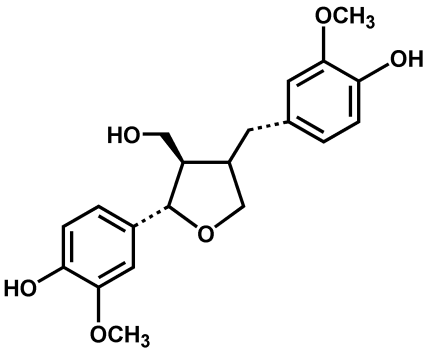
Secoisolariciresinol



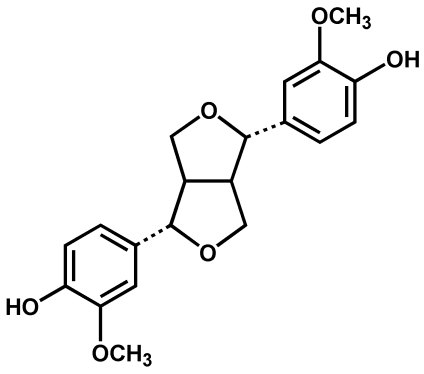
Isolariciresinol



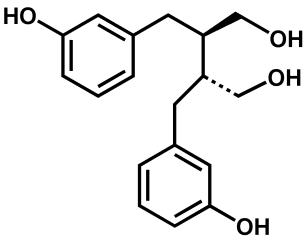
Matairesinol



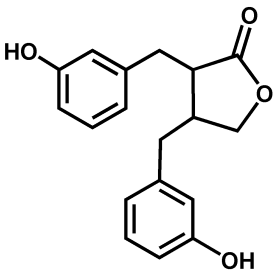
Lariciresinol



Pinoresinol

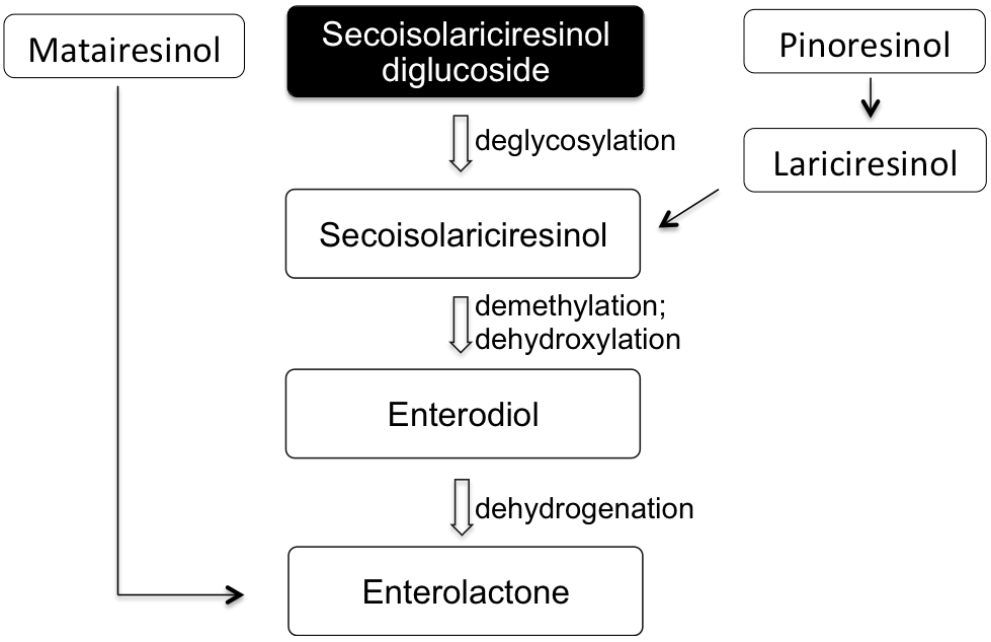


Enterodiol



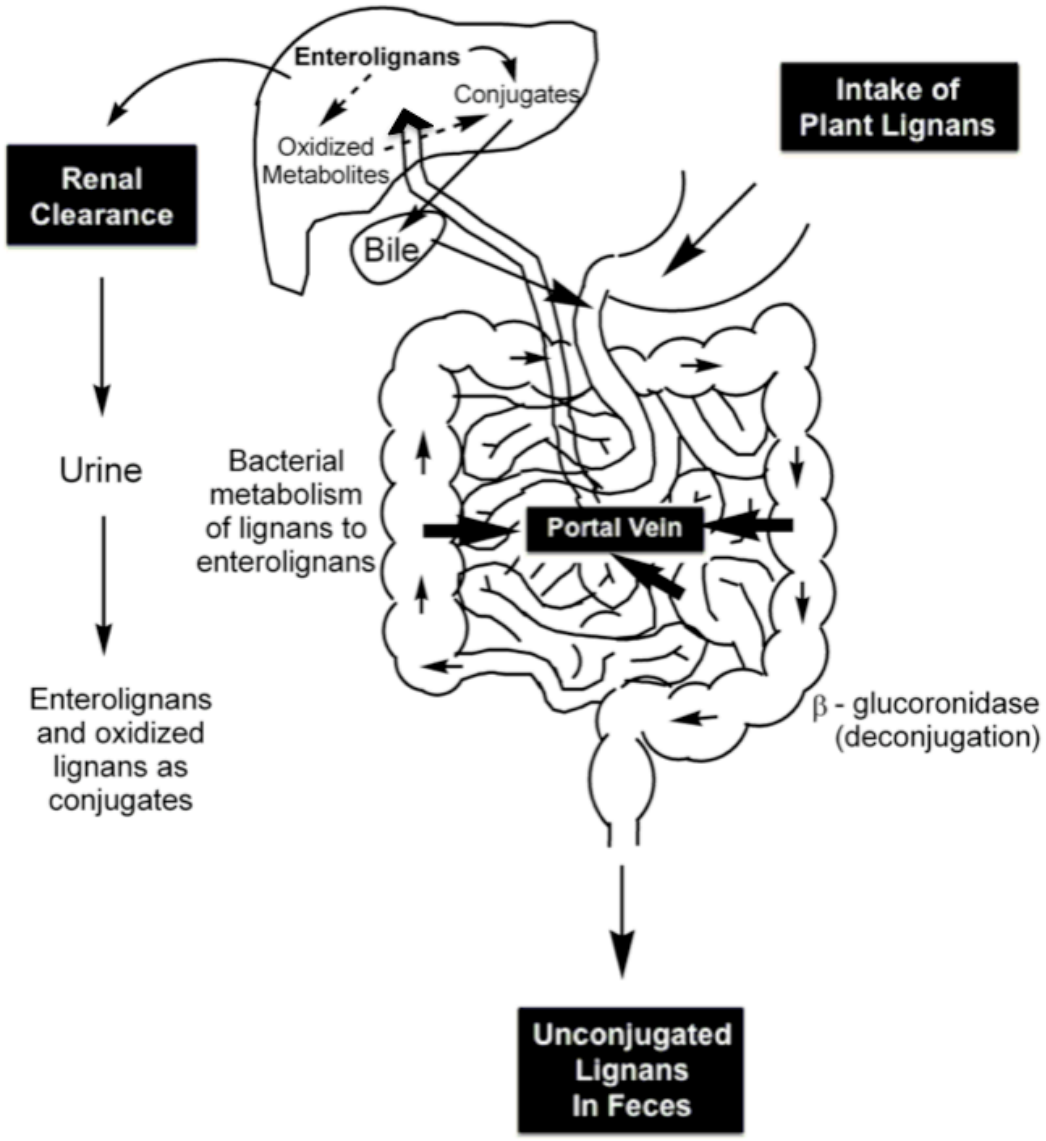
Enterolactone

Figure 9. Metabolism of plant lignans to enterodiol and enterolactone in humans.



Adapted from Clavel et al (7).

Figure 10. Enterohepatic circulation of flaxseed lignans.



Enterohepatic circulation of flaxseed lignans, bacterial metabolism and liver metabolites. Intake of plant lignans is followed by their bacterial metabolism to the mammalian lignans within the gut. Enterolignans may either be absorbed from the intestines, transported via the portal vein to the liver where they are conjugated to their glucuronide or sulphate conjugates, whereby they may either undergo enterohepatic circulation, or they are eliminated in urine. Enterolignans that are not absorbed are deconjugated and excreted in feces. (Adapted from Muir and Westcott (109) and Setchell et al (110).

2.3.3 Fiber

The total dietary fiber content of flaxseed is 27.3 g/100 g (58). Flaxseed consists of two types of fiber: insoluble and soluble. In 1 tablespoon (10.3 g) of WFX, ~2.1 g is insoluble fiber and ~1.0 g is soluble fiber (48). Insoluble fiber consists primarily of cellulose (56%, wt/wt) (111). Soluble fiber is the gel-like mucilage that can be easily extracted from flaxseed by soaking it in water (112). Mucilage fiber, also known as polysaccharide gum, comprises 8% of the overall flaxseed weight and consists of ~50-80% carbohydrates and 4-20% proteins and ash (112). The major components in the polysaccharide gum are neutral and acidic sugars. The neutral components are L-arabinose, D-xylose and D-galactose (3.5:6.2:1), whereas the acid components are a mixture of L-rhamnose, L-fucose, L-galactose and D-galacturonic acid (2.6:2:1.4:1.7) (112, 113).

The health benefits of consuming insoluble fiber are minimal; however, those of soluble fiber reflect potential for reducing several risk factors of CVD. Soluble fibers, or soluble flaxseed gums, are suggested to alter glucose absorption and may have cholesterol-lowering potential (114). *In vitro* data have demonstrated the ability for soluble flaxseed gums to bind bile acids (115), which suggests a possible mechanism for the cholesterol-lowering capacity of dietary flaxseed. As the liver converts more cholesterol into bile acids, this increases LDL-C uptake by the liver, removing it from circulation (116). Increased dietary fiber also increases satiety, therefore reducing food consumption and weight gain, which is also associated with an increased incidence of CVD. The discovery of new compounds in flaxseed mucilage continues (117-119), with many of the health benefits yet to be determined.

2.4 Cardiovascular health benefits of dietary flaxseed

2.4.1 Flaxseed in animal models

Animal models of cardiovascular disease provide valuable insights into disease processes often providing significant information regarding mechanism of action that cannot be obtained from clinical trials. Several animal models of CVD have examined the use of dietary flaxseed, or one of its extracts, in various disease processes and are discussed here.

2.4.1.1 Rabbit models

Most CVD research using rabbit models study the progression, prevention or regression of atherosclerotic plaques. It is well established that rabbits fed a hypercholesterolemic diet will develop atherosclerosis, making rabbits an ideal model to study atherogenesis (120). Previous data from our lab demonstrate that rabbits consuming a hypercholesterolemic diet along with milled flaxseed (MFX) had reduced atherosclerotic plaque development in the aorta and carotid arteries compared to animals on the same diet without flaxseed (121). Flaxseed as an intervention also improved endothelial vascular relaxation (121). In other studies, rabbits fed atherogenic diets at the onset and later switched to MFX supplemented diets had observed plaque regression after 14 weeks (122), but not after 4 weeks, despite significant reductions in TC and LDL-C (123). Dietary MFX also improved the recovery from an ischemic insult with an antiarrythmic effect, suggested to be due to a reduced QT interval (124).

The components in flaxseed that produce the anti-atherogenic and cholesterol-lowering benefits have been investigated. Prasad and colleagues have provided substantial evidence towards the cardiovascular benefits of dietary flaxseed lignans (125-130). Early

studies in hypercholesterolemic rabbits fed low ALA flaxseed (7.5 g/kg daily; containing about 2-3% of the fatty acid content as ALA) demonstrated reduced plaque development by 69% through the reduction in total-cholesterol (TC) and LDL-C (125). This is in contrast to a high ALA flaxseed (7.5 g/kg daily; containing about 51-55% of the fatty acid content as ALA) that lowered atherosclerosis by 46% without lowering circulating lipids. The null effect of flax oil (or ALA) to reduce serum lipids was substantiated in 2003 (131). Flax lignan complex reduced hypercholesterolemic atherosclerosis by decreasing oxidative stress, TC and LDL-C and by increasing HDL-C (127) and not through regression (128), but by slowing down plaque progression through reductions in oxidative stress (130). When just SDG and not the complete lignan complex was fortified in the standard chow following 2 months on an atherogenic diet, there were reduced atherosclerotic lesions that correlated with decreases in oxidative stress. In addition, the prevention of lesion progression was associated with declines in aortic oxidation and not to decreases in measured serum lipids (129).

The cardioprotective effects of MFX may also be attributed to the incorporation of omega-3 fatty acids into the tissue. All tissues (plasma, brain, liver, kidney, gastrocnemius muscle, heart, aorta and carotid artery) examined in rabbits fed a 10% (wt/wt) MFX diet exhibited significant increases in ALA (132). This may influence tissue/cell viability and may have many important functional implications. Adipose tissue was also examined to determine the effects of dietary flaxseed on adipokine signaling. Leptin expression was positively correlated with circulating ALA concentrations, which was inversely correlated with atherosclerosis (133).

2.4.1.2 Mouse models

Studies using mice have also provided valuable details regarding the anti-atherogenic effects of consuming milled flaxseed. The LDLr$^{-/-}$ model is one transgenic mouse model that can develop diet-induced atherosclerotic plaques, closely mimicking a human model of familial hypercholesterolemia (134). Mice fed a 10% (wt/wt) MFX supplemented atherogenic diet had attenuated circulating SFA and cholesterol levels and reduced aortic and aortic sinus plaques compared to those on an atherogenic diet (135). The mechanism may be via an anti-inflammatory or anti-proliferative pathway (135). In this same mouse model, several atherogenic and trans-fat diets were both prepared as controls and with the addition of either 10% MFX, 4.4% flaxseed oil rich in ALA, 7% partially defatted flax meal (PDFM) or 0.44% flax lignan (136). When only MFX or ALA were included in these dietary formulations, plaque formation was significantly reduced compared to the atherogenic control diets. These data strongly suggest that dietary fiber and lignan do not play a preventative role in atherogenesis, at least in this mouse model. The anti-atherogenic and anti-inflammatory benefits of ALA were further substantiated in ApoE deficient mice fed atherogenic diets supplemented with ALA (high or low doses) (137). Only high ALA doses decreased lesion area and reduced inflammatory markers, not low doses. Yet in another study, when atherogenic diets were fortified with either PDFM or SDG, only the PDFM diet had anti-thrombotic and anti-atherogenic action (138). PDFM contained about 33% of the ALA content found in MFX due to the tightly bound endosperm adhering to the flaxseed hull (55), providing a possible explanation for the protective effects of this diet.

The cholesterol-lowering benefits of flaxseed have been demonstrated in several studies. MFX decreased plasma TC by 19% in ApoE and 22% in LDLr$^{-/-}$ deficient mice

(139). Therefore, evidence for cholesterol lowering likely proceeds through processes related to cholesterol absorption and/or bile acid reabsorption and not through hepatic-mediated lipid lowering. Recently, SDG given at very high doses (20 mg/kg) was found to decrease serum cholesterol, LDL-C and triglycerides (140). In a model of obesity, the compounds (-)-SECO and (-)-ENL inhibited body weight, increased adiponectin and serum leptin concentrations (141), factors associated with reductions of CVD.

2.4.1.3 Rat models

The majority of studies using rats treated with dietary flaxseed have demonstrated antihypertensive benefits (142). In spontaneously hypertensive rats (SHRs) fed a whole flaxseed diet, a moderate but insignificant decrease in systolic blood pressure (SBP) was observed relative to control (143). The first study to examine flax oil as an antihypertensive observed a significant decrease in SBP within hours of ingestion in SHRs versus a high oleic sunflower oil control (144). Subsequent research on dietary oils enriched in ALA has shown a consistent pattern of BP reduction, as Sprague Dawley (SD) offspring from mothers fed dietary ALA had attenuated BP compared to rats consuming a control diet enriched in safflower oil (145, 146). Dietary supplementation with SDG induced a significant lowering of SBP in SD rats, but only when administered in the presence of a high fat diet (147).

Additionally, rat models have clearly demonstrated the cholesterol-lowering effects of dietary flaxseed. Healthy male Wistar rats fed a flaxseed flour diet had significantly lower blood lipids, blood glucose levels, aortic thickness, body weight and visceral fat compared to a control group fed casein (148, 149). The reduction of TC and LDL-C by FXO has been readily observed in rat models of hyperlipidemia (150-153). The mechanism of action was

suggested to be through activation of peroxisome proliferator-activated receptors gamma (PPAR-γ) dependent pathways by FXO thereby modifying hepatic lipid metabolism (151) or through anti-inflammatory action (154). However, when whole flaxseed, FXO and FXO + sesamin were compared with defatted flaxseed and a sesame seed diet, only the diets containing ALA were capable of reducing plasma TC concentrations (153). When flaxseed meal was fed to obese SHRs, cholesterol dropped 41% compared to lean control SHRs and hepatic lipids were significantly reduced in both lean and obese animals (155). The cholesterol lowering effects of flaxseed have also been attributed to the bioactive compound SDG. In hypercholesterolemic Wistar rats (induced via a 1% cholesterol diet), both SDG and SECO reduced body-weight gain, hepatic lipids and circulating levels of TC and LDL-C (156), yet this suggested benefit of SDG has only been demonstrated in this one study, requiring further evidence to substantiate this finding. The anti-diabetic action of SDG in rats, however, is well established.

Diabetic rat models that have been studied in relationship to dietary treatment with SDG include streptozotocin (STZ) injected SD rats, alloxan injected Wistar rats, BioBreeding rats that spontaneously develop diabetes and Zucker diabetic fatty rats. In STZ injected SD rats, SDG inhibited the incidence of diabetes by 75% and reduced serum and pancreatic oxidation (157). In BioBreeding rats, SDG treatment reduced the incidence of diabetes by 51% (158). In Zucker diabetic rats, a model of type 2 diabetes mellitus, SDG attenuated the progression of diabetes compared to untreated animals by a suggested antioxidative mechanism (159). In an additional study involving Wistar rats made diabetic via an alloxan injection, dietary MFX resulted in hypoglycemic action and reductions in TC and LDL-C (160).

2.4.1.4 Hamster models

All flaxseed interventions conducted in Golden Syrian hamsters have focused primarily on cholesterol lowering. In female ovariectomized hamsters, MFX successfully attenuated the progression of plaque formation, reduced lesion areas and the rise in TC that is characteristic of ovariectomy (161). It was also noted that TG levels were elevated significantly in the flax fed animals. When a MFX diet was compared with a FXO diet, only MFX was capable in preventing the rise in circulating TC levels (162). It was concluded that improved synthesis of bile acids is a possible mechanism of cholesterol lowering by dietary MFX. When male hamsters were fed a hyperlipidemic diet fortified with ALA, serum lipids decreased by 17-21% relative to the control diet (163). When FXO was used instead of ALA a similar response in serum lipids was observed, but also reductions in liver size, hepatic cholesterol levels and TGs (164). This study suggested that in hyperlipidemic hamsters, dietary MFX may attenuate nonalcoholic fatty liver that results from diets high in cholesterol. A similar response in blood lipids was noted in diabetic hamsters (165).

2.4.2 Cardiovascular health benefits of dietary flaxseed in human studies

A variety of human populations have been studied to determine the health benefits of dietary flaxseed. In doing so, a variety of inclusion and exclusion criteria have been established within the study design. Gender differences, subject age and health status must all be identified, in addition to the form and dose of the flaxseed intervention being used (i.e., WFX, MFX, FXO, ALA, PDFM or SDG supplements). Defining the health status of a population is often not black and white. Where the term “unhealthy” may be defined by disease status and administered medications, “healthy” could be vague and broadly defined.

For the purpose of the following discussion, "healthy" will refer to populations without chronic disease, non-smokers and individuals not being administered antihypertensive, anticoagulant or cholesterol lowering medications. However, for clarity, each study design should be carefully consulted so as to understand what criterion defines "healthy" or "unhealthy" for the representative population.

2.4.2.1 Antihypertensive effects

Several randomized, controlled clinical trials have demonstrated the antihypertensive properties of dietary flaxseed and have been outlined in **Table 3**. The Flax-PAD trial involved patients with PAD consuming 30 g/d of MFX for 24 weeks (74). An observed 10 mm Hg drop in SBP and 7 mm Hg drop in DBP occurred as a result of the flaxseed intervention. When a subgroup of hypertensive patients was examined, SBP dropped 15 mm Hg (74). In adults with metabolic syndrome (MetS), participants that received lifestyle counseling and MFX daily for 12 weeks had significant reductions in SBP (-8.8 mm Hg) and DBP (-5.0 mm Hg) compared to baseline values, but not compared to the control group that just received lifestyle counseling (166). When healthy menopausal females ingested 40 g/d MFX over 52 weeks SBP was reduced by 5.0 mm Hg and DBP by 4.1 mm Hg compared to baseline levels (167).

ALA has been suggested to be the bioactive ingredient in whole flaxseed that is responsible for lowering SBP and DBP (168). Several randomized trials that demonstrate the antihypertensive action of ALA or flax oil include studies done in hyperlipidemic patients (70, 72, 169) and one in healthy adults (73). When healthy, middle-aged adults consumed 2.6 g/d of ALA for 12 weeks, SBP dropped 5 to 8 mm Hg and DBP by 3 mm Hg (73). However,

4 weeks later, there were no longer observed differences suggesting that continued ingestion of ALA is required for antihypertensive benefits. In dyslipidemic adults, 8 g/d of ALA over 12 weeks reduced SBP by 10 mm Hg and DBP by 8 mm Hg compared to a control diet consisting of 11 g/d of LA (72).

To determine if lignans within flaxseed elicit antihypertensive action, three varieties of flaxseed were compared in postmenopausal women with vascular disease using a randomized, Latin square, double-blind, three-way crossover design (59). Patients consumed 30 g/d of MFX 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 varieties attenuated BP when individuals were given a stressful cognitive task. This study suggests that both ALA and lignans are important for attenuating BP.

In a randomized controlled trial involving adults >50 years of age participating in a walking program, normotensive subjects (14% had MetS) were asked to consume 543 mg/d of SDG from flaxseed lignan for 6 months (107). When the data was stratified by gender, only males demonstrated a decrease in DBP when consuming a flax lignan diet compared to those in the placebo (tablet containing 550 mg of 90 % maltodextrin and 10 % caramel colour) group. This significant group × sex × time interaction existed only for DBP and was not observed in females. When all individuals (no gender exclusions) with MetS were sub-grouped, the flax lignan group once again demonstrated a significant 7 mm Hg reduction in DBP over time compared to placebo (**Table 3**).

Recently, a high SDG lignan supplement was assessed for its hypotensive effects in a healthy population >48 years of age (108). BeneFlax is a commercially available source of

SDG lignan that provides 543 mg of SDG in a single tablet. Individuals consumed one BeneFlax tablet, or a placebo, daily for 6 months in this randomized, double blind, placebo-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).

Table 3. Characteristics of the clinical studies reporting blood pressure data as a result of consuming whole flaxseed, flax oil, flaxseed lignans or flax fiber.

Author, Publication year and reference	Study Design	Population (n)	Age (years)	Dietary Intervention	Placebo or Control	Treatment Duration	SBP (mm Hg)	DBP (mm Hg)	Other Outcomes
Whole Flax									
Rodriguez-Leyva et al, 2013 **(74)**	RP	PAD patients (110)	67 ± 9	30 g/d ground FX added as sprinkles or contained in baked food products (bagels, snack bars, buns, muffins, pasta)	30 g/d whole wheat as sprinkles or in baked food products.	24 wk	-10*	-7**	- 15 mm Hg (SBP) in hypertensive patients
Wu et al, 2010 **(166)**	RP	Adults with MetS (283)	49 ± 8	LC + 30 g/d FX baked into bread.	LC only	12 wk	-8.8* (vs baseline)	-5.0* (vs baseline)	Central obesity status improves with LC+FX.
Dodin et al, 2005 **(167)**	RP	Healthy, menopausal females (179)	54 ± 4	40 g/d ground FX: 20 g/d provided in 2 slices of bread + 20 g/d provided as sprinkles.	40 g/d ground wheat germ: 2 bread slices + sprinkles	52 wk	-5** (vs baseline)	-4.1** (vs baseline)	FX reduced total cholesterol & HDL compared to placebo.
Stuglin et al, 2005 **(170)**	None	Healthy adult males (15)	27 ± 2	32.7 g/d ground FX baked in muffins.	None	4 wk	-1.2 (NS)	-1.2 (NS)	
Spence et al, 2003 **(59)**	RC	PM females with vascular disease (35)	62 ± 8	30 g/d ground FX a) high lignan, low ALA; b) low lignan, high ALA; c) intermediate lignan and ALA FX was added to cereal, salads, juice or other foods.	No suitable placebo could be found.	12 wk on each diet	a) -2.9** b) -4.6** c) -5.4**	a) -0.8 (NS) b) -2.1 (NS) c) -2.7 (NS)	MAP decreased by -1.1* (a), -2.4* (b) and -3.7* (c) mm Hg
Flax Oil									
West et al, 2010 **(70)**	RC	HC adults (20)	49 ± 2	a) 19 g FXO (10.5 g ALA) + 37g W + 15g WO [6.5 % ALA] b) 0 g FXO + 37g W + 15g WO [3.5 % ALA] Given in baked goods, salad dressings, etc.	Average American diet (0.8 % ALA/d)	6 wk on each diet	-2.1 (NS)	-2 to -3*** for diets (a) and (b)	Diets (a) & (b) ↓TPR by 4%*. FMD ↑34% on (a).
Takeuchi et al, 2007 **(73)**	RP	Healthy adults (127)	46 ± 9	2.6g/d ALA in a 14 g enriched oil (rapeseed oil + FXO + rice oil) Given in bread rolls.	14 g of common blended oil (rapeseed oil + soybean oil)	12 wk	-5 to -8 after 4*, 8* & 12* wk	-3 after 12* wk	NC in SBP or DBP after 16 wk follow-up.
Paschos et al, 2007 **(72)**	RP	Dyslipidemic adults (87)	52 ± 1	15 ml FXO (8 g/d ALA; n=59) 1 tsp (5ml) with each meal.	15 ml SO (11 g/d LA; n=28)	12 wk	-10* (3.1%)*	-8* (-6.3%)*	-8 mm Hg* -6.0%* in MAP.
Nestel et al, 1997 **(169)**	RC	Obese adults (15)	54±6	a) 20 g/d FXO - ALA low fat diet b) 20g/d Sunola Oil - Oleic low fat diet	Saturated fat diet (Subjects started and ended on this diet)	4 wk on each diet	NM	NM	↓ MAP by 13* mm Hg and ↑ systemic arterial compliance w/ ALA***.

Author, Publication year and reference	Study Design	Population (n)	Age (years)	Dietary Intervention	Placebo or Control	Treatment Duration	SBP (mm Hg)	DBP (mm Hg)	Other Outcomes
Flax Lignan									
Billinsky et al, 2013 **(108)**	RP	Healthy adults (94)	61 ± 7	543 mg/d lignan tablet (BeneFlax)	Placebo tablet	24 wk	<65 y: -2 NS ≥65 y: +4 NS	<65 y: -2 NS ≥65 y: 0 NS	No hypotensive effect in healthy adults.
Barre et al, 2012 **(102)**	RC	Adults with T2DM (16)	66 ± 2	600 mg SDG/d in 4 capsules	4 placebo capsules	12 wk on each diet	0.6 (NS)	-3.4 (NS)	FX lignan ↓ WC, and prothrombotic state.
Cornish et al, 2009 **(107)**	RP	Older adults (81)	62 ± 1	543 mg/d SDG in tablet form	Placebo tablet (550 mg of 90% maltodextrin and 10% caramel colour.	24 wk	NC	-4* in males; -6.7** in patients with MetS	FX lignan reduced MetS score in males.
Pan et al, 2007 **(103)**	RC	Adults with T2DM and mild HC (68)	63 ± 7	FX lignan capsules (360 mg/d)	Placebo capsules	12 wk on each diet	+0.3 (NS)	-2 (NS)	FX lignan improved glycemic control (HbA_{1c}**)
Hallund et al, 2006 **(101)**	RC	Healthy PM females (22)	61 ± 7	500 mg/d SDG in a low fat muffin	A low fat muffin	6 wk on each diet	-2 (NS)	-2 (NS)	
Flax Fiber									
Thakur et al, 2009 **(114)**	RP	T2DM patients (120)	49 ± 5	5g of FX gum in wheat flour chapattis	Wheat flour chapattis	12 wk	-16 ± 4 (NS)	-8 ± 2 (NS)	-10 ± 4 mm Hg (NS) in MAP. TC*, LDL* and FBS* all were reduced with FX

ALA, alpha-linolenic acid; BMD, bond mineral density; DBP, diastolic blood pressure; FBS, fasting blood sugar; FMD, flow-mediated dilation; FX, flaxseed; FXO, flax oil; HC, hypercholesterolemic; HDL, high density lipoprotein; LA, linoleic acid; LC, lifestyle counselling; LDL, low density lipoprotein; MAP, mean arterial pressure; MetS, metabolic syndrome; N/A, not applicable; NC, no change; NM, not measured; NS, not significant; PAD, peripheral artery disease; PM, postmenopausal; RP, randomized parallel design; RC, randomized crossover design; SBP, systolic blood pressure; SO, safflower oil; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TPR, total peripheral resistance; W, walnuts; WC, waist circumference; WO, walnut oil. Statistical significance: **P*<0.05; ***P*<0.01; ****P*<0.001.

The antihypertensive action of dietary fiber is well established in several meta-analyses (171, 172). In data from 24 trials published from 1966 to 2003, an average fiber dose of 11.5 g/d can decrease SBP by -1.13 mm Hg and DBP by -1.26 mm Hg (172). Based upon 8 clinical trials, 7.2 to 18.9 g/d of dietary fiber provides maximal reductions (-3.4 mm Hg in SBP and -1.97 mm Hg in DBP) (171). Fiber doses >19 g/d do not provide additional BP lowering. Soluble fiber produces most of the antihypertensive benefits, accounting for 85% of the fiber-associated decreases in SBP and 59% in DBP (172). 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 mean arterial pressure (MAP) (-16, -8 and -10 mm Hg) (114). Higher doses of soluble fiber may be required to elicit significant anti-hypertensive action.

2.4.2.2 Cholesterol lowering effects

Health Canada has recently approved a health claim recognizing the cholesterol lowering benefits of consuming flaxseed in adults (10). The suggested dose was 40 g/d and was based upon several studies using this amount (167, 173-175). Of the studies reported in adults using whole flaxseed, the effects of TC and LDL-C lowering are noted, with minimal influence on HDL-C and TG levels (**Table 4**). This is consistent with reports of flaxseed reducing circulating cholesterol in hemodialysis patients (176), in patient populations with hyperlipidemia (174, 177-179) and postmenopausal women (173, 180). In healthy control populations, the effects of flaxseed are less consistent with a modest cholesterol-lowering effect at higher flaxseed concentrations (40 to 50 g/day) (167, 181-183) and no effect at doses of 30 g/day (5, 184). However, in children, the outcome may be very different. In

hypercholesterolemic children given 30 g/d of MFX, non-significant decreases in TC and LDL-C were observed, but more alarmingly was the significant decrease in HDL-C (-15%) and increase in TG (+26%) (185). The cholesterol-lowering effects of flaxseed in children may be very different from those observed in adults.

Most of the published evidence in humans does not support FXO, and therefore ALA, as having a primary role in cholesterol lowering (81, 179). FXO reduced TC and LDL-C in only one study involving healthy adults (186), but not in five others (5, 81, 184, 187, 188). In a clinical trial involving unhealthy obese adults, there were no changes in TC, LDL-C and TG, yet there was a significant reduction in HDL-C (169). In a recent study involving hemodialysis patients, the FXO group significantly decreased TC (-7.6%) and LDL-C (-11.1%) and increased HDL-C (8.1%) when compared to baseline values within the same treatment group (77). A drawback of this study was that baseline cholesterol values between the two groups were significantly different from each other, with the FXO group being substantially higher than the placebo group. Alternatively, higher doses of flax lignans in healthy (99) or unhealthy (100) individuals, or defatted flaxseed in hyperlipidemic patients (178), have been suggested to play a role in lowering plasma cholesterol (**Table 4**). Type 2 diabetic patients consuming 5 g/d of flaxseed gum for 12 weeks noted significant attenuations in TC and LDL-C without any changes in HDL-C or TG (114). Soluble mucilage fiber may play a valuable role in cholesterol lowering which is consistent with other conclusions in the literature (81, 173, 189).

Table 4. Characteristics of the clinical studies reporting plasma or serum cholesterol and triglyceride values as a result of consuming whole flaxseed, flax oil, flaxseed lignans or flax fiber.

Author and reference	Study Design	Population (n)	Age (years)	Dose of Flaxseed	Placebo or Control	Study Period	Plasma Bioactives	TC (%)	LDL-C (%)	HDL-C (%)	TG (%)	Other lipid-based parameters
Whole Flax												
Edel et al. (6)	RP	PAD patients (110)	67 ± 9	30 g/d MFX	Whole wheat	52 wk	+ ALA‡ + END‡ + ENL‡	-11%NS(4 wk & 24 wk)	-15% NS (4 wk & 24 wk)	NC	NC	N/A
Wong et al. **(185)**	RP	HC in children (32)	13 ± 2	30 g/d MFX	Whole wheat flour	4 wk	NM	-4% NS	-5% NS	-15%‡	+26%*	N/A
Khalabarti-Soltani et al. **(176)**	RP	HD patients (38)	54 ± 4	40 g/d MFX	Normal diet	8 wk	+ ALA†	-15% †	-16.9% †	+16.2% †	-38.5% †	N/A
Patenaude et al. (5)	UC	Younger and older healthy adults (20)	18 – 29 & 45 - 69	30 g/d MFX (6 g of ALA)	None	4 wk	+ ALA*	NC	NC	NC	NC	N/A
Bloeden et al. **(174)**	RP	HC adults (62)	44 - 75	40 g/d MFX	Wheat bran	10 wk	+ALA‡	NC	-13%** (5wk) -7% NS (10wk)	(M): -16%* (5wk) -9%* (10wk)	NC	-14% Lp[a]
Patade et al. **(180)**	RP	HC, PM females (55)	47 - 63	30 g/d MFX	Oat bran fiber	12 wk	NM	-7%*	-10%*	NC	NC	N/A
Dodin et al. **(167, 183)**	RP	MP, healthy, females (179)	54 ± 4	40 g/d MFX	40 g/d ground wheat germ	52 wk	+ALA‡	-5.3%*	NC	-5.3%*	NC	+apo A-I*, +apo B*, +Lp[a]‡, -LDL particle size‡
Austria et al. **(184)**	UC	Healthy adults (20)	18 - 49	30 g/d MFX; 30 g/d WFX	None	12 wk	+ALA† (MFX)	NC	NM	NM	NC	N/A
Mandasescu et al. **(177)**	RP	Mildly HC adults (40)	NR	20 g/d MFX	Low fat diet	60 d	NM	-17%*	-3.9%*	NC	-36%*	-33.5%* TC/HDL-C ratio
Stuglin et al. **(170)**	UC	Healthy adult males (15)	27 ± 2	32.7 g/d MFX	None	4 wk	NM	NC	NC	NC	+29%*	N/A
Lucas et al. **(175)**	RP	PM females (58)	54 ± 8	40 g/d MFX + 1 g Calcium + 400IU VitD	Wheat + 1 g Calcium + 400IU VitD	12 wk	NM	-6%*	-4.7%NS	-4.8%NS	-13%NS	-6% apo A-1†, -7.5% apo B†, -6% nonHDL*
Lemay et al. **(190)**	RC	HC, MP females (25)	58 ± 5	40 g/d crushed FX	HRT	8 wk	NM	NC	NC	NC	NC	N/A
Clark et al. **(191)**	RC	Adults w/ LN (15)	NR	30 g/d MFX	Time points with and w/o diet	52 wk	+ ALA	NC	NC	NC	NC	N/A

Author and reference	Study Design	Population (n)	Age (years)	Dose of Flaxseed	Placebo or Control	Study Period	Plasma Bioactives	TC (%)	LDL-C (%)	HDL-C (%)	TG (%)	Other lipid-based parameters
Arjmandi et al. **(173)**	RC	HC, PM females (38)	56 ± 7	38 g/d WFX	Sunflower seed	6 wk	NM	-6.9%†	-14.7%‡	NC	NC	-7.4%* Lp[a]
Cunnane et al. **(182)**	RC	Healthy adults (10)	25 ± 3	50 g/d MFX	Wheat flour	4 wk	+ALA*	-6%*	-9%*	NC	NC	N/A
Clark et al. **(192)**	UC	Adults w/ LN (9)	32 - 72	15, 30 and 45 g/d MFX	None	4 wk at each dose	+ ALA* (30g)	-11%* (30g); -9%* (45g)	-12%* (30g)	NC	-4%NS (45g)	N/A
Cunnane et al. **(181)**	UC	Healthy females (9)	30 ± 4	50 g/d MFX	None	4 wk	+ ALA*	-9%*	-18%*	NC	NC	N/A
Flax Oil												
Kontogianni et al. **(186)**	RC	Healthy adults (37)	26 ± 6	13.8 g/d FXO (8 g of ALA)	13.8 g/d Olive oil (0.13 g ALA)	6 wk	+ ALA †	-5.0%† vs baseline	-6.7%† vs baseline	NC	NC	N/A
Lemos et al. **(77)**	RP	HD patients (114)	59 ± 13	2.0 g of FXO + α-tocopherol (7 mg)	2.0 g of MO + α-tocopherol (7 mg)	17 wk	NM	-7.6%† vs baseline	-11.1%‡ vs baseline	8.1%† vs baseline	NC vs baseline	N/A
Patenaude et al. (5)	UC	Younger and older healthy adults (20)	18 - 29 & 45 - 69	12 g/d FXO (6 g of ALA)	None	4 wk	+ ALA*	NC	NC	NC	-21%* (oil diet; 18-29 group)	N/A
Austria et al. **(184)**	UC	Healthy adults (10)	18 - 49	12 g/d FXO (6 g of ALA)	None	12 wk	+ALA‡	NC	NM	NM	NC	N/A
Kaul et al. **(187)**	RP	Healthy adults (86)	35 ± 2	2 g/d FXO	Placebo (fish oil and hempseed oil)	12 wk	+ ALA* (6wk)	NC	NC	NC	NC	N/A
Harper et al. **(81)**	RP	Healthy adults (56)	49 ± 11	5.2 g/d of FXO (3 g/d of ALA)	Olive oil	26 wk	NM	+8.3%*	NC	NC	NC	N/A
Schwab et al. **(188)**	RC	Healthy adults (14)	71 ± 9	30 mL/d FXO	30 mL/d hemp oil	4 wk	+ ALA‡	NC	NC	NC	-9%*	NC in apo A-I -apo B†
Nestel et al. **(169)**	RP	Obese adults (15)	54 ± 6	20 g/d FXO	Sunola sunflower oil	4 wk	+ALA‡	NC	NC	-8.3%*	NC	N/A
Flax Lignan												
Almario et al. **(99)**	RP	Healthy adults (37)	54 ± 7	High lignan (HL; 0.41 g) flax bar (3.0 g ALA)	Low lignan (LL; 0.15 g) flax bar (3.0 g ALA)	6 wk	NM	-12%* (HL)	-15%* (HL)	-trendNS	NC	-25% oxLDL* (HL)

Author and reference	Study Design	Population (n)	Age (years)	Dose of Flaxseed	Placebo or Control	Study Period	Plasma Bioactives	TC (%)	LDL-C (%)	HDL-C (%)	TG (%)	Other lipid-based parameters
Fukumitsu et al. **(100)**	RP	HC males (30)	35 ± 3	SDG 20mg; SDG 100 mg	Placebo capsules	12 wk	NM	NC	NC	NC	NC	-16% LDL-C/HDL-C ratio* (100 mg)
Zhang et al. **(104)**	RP	HC adults (55)	54 - 58	300 or 600 mg/d SDG	Placebo w/o SDG	8 wk	+END* +ENL* +SECO*	-22%†	-24%†	NC	NC	N/A
Hallund et al. **(101)**	RC	PM, healthy females (22)	61 ± 7	500 mg/d SDG	Placebo w/o SDG	6 wk	+END‡ +ENL‡	NC	NC	NC	NC	N/A
Jenkins et al. **(178)**	RC	HC adults (29)	57 ± 2	50 g/d PDFX (20 g/d fiber)	Wheat bran	3 wk	NM	-4.6%‡	-7.6%‡	NC	+10%*	-5.4% apo B*** -5.8% apo A-I**
Flax Fiber												
Thakur et al. **(114)**	RP	T2DM adults (120)	49 ± 5	5 g of FX gum	Wheat flour	12 wk	NM	-10.4%*	-16.4%*	NC	NC	N/A

Abbreviations: ALA, alpha-linolenic acid; apo A-I, apolipoprotein A-I; apo B, apolipoprotein B; END, enterodiol; ENL, enterolactone; FX, flaxseed; FXO, flax oil; HC, hypercholesterolemic; HD, hemodialysis; HDL-C, high density lipoprotein-cholesterol; HL, high lignan; HRT, hormone replacement therapy; LA, linoleic acid; LDL-C, low density lipoprotein-cholesterol; LL, low lignan; LN, Lupus Nephritis; MetS, metabolic syndrome; MO, mineral oil; MP, menopausal; N/A, not applicable; NC, no change; NM, not measured; NS, not significant; oxLDL; oxidized low density lipoprotein; PAD, peripheral artery disease; PDFX, partially defatted flaxseed; PM, postmenopausal; RP, randomized parallel design; RC, randomized crossover design; SECO, secoisolariciresinol; SDG, secoisolariciresinol diglucoside; SO, safflower oil; T2DM, type 2 diabetes mellitus; TC, total cholesterol; UC, uncontrolled; WFX, whole flaxseed.

2.4.2.3 Other Cardiovascular Related Benefits of Consuming Flaxseed

Dietary flaxseed has other cardiovascular related benefits beyond blood pressure and cholesterol lowering. It has been studied for effects on body morphometrics with significant improvements noted by dietary MFX with weight loss and attenuated body mass index (BMI) (167), on reduced waist circumference (78), in central obesity status when coupled with lifestyle counseling (166) and with dietary SDG (600 mg/d) in reducing waist circumference (102). Dietary flaxseed, but not flaxseed oil, may have beneficial effects in diabetic control. MFX at doses of 40-50 g/d decreased glucose in hypercholesterolemic menopausal females (190) and postprandial blood glucose levels in healthy women (181). Flaxseed gum attenuated fasting blood glucose (114) and SDG from flaxseed (360 mg/d) increased glycemic control (HbA1c) in patients with type 2 diabetes mellitus (T2DM) (103). FXO did not appear to have any effect on blood glucose, insulin, HbA1c or on glycemic control in patients with T2DM (78, 79). The anti-inflammatory effects of 30 g/d of flaxseed flour have also been observed with decreases in CRP and serum amyloid A (SAA) (193) and in CRP with flaxseed derived lignan supplements (106). To date, studies examining the cardiovascular benefits of dietary flaxseed involve intervention periods as short as 4 weeks (181) up to a maximum of 1-year (167), with the majority of studies lasting 12 weeks (102).

3. PLASMA BIOAVAILABILITY OF THE FLAXSEED BIOACTIVES ALA AND SDG-DERIVED ENTEROLIGNANS

The term bioavailability represents the amount of a circulating bioactive ingredient that is available for uptake by peripheral tissues following administration of a bioactive or a bioactive-precursor. ALA from flaxseed is bioavailable in circulation, yet its bioavailability depends on the form in which it is ingested. Approximately 6 g of ALA is contained in 30 g of MFX or WFX and in 12 g of FXO (184). When healthy, younger adults ingested ALA in one of each of these forms for 4 and 12 weeks, circulating concentrations following absorption varied substantially despite the same quantity of ALA in each flaxseed intervention (184). When ALA was ingested as FXO, plasma concentrations were significantly greater than those measured in the MFX and WFX groups. However, milling flaxseed significantly increased the circulating ALA concentrations from baseline values and from those measured in the WFX group at equal time points. ALA concentrations were not elevated at either time point when flaxseed was consumed as WFX. This study clearly demonstrated that milling flaxseed enhances the absorption of ALA and the plasma bioavailability of ALA. Since flaxseed oil is susceptible to rancidity, MFX is a valuable alternative for increasing circulating levels of ALA, with the combined advantage of dietary lignans and fiber.

Increased plasma ALA concentrations as a result of MFX consumption have been reported in a number of human studies. When doses as low as 13 g MFX/d were ingested, plasma ALA concentrations increased 1.4 times from baseline values and only 1.6 times when the dose was doubled (194). When 30 g/d of MFX was ingested, ALA concentrations once again doubled from those reported at baseline regardless of shorter intervention periods

(4 or 12 weeks) (5, 184) or longer ones (6 months) (74). Even higher doses of 40 g/d only doubled ALA concentrations from baseline values after 10 weeks (174) and even after one year (183) further suggesting that higher doses and longer ingestion periods do not improve plasma ALA bioavailability beyond lower doses and shorter treatment periods, but simply sustain it. In another study, participants ingested 50 g/d of MFX where ALA in plasma phospholipids increased 1.3 times and doubled in plasma triglycerides after 2 and 4 weeks (182). This evidence from multiple studies clearly suggests the upper-limit threshold of plasma ALA concentrations following ingestion of various doses of MFX (13 g/d up to 50 g/d) without improvements resulting from longer ingestion periods. However, dietary MFX is also a rich source of lignans whose metabolites have their own associated health benefits and are also present in circulation.

Whole forms of flaxseed contain plant lignans, whereas flaxseed oil or its extracts do not. Milling whole flaxseed, compared to unmilled WFX or even crushed flaxseed, substantially improves enterolignan plasma bioavailability (195). After 10 days of daily dietary supplementation with 0.3 g of MFX per kg of body weight (ie., an adult weighing 160 lbs would consume 21.8 g/d of MFX), plasma concentrations of END and ENL were significantly increased (195).

Large inter-individual variations are observed when measuring enterolignan concentrations whereas this is not observed when measuring ALA levels following ingestion of flaxseed. This is largely due to differences in metabolism. Lignans require gut microflora to facilitate their conversion to enterolignans unlike dietary fat which requires bile salts and pancreatic lipase to liberate the fatty acids (ie., ALA). Individual variations in gut microflora play a primary role in the amounts of END, ENL and total enterolignans produced (8, 9)

lending to variability in reported plasma concentrations (195-197). These same studies used 20 to 25 g/d of MFX and reported increases in plasma END concentrations ranging from 54 to 840 times those reported at baseline. ENL increases were approximately 20-fold. Baseline plasma END concentrations tended to be negligible in individuals not consuming lignan-rich foods or vegetarian diets, which explains the extremes in fold-increases observed for END. Alternatively, baseline concentrations of ENL are typically above the limits of instrument detection having upper-end concentrations of 20 nM in Canadian and Australian females consuming standard diets (197, 198). To maintain significant circulating levels of enterolignans, continuous ingestion of lignan-rich foods, like flaxseed, is advised (198, 199).

CHAPTER III: RATIONALE AND HYPOTHESES

Rationale

Dietary flaxseed contains many valuable nutrients that are beneficial to our cardiovascular health. Studies involving humans ingesting MFX have predominantly examined the health effects resulting from ALA, despite the presence of other potentially beneficial cardioprotective constituents, namely fiber and lignans. The lignan metabolites, enterolignans, have been far less studied than ALA and fiber, which is a major gap that this research intends to address. Yet, as MFX contains each of these bioactives, the presence of each component, along with any potential synergisms, will be explored. The antioxidative and estrogenic/anti-estrogenic potential of enterolignans has been established in various model systems; however, evidence in humans is lacking. Prior to assessing the overall health benefits of enterolignans in humans, it is first necessary to identify and quantify their presence in circulation under a variety of human conditions. As a result, our aim is to supplement the diets of both healthy and unhealthy human populations with MFX to determine if factors such as flaxseed dose, participant age and cardiovascular disease status influence circulating enterolignan concentrations and metabolism.

The flow of experiments to assist in developing this thesis project proceeds as follows. Firstly, current methods of measuring plasma enterolignans are lengthy and cumbersome, so we need to design and validate a SLE and GC/MS-μSIS method to extract and quantify END and ENL that could be applied to a series of human studies. Secondly, many scientific studies employ MFX as a therapeutic intervention, yet the required MFX dose to significantly elevate plasma ALA and enterolignan concentrations has not yet been defined. Therefore, we will explore the effect of MFX dose on plasma concentrations of

these bioactives in healthy adults. Thirdly, as aging is associated with declines in metabolism and alterations in gut bacterial strains, it is necessary to study the influence of age on lignan metabolism and therefore enterolignan bioavailability in a healthy population of both younger and older adults prior to looking at a diseased state. Fourthly, the effect of MFX and its bioactive ingredients on circulating cholesterol levels in older patients with pre-existing CVD is unknown. PAD is predictive of future cardiac events. A major risk factor of PAD is hypercholesterolemia making this an ideal diseased state to not only investigate MFX metabolism and enterolignan bioavailability, but also cholesterol lowering. MFX can lower cholesterol levels in healthy adults as has been established in a health claim by Health Canada; however, it has never been explored in patients with PAD administered concurrent CLMs. Therefore, quantities of ALA, END and ENL in plasma can then be directly correlated to measured blood lipids to provide insight into potential mechanisms. Additionally, we want to determine if MFX administered concurrently with CLMs influences cholesterol-lowering in these patients.

Hypotheses

1. We hypothesize that supported liquid extraction (SLE) will be a powerful tool for extracting and concentrating enterolignans from human plasma.
2. We hypothesize that consuming dietary MFX in increasing amounts (10 g/d up to 40 g/d) will increase circulating concentrations of the flaxseed bioactives ALA, END and ENL in a dose-dependent manner.
3. We hypothesize that supplementing the diet with MFX will alter circulating concentrations of enterolignans in an age-dependent manner and that plasma ENL

concentrations will be greater in older adults than younger adults due to longer transit times and therefore conversion times of END to ENL in the gut.

4. We hypothesize that dietary MFX will reduce total and LDL-C in patients with PAD and that it will not inhibit the action of CLMs, but instead provide additional cholesterol lowering action beyond that of CLMs alone.

CHAPTER IV: OBJECTIVES

1. To develop and validate an analytical extraction method using SLE to isolate lignan metabolites from human plasma. We will also develop an analytical method using GC/MS to quantify enterolignan concentrations (Chapter V, pages 53-86).
2. To determine the optimal dose of MFX to provide the highest circulating concentration of ALA and enterolignans in healthy adults (Chapter VI, pages 87-130).
3. To determine if the ALA metabolites EPA, DPA and DHA rise as a result of increased MFX dose (Chapter VI, pages 87-130).
4. To determine which dose of MFX provides the best cholesterol-lowering benefits, without inhibiting platelet aggregation or producing adverse events (Chapter VI, pages 87-130).
5. To determine if younger and older healthy adults metabolize lignan metabolites from flaxseed ingestion in a similar manner (Chapter VII, pages 131-166).
6. To determine if MFX consumption for one year can lower circulating concentrations of total-cholesterol and LDL-cholesterol in patients with PAD compared to a control group (Chapter VIII, pages 167-206).
7. To determine if body morphometrics change as a result of consuming either the MFX or control dietary interventions for one year (Chapter VIII, pages 167-206).
8. To determine if cholesterol-lowering medications were prescribed less in patients consuming a MFX dietary intervention compared to those in the control group (Chapter VIII, pages 167-206).
9. To determine if dietary MFX improves cholesterol-lowering in a subgroup of patients administered CLMs compared to a control group (Chapter VIII, pages 167-206).

CHAPTER V: Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults

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Abbreviations: ALA, alpha-linolenic acid; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; END, enterodiol; ENL, enterolactone; GC/MS-μSIS, gas chromatography/mass spectrometry-micro selected ion storage; HPLC-CEAD, high performance liquid chromatography-coulometric electrode array detection; LLE, liquid liquid extraction; LOD, limit of detection; LOQ, limit of quantitation; SDG, secoisolariciresinol diglucoside; SIM, single-ion monitoring; SLE, supported liquid extraction; SPE, solid phase extraction; TMS, trimethylsilylated; TR-FIA, time resolved fluoroimmunoassays.

ABSTRACT

Dietary interventions involving foods that are enriched in lignans, such as flaxseed, are drawing attention due to their beneficial protective effects in various diseases and human conditions. Accurate quantitation of key lignan metabolites such as enterodiol (END) and enterolactone (ENL) is necessary in order to identify factors that may influence overall bioavailability. Here we describe the validation of a novel supported liquid extraction (SLE) method for isolation of plasma enterolignans, END and ENL, using $^{2}H_{6}$-labeled isotopes with gas chromatography-mass spectrometry in micro selected ion storage (GC/MS-μSIS) mode. Following enzymatic hydrolysis and SLE extraction with 70:30 diethyl ether: ethyl acetate, enterolignans were rapidly separated within 8 minutes. SLE in combination with GC/MS-μSIS gave high recoveries of 96.4% and 96.0% for END and ENL, respectively. Intra-assay precision ranged from 2.5 to 5.9% for both compounds whereas the inter-assay precision was 2.6 to 6.9%. SLE was also directly compared to liquid liquid extraction (LLE). Both techniques offered high precision and accuracy; however, SLE consistently enabled successful analyte extractions and derivatizations, unlike LLE, which had an ~4% failure rate. SLE was also tested in a study where dietary milled flaxseed supplementation (30 g/day for 1 month) and enterolignan bioavailability was examined in a healthy, human population (n=10). Plasma total enterolignan levels significantly increased ($p = 0.002$) at 4 weeks relative to baseline. Average concentrations for END and ENL were 209 nM and 304 nM, respectively.

Keywords: Enterolignans, plasma, supported liquid extraction, gas chromatography, mass spectrometry, flaxseed

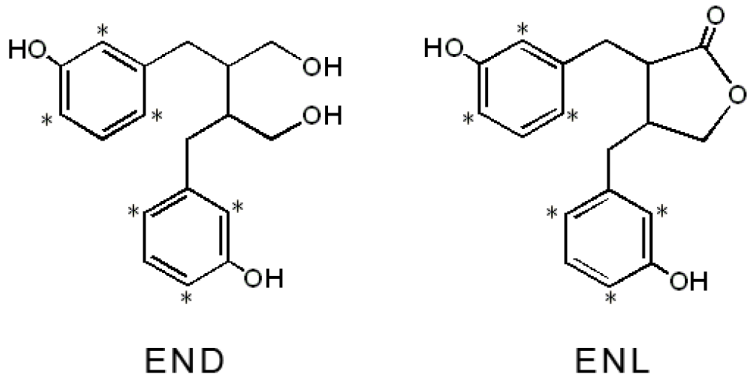
INTRODUCTION

Epidemiological studies have shown the beneficial effects that diets rich in whole grains [1], seeds (flaxseed and sesame) [2-4], fruits and vegetables [5] and beverages (teas and wine) [6] have on lowering cardiac risk factors. These foods are very high in plant lignans which have protective effects against various cancers and cardiovascular diseases [7, 8]. Flaxseed or linseed (Linum usitatissimum) is not only one of the richest sources of the cardioprotective n-3 fatty acid, alpha-linolenic acid (ALA), but also of polyphenolic plant lignans. Secoisolariciresinol diglucoside (SDG) is the most prevalent lignan found in flaxseed followed by matairesinol, lariciresinol and pinoresinol [9]. In nature, lignans exist primarily as glycosides [10] and upon consumption are metabolized by microflora in the intestines to form the enterolignans enterodiol (END) and/or enterolactone (ENL) (**Figure 11**). They are then absorbed by the gut and repackaged in the liver as β-glucuronide or sulfate conjugates, which then circulate within the bloodstream or are eliminated in urine or feces [11]. Information on inter-individual differences in END and ENL production have been reported in several studies in humans [11-13] and are suggested to be influenced by the nature of the host bacteria in the intestinal tract [14]. In terms of the beneficial properties of enterolignans it has been suggested that they may behave as antioxidants *in vivo* as these properties have already been demonstrated *in vitro* [15].

Traditional methods of extracting enterolignans from plasma involve lengthy liquid liquid extraction (LLE) [16] or solid phase extraction (SPE) steps [17]. Supported liquid extraction (SLE) is a relatively new technology that offers advantages of high recoveries, removal of the matrix effect, reduced sample preparation times, loss of emulsions and reduced sample volumes. SLE and LLE are comparable in analyte recoveries yet SLE offers

improvements due to matrix cleanup qualities [18]. SLE is conceptually analogous to traditional LLE; however, it uses an inert support material of modified diatomaceous earth, with a high surface area for extraction interface and a modified flow-through technology [19]. Unlike SPE, SLE does not require solvent preconditioning and is directly loaded with aqueous sample to which water absorbs to the column bed, thus effectively removing it from the equation. Analytes are eluted from both SPE and SLE columns using solvents; however, the initial loading conditions, as predetermined by the stationary phase, differentiate the flow-through technology of the two techniques. Current applications involving SLE related to plasma bioanalysis include matrix purification [20], circulating drugs [20-23], steroid hormones [19], vitamin D [24] and phospholipid removal [25].

Figure 11. Chemical structures of END, $^{2}H_{6}$-END, ENL and $^{2}H_{6}$-ENL.



* Refers to the location of the deuterium ions in [2,2^{1},4,4^{1},6,6^{1}-$^{2}H_{6}$] enterodiol ($^{2}H_{6}$-END) (596 g/mol) and [2,2^{1},4,4^{1},6,6^{1}-$^{2}H_{6}$] enterlactone ($^{2}H_{6}$-ENL).
Image reprinted with permission from Elsevier on April 2, 2015; © 2013 Edel, Aliani and Pierce. Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults (2).

Detection and quantitation of human plasma enterolignans have been accomplished primarily using liquid chromatography-mass spectrometry (LC/MS) [26] or gas chromatography-mass spectrometry (GC/MS) [11, 27-32] with more recent procedures including more tandem MS techniques [12, 13, 16, 17, 33-36]. Other methods such as time resolved fluoroimmunoassays (TR-FIA) [37-39] and liquid chromatography with coulometric electrode array detection (HPLC-CEAD) [40] have been employed; however, they lack specificity compared to techniques using mass spectrometry. To our knowledge, SLE has never been applied to the extraction of plasma enterolignans and has only been used once as a sample preparatory tool in conjunction with GC/MS [41].

Compared to GC/MS operated in full scan mode, micro selected ion storage (μSIS) offers improved ion sensitivity and selection. Full scan analyses offer qualitative information yet suffer in their limits of detection (LOD) due to interfering background noise. μSIS is a similar technique to single-ion monitoring (SIM) where narrow selection windows are used to extract ions of interest at specified time points. Incorporating this technique with isotopically labeled compounds as internal standards enables accurate analyte quantitation by removing any errors that may have arisen during sample preparation and/or GC/MS analysis.

This manuscript details the validation of an SLE method to extract END and ENL from human plasma, followed by silylation and quantitation using GC/MS-μSIS. A direct comparison of SLE to LLE will demonstrate the preserved analytical integrity of this new method, with the benefit of increased sample throughput. Application towards plasma enterolignans at baseline and 4-weeks post daily ground flaxseed consumption (30g/d) in a healthy adult population will also be discussed.

MATERIAL AND METHODS

Chemicals, materials and reagents

HPLC grade (≥95%) standards of enterodiol and enterolactone were purchased from Sigma Aldrich (Oakville, Ontario, Canada). Hexadeuterated internal standards of $^{2}H_{6}$-enterodiol ($^{2}H_{6}$-END) and $^{2}H_{6}$-enterolactone ($^{2}H_{6}$-ENL) were purchased from Dr. Wähälä (University of Helsinki, Finland). Aqueous sodium acetate buffers (0.5M and 0.1M; pH 5.0) and 2600 units of β-glucuronidase-sulfatase from *Helix pomatia* (EC 3.2.1.31) (G1512) (made up in 0.5M sodium acetate buffer) were prepared fresh daily (Sigma Aldrich).

Pyridine and ethyl acetate were both Chromasolv Plus grade for HPLC (≥99.9%), diethyl ether and methanol were Chromasolv grade for HPLC (≥99.9%) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (derivatization grade) were all purchased from Sigma Aldrich. Buffers were made using doubly distilled water collected at 18 megohm-cm from a Barnstead E-Pure system (Dubuque, Iowa, USA). Rabbit plasma from male New Zealand white rabbits (2.8 ± 0.1 kg, Southern, Rose Rabbitry) was used for preliminary solvent optimization experiments. Human plasma (HPEK2-0500) for preparation of standards, blanks and spikes for all of the validation studies had K_2-EDTA as the anticoagulant and was purchased from Cedarlane Laboratories Limited (Burlington, Ontario, Canada). Human plasma from healthy adults consuming a daily dosage of flaxseed is described in the next section. All plasma samples were frozen at -80°C and thawed at room temperature prior to using.

Isolute SLE+ 1 mL supported liquid extraction columns were purchased from Biotage (Charlotte, North Carolina, USA). All glass vials and vial inserts were silanized and screw

caps were PTFE-lined. These were all purchased from VWR International (Mississauga, Ontario, Canada).

Human plasma samples from flaxseed study

This study was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Healthy human volunteers were between 18 and 49 years of age, were equally male and female and were not taking cholesterol lowering or hypertension drugs, antihistamines or nutritional supplements. Plasma was obtained at baseline and 4 weeks following daily dietary supplementation with a muffin containing 30g of ground flaxseed. Muffins were baked by the Canadian International Grains Institute in Winnipeg, Manitoba and stored for a maximum of one month at -20°C. Two types of muffin flavors were prepared: banana chocolate chip and orange cranberry. Formulation details and a flavor profile are available for the orange cranberry muffin [42]. Individuals were asked to continue with their same dietary pattern with no restrictions placed on consumption of lignan containing foods, other than those containing flaxseed. Baseline plasma enterolignan levels would be indicative of typical lignan consuming patterns prior to dietary intervention. Plasma was collected into EDTA containing tubes, spun at 1800 × g at 4°C for 5 mins and then aliquoted into cryovials and stored at -80°C until analysis. Throughout the 4-week study, participants were allowed to withdraw at any time without consequence.

Preparation of standards and blank

Pure standards of END, ENL and their $^{2}H_{6}$-isotopically labeled internal standards (**Figure 11**) were accurately weighed and dissolved in methanol then stored at -20°C until needed. Blanks and standards were prepared by first adding 13.8 μL of a 17.3 μM cocktail of

internal standards to each silylated glass tube. The final concentration of internal standard at the time of injection was 1000 nM. Eight standards were then made, equally distributed throughout the concentration range (33 to 1000 nM) by the addition of aliquots from either a 170 or 1000 nM END and ENL stock standard solution. Methanol was removed under a gentle stream of N_2 (g) and then 300 µL of control human plasma was added. Sample blanks contained only the internal standard and plasma. Calibration standards were run in duplicate at the start and end of each sample set with the combined averages of each of these injections enabling the final construction of the calibration curves. Final sample concentrations were calculated using the slope of the line equation (y=mx+b) where y represented the ratio of enterolignan area to its corresponding $^2H_6$-internal standard area and x was the concentration being solved for.

Enterolignan hydrolysis

Kuijsten et al previously demonstrated validated conditions for hydrolyzing enterolignans in plasma [16]. Briefly, all standard/sample enterolignan hydrolyses and isolations were carried out in 4 mL silanized tubes containing 1 mM of internal standards, devoid of methanol. Three hundred mL of plasma was added to each tube, followed by the addition of 300 µL of 0.1 M sodium acetate buffer, pH = 5.0. Concentrated hydrochloric acid (12 M) was used to adjust the pH. Sixty mL of freshly prepared β-glucuronidase-sulfatase from *H. pomatia* (2600 units made up in 0.5 M sodium acetate buffer, pH 5.0) was then added to each tube. Total volume of sample (300 µL), buffer (300 µL) and enzyme (60 µL) yielded 660 µL. An additional optimization experiment was carried out using 240 µL of 0.1 M sodium acetate buffer instead of 300 µL to assess if altering the sample-to-buffer ratio from 1.2:1 to 1:1 would impact the effectiveness of the separation. Individual tubes were

capped, gently vortexed (3 × 5 sec) and then incubated at 37°C for 4 hours in the dark using a Model 2000 Micro Hybridization Chamber (Robbins Scientific). Enterolignan aglycones were then isolated from the matrix using either LLE or SLE.

Extraction of mammalian enterolignans using LLE

LLE was done using a slight modification of the extraction procedures described by Kuijsten [16]. Following incubation, standards/samples were left at room temperature in the dark for 15 minutes prior to extraction. Each standard/sample was extracted twice with 1.5 mL of diethyl ether. After each 1.5 mL addition of diethyl ether, samples were vortexed 3 x 5 sec each and then centrifuged at 2300 x g for 10 mins at 10°C. The upper diethyl ether layer was removed and added to a new 4 mL silanized tube. Both ether extracts were combined. The resulting diethyl ether was evaporated under a gentle stream of N_2 (g) at room temperature liberating the enterolignan mixture. Samples were derivatized immediately using the method described in the 'derivatization procedure' section.

Optimization experiments for extraction of mammalian enterolignans using SLE

Following incubation, samples were left at room temperature in the dark for 15 minutes prior to loading onto the SLE columns situated on a vacuum manifold (Vac Elut SPS 24 Manifold). Once total sample volume was loaded (660 µL), vacuum from the in-house system (19" Hg) was applied for 10 seconds, which enabled immediate entry of the sample onto the column. Following a 6 min waiting period, enterolignans were eluted by gravity into 8 ml silanized vials using a number of different test solvents including diethyl ether, ethyl acetate, dichloromethane, 1% methanol in ethyl acetate, 70:30 (v/v) diethyl ether:ethyl acetate or 50:50 (v/v) diethyl ether:ethyl acetate (4 x 1.25 mL). Once all of the organic solvent had eluted or a maximum time of 6 mins elapsed, vacuum was gently pulled through

the system for 1 minute and then more forcefully for an additional 10 seconds to ensure complete sample elution. Evaporation of the solvent was accomplished using a gentle stream of N_2 (g) with the tube bases warmed to 37°C using aluminum beads contained in a Lab-Line brand Lo-Boy Tissue Float Bath. These samples were derivatized as described in the 'derivatization procedure' section. Each solvent was assessed for its ability to quantitatively extract enterolignans from the SLE column. Three criteria in selecting the elution solvent required that the averaged internal standard areas for each compound have RSD values ≤15%, the calibration linearities were ≥0.98 and percent recoveries were ≥90%. Overall elution and drying times were taken into consideration as well.

Method validation

The solvent that met the above criteria was then used for all method validation experiments of which derivatization limits, carryover, sensitivity, linearity, precision and accuracy were all assessed. Successful derivatization was associated with a high linear correlation when larger concentration standards were added to the already existing calibration range. The highest concentration standard that was examined was 2300 nM. A correlation value above 0.98 indicated successful derivatization of the enterolignans and thus suitability of the derivatization method at these concentrations. To assess the reproducibility of SLE to routinely produce high analyte recoveries in a consistent manner, 5 replicates of both a medium (170 nM) and high (1000 nM) enterolignan mixture were spiked in control human plasma. Additionally, spiked plasma samples containing 250 nM and 830 nM of enterolignan mixture were prepared in triplicate and calculated for their percent recoveries. Carryover was determined by running a blank sample prepared in control human plasma directly after a high concentration standard. The sensitivity of the method was calculated by

determining the signal-to-noise ratio of a low concentration standard made using a standard solution that was further diluted with derivatization solvent.

Derivatization procedure

Silylation of standards/samples for GC/MS analysis was accomplished immediately following LLE and SLE using a 1:1 (v/v) pyridine:BSTFA mixture (120 μL of each agent). Derivatization proceeded by heating at 90°C in a Single-Wall Transite Oven (Blue M Electric Company) for 30 mins and then remained at room temperature in the dark overnight. Blanks, standards and samples were analyzed within 24 hours.

LLE and SLE extraction comparison

In order to determine the effectiveness of switching to SLE from LLE, it was important to compare both sample preparation techniques in tandem. A condensed calibration made up using 3 standards ranging from 170 to 1000 nM was prepared in control human plasma in triplicate and enterolignans were extracted using both techniques. The two methods were assessed for linearity across the calibration range, percent recoveries from 330 nM spikes to plasma and relative standard deviations of internal standard areas. Diethyl ether was used as the extraction solvent for LLE and 70:30 (v/v) diethyl ether:ethyl acetate was used for SLE. Cost and time analyses were calculated as well for a clinical trial involving ~ 300 samples.

Gas chromatography/mass spectrometry conditions

A Varian 450-Gas Chromatographic instrument connected to a 240-Mass Spectrometry detector from Agilent Technologies was used to separate and detect the trimethylsilylated (TMS) enterolignans. A splitless injection of 1 μL of standard/sample was injected using a Varian CP-8400 autosampler. The injector temperature was maintained at

240°C throughout analyses. A Varian FactorFour capillary column (VF-5ms 30m x 0.25mm x 0.25μm) from Agilent Technologies was used. The sample was carried at 1.0 mL/min using ultra-high purity helium as the carrier gas through the column which was rapidly heated from 120 to 290°C at a rate of 50°C/min in 3.4 min. The temperature was then held at 290°C for 4.6 mins completing the run at 8 mins. The external ion trap was operated in positive EI mode (70V) using micro-Selected Ion Storage (μSIS) mode. **Table 5** identifies the retention times as well as the target and qualifier ions that were used to identify the enterolignans and their corresponding internal standards. ENL and $^{2}H_{6}$-ENL (target m/z's of 180 and 183, respectively) had an isolation window of 2 m/z whereas END and $^{2}H_{6}$-END (410 and 416) was 3 m/z. Trap and manifold temperatures were operated at 150 and 50°C, respectively, with the transferline and ion source temperatures set to 250°C. Peak areas of analytes were integrated using MS Workstation version 6.9.3 software (Agilent Technologies). Quantitation was based upon the most abundant fragment ion, with confirmation based upon the next most abundant fragment. The relative area of each standard compared to its corresponding stable isotope was used for quantitation.

Stability of derivatized enterolignans

Derivatized samples were typically analyzed within 24 hours; however, stability of analytes beyond this was investigated further. Samples were immediately recapped and stored at -80°C following each GC/MS analysis. Upon reinjection, samples were allowed to return to room temperature, vortexed (3 x 5 sec) and reinjected at 48 and 144 hours post-initial analysis.

Table 5. Target and qualifier ions of trimethylsilylated (TMS) forms of END, ENL and their hexadeuterated internal standards.

Compound	Retention Time (min)	Compound Mw[a] (g/mol)	Ion Monitored	Target Ion (m/z)	Qualifier Ion (m/z)
Enterolignans					
END	5.42	590	[M-180]	410	180
ENL	6.36	442	[M-262]	180	442
Internal Standards					
$^{2}H_6$ - END	5.40	596	[M-180]	416	183
$^{2}H_6$ - ENL	6.33	448	[M-265]	183	448

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Statistical analysis

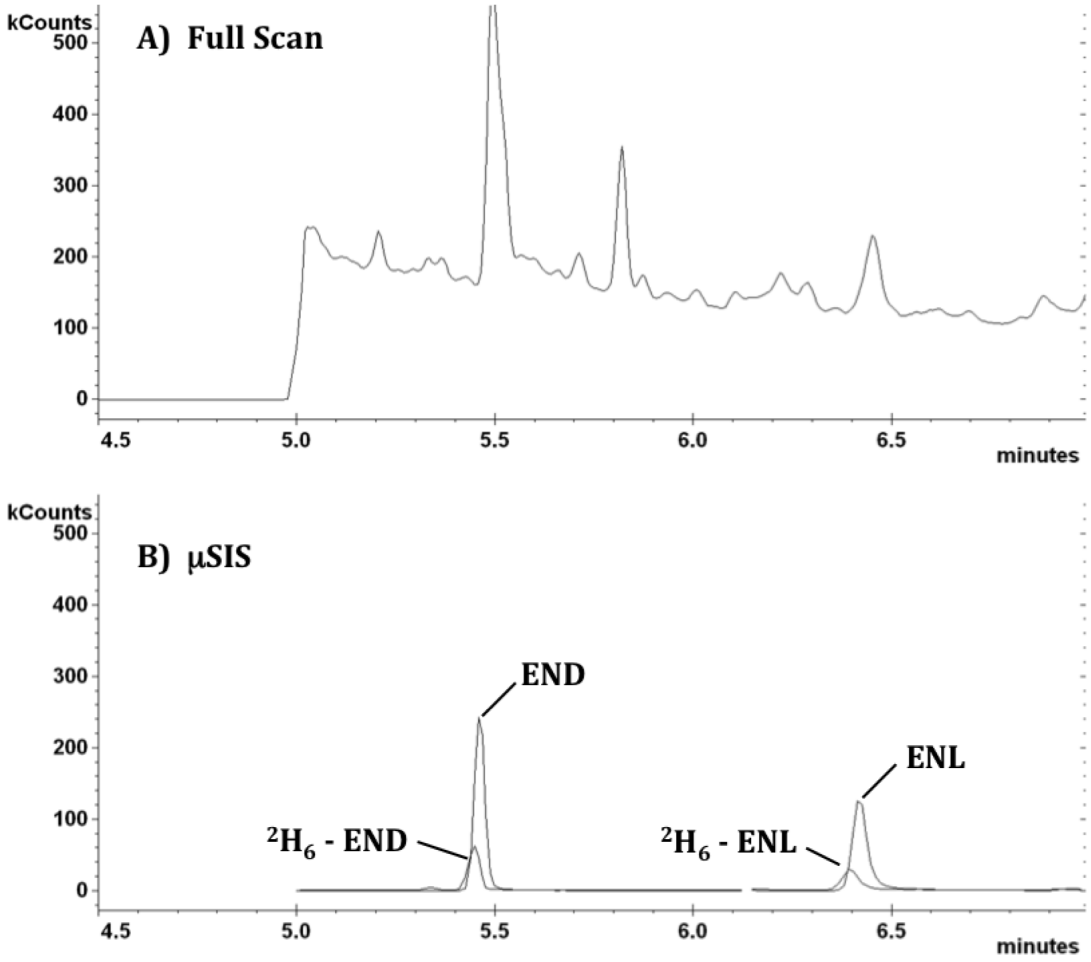
Statistical significance was calculated with Sigma Stat software (version 2.03, SPSS) using the paired Student's t-test. Differences between means were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Chromatographic identification of enterolignans and internal standards

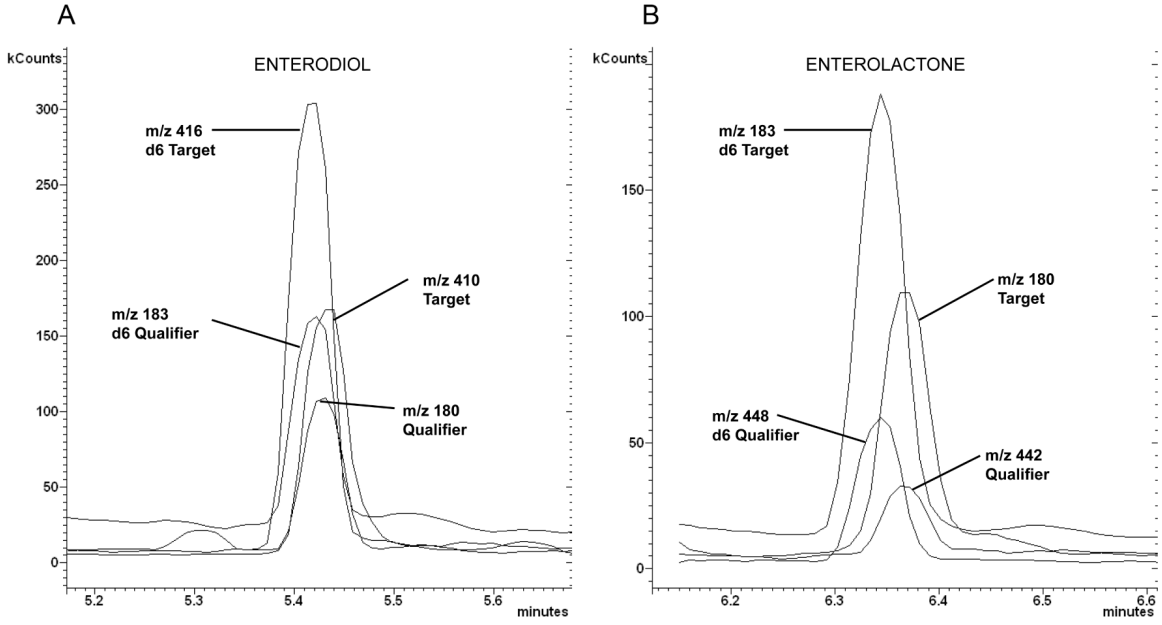
Confirmation of pure forms (without matrix effects) of TMS derivatives of END, ENL and their corresponding internal standards was first accomplished using higher concentrations of each compound and analyzed individually using GC/MS in full scan mode (**Appendix 1**). $^{2}H_{6}$-END and END eluted at 5.40 and 5.42 mins, respectively whereas $^{2}H_{6}$-ENL and ENL had retention times of 6.33 and 6.36 mins (**Table 5**). Application of μSIS as an ion storage mode greatly improved instrument sensitivity of enterolignans compared to full scan mode as seen in **Figure 12**. This technique is used in ion trap mass spectrometers to store desired ions and reject unwanted ones thus removing interfering matrix ions and enhancing overall signal-to-noise. μSIS offered a two-fold increase in ion sensitivity relative to SIS mode. Improved limits of detection (LOD) of μSIS- compared to SIS-MS mode has been demonstrated previously in a MS detection comparison study involving pesticides [43]. Target ions were the most abundant fragment ions formed and were used for quantitation using μSIS analysis whereas qualifier ions were used as confirmation ions to ensure the absence of interfering compounds (**Figure 13**). Mass spectral data agrees with that previously published for both labeled [44] and unlabeled compounds [45] (**Appendices 2 & 3**). No detectable analyte carryover was observed following analysis of the highest standard (1300 nM) as monitored by subsequent blank solvent injections.

Figure 12. Full scan (A) and μSIS (B) GC/MS chromatograms of a standard containing 2.3 μM END and ENL with 1.0 μM of both internal standards.



For the chromatograms displayed in Figure 12B, the following m/z's were used: 410 for enterodiol (END), 416 for $^2H_6$-END, 180 for enterolactone (ENL) and 183 for $^2H_6$-ENL. *Image reprinted with permission from Elsevier on April 2, 2015; © 2013 Edel, Aliani and Pierce. Supported liquid extraction in the quantitation of plasma enterolignans using isotope dilution GC/MS with application to flaxseed consumption in healthy adults (2).*

Figure 13. GC/MS chromatographic spectra of TMS derivatives of $^{2}H_{6}$-END, $^{2}H_{6}$-ENL (1000 nM), END and ENL (270 nM) in µSIS mode.



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Other groups using LC/MS/MS and GC/MS-SIM have noted the presence of phytoestrogens coming from the enzyme β-glucuronidase from *H. pomatia* [34,46]. Although different sources of the enzyme were used in each of these studies, Grace [34] detected small levels of genistein and trace levels of O-desmethylangolensin within the enzyme itself and Adlercreutz [46] determined that the contribution was from daidzein. In our study, small interfering shoulder peaks were observed at both retention times of END and ENL when examining the sample blank. In order to determine the source of this compound, alternate blanks were prepared in which one contained plasma alone and the other plasma and enzyme. Internal standards were not added to either blank; however, buffers were present. All sample pretreatment was continued as usual with final analysis using GC/MS in

both full scan and μSIS modes. No detectable differences were visible in full scan mode due to both the poor sensitivity of this technique and the low levels of this interference within the sample. In μSIS mode, the interfering peaks did not appear when plasma alone was used, but did when plasma and enzyme were combined. The most abundant m/z's of these peaks were at 183.0 and 411.2 for END and 180.1 and 449.2 for ENL when examined in μSIS mode. This pointed to the enzyme as being the source of interference. To correct for this, sample blanks were prepared with every sample set and subtracted from the measured enterolignan values.

Optimal solvent and buffer selection with enterolignans and Isolute SLE+

Preliminary investigation into selection of appropriate solvent systems began by examining spiked rabbit plasma samples at levels within our mid-to-upper concentration range. Four separate sets of calibration standards (330, 660 and 1000 nM) were prepared and loaded onto Isolute SLE+ columns. Four solvent systems were investigated for their ability to effectively desorb all of the compounds of interest. These included diethyl ether, ethyl acetate, 1% methanol with ethyl acetate and dichloromethane. Diethyl ether was chosen due to its previously demonstrated high extraction capabilities with enterolignans when using LLE [16], the low levels of interfering plasma phospholipids that are eluted using this solvent with SLE [47] and its high volatility. The remaining three solvents were recommended by the manufacturer as part of their SLE+ method development guidelines [48]. Optimal extraction conditions were initially assessed by comparing the relative standard deviations (RSD values) of the three sets of duplicate internal standards and of the linearity coefficients (R^2). Dichloromethane was immediately disqualified as it did not solubilize END or $^2H_6$-END. $^2H_6$-END and $^2H_6$-ENL both had the lowest RSD values when using 100% ethyl

acetate (4.6% and 4.0%, respectively) and 1% methanol in ethyl acetate (5.3% and 3.3%). Diethyl ether gave slightly higher RSD values of 10.1% and 8.5%. Linearity coefficients across this calibration range were similar for diethyl ether and ethyl acetate for both compounds as defined by R^2 values ≥0.99, whereas the use of 1% methanol in ethyl acetate yielded a poor R^2 value of 0.97 for END and 1.00 for ENL. The addition of methanol was initially selected due to the known solubility of enterolignans in this solvent. However, as methanol and water are miscible, it was decided to abandon the use of this solvent so that water would not be brought back into the final sample eluent. Only diethyl ether and ethyl acetate were further investigated due to their immiscibility with water and their effectiveness in solubilizing enterolignans. A major drawback of using ethyl acetate was the considerably longer drying times following SLE compared to diethyl ether (70 versus 30 mins). Subsequent experiments optimized the ratio of diethyl ether to ethyl acetate and incorporated moderate heat (37°C) during solvent evaporation as a means to reduce overall extraction times.

Diethyl ether to ethyl acetate ratios of 70:30 (v/v) and 50:50 (v/v) were compared along with their parent solvents as controls in the next set of optimization experiments, now using human plasma and moderate warming to reduce evaporation times. Each of these experiments was performed in triplicate on separate days (**Table 6**). Linearity was high in each of the groups (>0.991). Relative standard deviations were attenuated in the 70:30 diethyl ether:ethyl acetate group with $^2H_6$-END as 7.2% and $^2H_6$-ENL as 8.7%. The other solvent systems yielded deviations ranging from 8.5 to 21.1% for both compounds. Percent recoveries were very high for each of the extraction solvents ranging from 94.8 to 104.3%. Evaporation times were much lower using 70:30 diethyl ether:ethyl acetate (30 mins) relative

to the 50:50 group which was 45 mins. Further experiments were carried out using 70:30 diethyl ether:ethyl acetate which exhibited high recovery and linearity values, had the lowest relative standard deviations for each of the compounds of interest and had a lower evaporation time.

Table 6. Solvent optimization in the extraction of enterolignans from human plasma with solvent removal at 37 °C.

Compound	100% Ether	70%:30% Ether:EtOAc	50%:50% Ether:EtOAc	100% EtOAc
RSD[a]				
$^2H_6$ - END	14.0 ± 5.9 %	7.2 ± 1.7 %	11.0 ± 5.21%	8.5 ± 2.4 %
$^2H_6$ - ENL	21.1 ± 2.5 %	8.7 ± 0.9 %	11.5 ± 4.1 %	10.6 ± 1.7 %
Linearity (R^2)				
END	0.991 ± 0.007	0.998 ± 0.001	0.999 ± 0.000	0.997 ± 0.002
ENL	0.995 ± 0.001	0.998 ± 0.001	0.999 ± 0.001	0.995 ± 0.006
% Recovery[b] (%)				
END	103.2 ± 8.8	95.0 ± 6.8	99.1 ± 3.7	95.6 ± 3.4
ENL	104.3 ± 12.9	97.7 ± 9.0	94.8 ± 7.0	101.5 ± 5.5
Drying Time[a] (min)	18	30	45	60

Results are represented as the mean ± S.D.
Ether refers to diethyl ether and EtOAc refers to ethyl acetate.
Results are based upon five calibration standards ranging from 100 to 1000 nM and measured as TMS derivatives using GC/MS.
[a] Based on areas of internal standards (N=5).
[b] Calculated from an N=3. Spike was 420 nM.
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Manufacturer recommendations for Isolute SLE+ [49] suggest using equivalent v/v ratios of buffer to biological fluid so as to control sample viscosities thus promoting even flow through the column. Modeling the hydrolysis conditions established by Kuijsten et al

[16] the ratio was 1.2:1. To test a 1:1 ratio we used 240 μL of buffer. RSD and R^2 values were similar for both (**Appendix 4**). Further validation experiments were done using 300 μL of buffer.

Method validation

Using 70:30 diethyl ether:ethyl acetate for all further elutions, the accuracy and precision of the method was determined. To assess the precision of SLE as a sample preparatory tool, multiple replicates (n=5) of low (170 nM) and high (1000 nM) enterolignan standards were prepared by spiking human control plasma. At low concentrations of END, levels averaged 157 ± 13 nM whereas ENL was 173 ± 13 nM yielding relative standard deviations of 5.8 and 5.9%, respectively. High concentrations averaged 1015 ± 28 nM for END and 1057 ± 46 nM for ENL with RSD values of 2.5 and 4.1% (**Table 7**). These values are comparable to those reported by other authors [16, 34].

The inter-day variability of both medium (270 nM) and high (670 nM) END and ENL standards was acceptable. The lower concentration group had deviations below 4.3% and the higher group was less than 6.9%. It was difficult to directly compare this with literature as most studies typically monitor inter-day variability using lower concentration standards. This study reports higher concentrations than those normally monitored as these are typical levels in plasma for populations consuming foods high in lignans, such as flaxseed [36]. Kuijsten noted that enterolignan RSD values ranged from 10-21% across two standard concentrations, with the highest concentration being 39.2 nM [16]. These deviations are higher than ours in part because of the lower concentration they are monitoring. Adlercreutz reported that in omnivorous and vegetarian women, errors ranged between 5.1 to 18.1% for END and between 1.4 and 10.7% for ENL [11]. Higher deviations were present for the

lower concentrations relative to the higher ones. Smeds and Hakala reported that enterolignans prepared in plasma compared to those that are not have much higher RSD values [17] which is possibly why Grace [34] and Valentin-Blasini [33] reported lower values (3.3 - 5.1%) as there was no matrix influence present in the preparation of their calibration curves.

Table 7. Intra- and inter-assay precision of mid- and high-concentration enterolignans as TMS derivatives quantified using GC/MS in μSIS mode.

Results are calculated based upon area ratios of the standard relative to its labeled isotope.

Enterolignan	Intra-assay Precision (n=5)		Inter-assay Precision (n=5)	
	[C] (nM)	RSD (%)	[C] (nM)	RSD (%)
END (mid)	170	5.8	270	2.6
END (high)	1000	2.5	670	6.9
ENL (mid)	170	5.9	270	4.3
ENL (high)	1000	4.1	670	3.3

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To assess the percent recoveries at both lower and higher concentrations that would be typical of those observed in healthy individuals consuming flaxseed or other high lignan diets, control human plasma samples were spiked at 250 and 830 nM (n=3). Percent recoveries were 96.4 ±3.4% for END and 96.0 ± 5.2% for ENL when the spike contained 250 nM of enterolignans. Recoveries were slightly higher at the higher concentration level with END as 103.3 ± 5.1% and ENL as 114.4 ± 8.8%. These recoveries compare quite nicely with values reported by Adlercreutz et al of 98.9% and 97.9% for END and ENL using GC/MS-SIM [11]. Lower values were reported by Nesbitt and colleagues (84.6 and 86.9%) [29]. Kuijsten et al reported recoveries of 97% for END and 99% for ENL when using 100

nM spikes when extraction was done using LLE with analysis by LC/MS/MS [16]. High recoveries were also exhibited when 170 nM spikes were added to serum samples and extracted using SPE and quantitated using LC/MS/MS (END 97.0% and ENL 97.6%) [34]. Both of these studies look at lower concentration spikes typical of low to moderate lignan intake diets. However, plasma samples from individuals consuming high lignan foods like flaxseed have enterolignan levels much higher than what these groups report as "high" lignans. Therefore, spikes of 250 and 830 nM are more relevant when investigating plasma profiles of subjects consuming higher lignan containing foods like flaxseed.

Instrument sensitivity was established using signal-to-noise (S/N) ratios involving spiked enterolignans in human plasma. The LOQ, defined as a S/N of 10:1, was 9.9 nM for END and 10.1 nM for ENL. A S/N of 3:1 defined the LOD and was 6.6 and 6.7 nM, respectively. Signal-to-noise was calculated using MS Workstation version 6.9.3 using the ratio of peak heights. Baseline noise was calculated via the software from peak to peak. As compliancy is one of the applications of this method for studying enterolignan bioavailability from flaxseed consumption, 30 nM was set as the lowest calibration standard for the remainder of the study.

Derivatization

To ensure that all enterolignans were being derivatized to completion, the linear range of the calibration was extended. Linearity was still being achieved up to 2330 nM for both END and ENL ($R^2 \geq 0.99$). Levels higher than this were not tested so no upper limits were defined. Therefore, calibration ranges within the limits of 33 to 2330 nM were acceptable for these two enterolignans; however, a maximum of 1000 nM was suitable for our analyses.

Sample stability

Samples were typically left at room temperature overnight following the 30 min heating derivatization step at 90°C and analyzed the next morning. However, sample stability was examined at 48 and 144 hours post initial analysis after having been recapped and stored at -80°C in-between. Comparison of internal standard areas at baseline and 48 hours showed no significant changes in both $^{2}H_{6}$-END and $^{2}H_{6}$-ENL (N=11). However, $^{2}H_{6}$-END was significantly attenuated at 144 hours relative to baseline (P = 0.020) as was $^{2}H_{6}$-ENL (P = 0.048).

Plasma enterolignan concentrations from flaxseed consumption

Isolute SLE+ in conjunction with GC/MS-μSIS was applied to the extraction and quantitation of plasma samples from healthy adults who were on a 4-week flaxseed dietary intervention. Baseline levels of END and ENL hovered tightly around the established LOQ for each compound, suggesting low consumption levels of lignan containing foods in young to middle-aged adults within the Winnipeg, Manitoba region. Following 4 weeks of daily consumption of muffins fortified with 30g of ground flaxseed, total enterolignan (END + ENL) levels increased significantly in all individuals relative to baseline levels (n=10) (P = 0.002). Final END and ENL concentrations ranged from 49 nM to a maximum of 401 nM after 4 weeks and from 35 to 1128 nM, respectively. The average value for END was 209 nM and 304 nM for ENL. Between subject variability was also very high which has been documented previously [12, 36]. As all subjects exhibited increased plasma enterolignans, these compounds offer potential as reliable biomarkers of plant lignan consumption and compliancy in intervention trials involving flaxseed [13, 50].

Plasma enterolignan concentrations have been reported in only a few studies where 25 to 30 g of flaxseed was used as the lignan source. These were short-term studies typically lasting only a maximum of 1 to 2 weeks. Saarinen et al report average values of 731 nM for END and 755 nM for ENL following 8 days consumption of 25 g crushed flaxseed with large between subject variability [36]. Another study involving females required similar consumption patterns of 25 g of ground flaxseed daily for 8 days. Measurement every 3 hours over the first 24-hour period revealed a steady increase in baseline levels for both END (22.45 to 46.29 nM) and ENL (6.90 to 19.65 nM) which increased to a maximum of 3- to 4-fold after 8 days [29]. In addition, Morton et al demonstrated that healthy postmenopausal Australian females consuming 25 g/d of flaxseed for 2 weeks had marked increases of END (582 nM) and ENL (379 nM) relative to baseline (3 nM and 17 nM, respectively) [30]. A number of factors play a role on the varying concentrations of circulating enterolignans resulting from flaxseed consumption. Some of these include the varying lignan contents within flaxseed cultivars, the type of flaxseed (milled or whole), dietary patterns, intestinal transit times, differences in gut micro flora and antibiotic usage, [12, 51, 52].

SLE and LLE validation comparison

Preliminary experiments in our laboratory were conducted using LLE with diethyl ether for the isolation of enterolignans from plasma. A reoccurring problem was that 1 out of every 24 samples (~4%), within at least every other sample set, never displayed internal standard or analyte peaks within the chromatogram. Repeated injections of the null sample followed by injection of a sample and standard that did previously show peaks within the same sample set verified proper injection syringe, autosampler and GC/MS function. At no time during sample preparation were there any observable differences between any of the

samples within a sample set during hydrolysis, extraction or derivatization. As all samples were extracted uniformly and sequentially, it was hypothesized that water from the aqueous layer may have been transferred with the organic layer erroneously or an emulsion may have existed. It is well known that water is a scavenger of silylating materials [53] so it was hypothesized that water may have been competing and possibly preventing this reaction from proceeding. However, additional experiments, with extra care given to not remove any of the lower aqueous layer, still resulted in this ~4% sample loss. Experiments to test whether or not water is impeding the derivation process have not been done. There are no reports to our knowledge of this problem with LLE; however, difficult emulsions associated with LLE are well documented [19, 54]. Morton et al reports using LLE with diethyl ether with final analysis of the BSTFA derivatized enterolignans using GC/MS [30]. No complications were reported. However, an additional separation step was used following LLE using a diethylaminohydroxypropyl Sephadex LH-20 short column with CO_2 bubbled methanol as the eluent. This additional separation step may have removed any residual water. An additional complication of this method was the large plasma volume of 1 mL which was required. As this sample loss problem could not be resolved without potentially adding an additional separation step like Morton used we determined that a new, single-step extraction method had to be developed that would additionally enable a smaller plasma volume to be used (300 µL instead of 1 mL). Since developing this extraction method using SLE over 400 plasma samples have been extracted with no samples lost as in the case with LLE.

Due to high sample loads that often accompany clinical investigations, it was of interest to validate our SLE method to see if it could rank competitively, if not better than, LLE as a sample extraction tool. Traditional LLE methods are time consuming and labor

intensive when repeated at high sample loads. Therefore, to see if there truly were advantages of switching to SLE from LLE the next series of experiments were performed in tandem using both methods (**Table 8**). A condensed calibration range was prepared from 170-1000 nM having linear correlations ≥0.99. High linearity's have previously been shown using diethyl ether as the mobile phase with both SLE (**Table 6**) and LLE [16]. RSD values were lower using SLE (<6.5%) than LLE (<7.4%) for each of the compounds at both mid and high concentration ranges and the percent recoveries were comparable ranging from 98.2 to 102.1%. These results indicate that switching from LLE to SLE will not result in any compromise in accuracy or precision of the separation. The next step was to calculate the time it would take to analyze ~300 clinical plasma samples and the estimated cost to complete this analysis. SLE dramatically reduced the number of weeks from 12 to 9 compared to LLE. This is a huge savings in labor expenses alone as higher throughput could be achieved. It was also interesting to calculate the overall cost of estimated consumables required to facilitate these extractions. The difference was estimated to be only $200. Therefore, SLE was chosen as the sample preparatory tool of choice for the extraction of enterolignans from plasma.

In addition to the extraction of plasma enterolignans from a number of other clinical samples within our facility, application of SLE towards extraction of urinary enterolignans is currently being investigated.

Table 8. Comparison of SLE and LLE in combination with GC/MS as an extraction technique for enterolignans from 300 μL of plasma (N=3).

Compound		Linearity[a,b] (R^2)	RSD (330)[a,c] (%)	RSD (170)[a,c] (%)	Percent Recovery[d] (%)	Analysis Time[e] (wks)	Estimated Cost[e,f] ($)
SLE						9	4500
	END	0.999 ± 0.001	6.5	2.6	101.9 ± 7.5	-	-
	ENL	1.000 ± 0.001	4.2	5.0	102.1 ± 7.5	-	-
LLE						12	4300
	END	0.997 ± 0.004	7.4	3.0	98.2 ± 6.8	-	-
	ENL	0.999 ± 0.002	5.7	7.3	100.7 ± 0.7	-	-

[a] Short calibration curves were used to construct this data (170, 330 and 1000 nM). Each standard was made in triplicate and injected twice.
[b] Values are represented as the mean ± S.D.
[c] % RSD's are based upon 330 and 170 nM standards.
[d] Percent recoveries are calculated based on 330 nM spikes. LLE spike recoveries were done in rabbit plasma.
[e] Calculations were based upon 282 clinical samples.
[f] Cost does not take into account GC/MS analysis or labor.
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CONCLUSIONS

This paper outlines the successful development and validation of a method to determine END and ENL in human plasma using SLE with quantification using GC/MS-μSIS. This is the first extraction of enterolignans from plasma using SLE as well as the first time SLE has been applied as a sample preparatory technique with plasma in conjunction with GC/MS analysis. The majority of published manuscripts involving SLE use it in combination with LC/MS/MS. The advantages of using SLE over traditional LLE are faster throughput, simplified extractions, reduced costs in terms of labor and elimination of emulsions leading to zero sample loss. This technique has now been applied to the extraction of enterolignans in larger clinical trials where only minimal volumes of plasma are available.

To date, there have been absolutely no samples lost due to interferences caused by emulsions in over 400 samples that have been analyzed. In addition, this technique offers an affordable and effective means to quantify END and ENL in plasma as well as providing a means to monitor compliancy in nutritional intervention trials where consumption of high lignan foods, like flaxseed, may be used. Accurate measurement of plasma enterolignans is essential for understanding their bioavailability so that associations with disease prevention can be more clearly identified.

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CHAPTER VI: The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults

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ABSTRACT:

Purpose: The primary endpoint was to determine the plasma concentration of alpha-linolenic acid (ALA), and its metabolites, following milled flaxseed consumption at four doses. Secondary outcomes focused on plasma enterolignan concentrations and the effects on tolerability, platelet aggregation, plasma lipids and urinary thromboxane levels.

Methods: Healthy, younger adults (n = 34; 18-49 years old) were randomized into four groups consuming 1 muffin daily for 30 days fortified with 10, 20, 30 or 40 g of milled flaxseed. Blood and urine were collected at baseline and 4 weeks.

Results: Plasma ALA concentrations increased with all flaxseed doses ($P < 0.01$), except the 20 g/d dose ($P = 0.10$), yet there was no significant dose-dependent response ($P = 0.81$). Only with the 30 g/d diet were n-3 polyunsaturated fatty acids ($P = 0.007$) and eicosapentaenoic acid (EPA) ($P = 0.047$) increased from baseline values. Docosapentaenoic acid and docosahexaenoic acid were not detected at any dose. Plasma total enterolignan concentrations significantly increased over time in all treatment groups, yet despite a dose-dependent tendency, no between-group differences were detected ($P = 0.22$). Flaxseed was well-tolerated, even at the highest dose, as there were no reported adverse events, changes in cholesterol, platelet aggregation or urinary 11-dehydro-thromboxane B_2.

Conclusions: In healthy, younger adults 10 g/d of milled flaxseed consumption is sufficient to significantly increase circulating ALA and total enterolignan concentrations; however, 30 g/d is required to convert ALA to EPA. Although all doses were well-tolerated, 40 g/d is too low to attenuate cholesterol in this population.

Key Words: alpha-linolenic acid • enterolignans • flaxseed • platelets • cardiovascular disease.

INTRODUCTION

Flaxseed (*Linum usitatissimum*), an oilseed predominantly produced in North America, has gained interest as a potential therapeutic agent in the treatment of risk factors of cardiovascular disease (CVD) (1, 2). Animal studies involving dietary flaxseed have demonstrated improved vascular reactivity (3), inhibition of atherosclerosis (4), regression of pre-existing atherosclerotic plaques (5), inhibition of arrhythmias during ischemia/reperfusion injury (6) and reduced plasma cholesterol (7). Human trials have shown anti-hypertensive (8, 9) and cholesterol-lowering effects (10, 11) of dietary flaxseed.

Several bioactive components within flaxseed are thought to provide these beneficial cardioprotective properties. Alpha-linolenic acid (ALA; C18:3n-3) is an n-3 fatty acid that is enriched in flaxseed. Approximately 50% of the overall total fatty acid content consists of ALA (1). Several clinical studies have demonstrated the anti-hypertensive properties of dietary ALA (12-14). Flaxseed also contains the highest amount of the plant-based lignan, secoisolariciresinol diglucoside (SDG) (15). Next highest food sources of SDG include asparagus (180-fold less) (16), rye and wheat (360- and 870-fold less, respectively) (17), followed by various nuts (15) and legumes (18). The SDG metabolites, enterodiol (END) and enterolactone (ENL) are enterolignans that undergo enterohepatic circulation *in vivo*. They are initially formed in the gut, are absorbed by the intestines, conjugated within the liver, excreted in bile and then reabsorbed and repackaged as sulfate or glucuronide conjugates in the liver (19, 20). These are the bioactive forms of SDG that circulate systemically (21) and demonstrate antioxidative properties *in vitro* (22) and *in vivo* (23). Clinical studies using dietary SDG or high-lignan flaxseed have demonstrated improved glycemic control (24), reduced plasma cholesterol and glucose concentrations (25) as well as

lowered diastolic blood pressure (26). Flaxseed is also 28% dietary fiber by weight (27). Dietary fiber can reduce circulating levels of cholesterol (28), which is also a predictive risk factor of CVD.

Since flaxseed contains these important bioactives in large quantities, gaining an understanding of the factors that will optimize their presence in plasma is critical to optimize their health-related actions. Flaxseed form is one factor that can influence plasma concentration. Plasma ALA and enterolignan concentration is improved when the seed is milled compared to its whole form (29, 30). However, the optimal dose of milled flaxseed to provide significant concentrations of ALA and enterolignan within a healthy human population has not yet been defined. Most human studies use upwards of 25 g/d of flaxseed (8, 27, 29, 31-35) with as much as 50 g/d (36, 37). Studies using flaxseed at doses <25 g/d are limited (38-41). Therefore, the primary endpoint of this study was to investigate the concentration of ALA in plasma as a function of the dosage of milled flaxseed with secondary endpoints focused on enterolignans and the associated effects of flaxseed ingestion on blood lipids, platelet aggregation, urinary thromboxane levels and tolerability. Although flaxseed has attained *generally recognized as safe* (GRAS) status (42), other reports using milled flaxseed in a dose-dependent manner do not include incidences of gastrointestinal related adverse events (41, 43, 44) other than increased laxation (40). We hypothesized that plasma ALA, its longer chain metabolites eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) but not docosahexaenoic acid (DHA), and enterolignan concentrations would be significantly elevated in a dose-dependent manner. Additionally, adverse event reporting and attenuations in platelet aggregation, total- and LDL-cholesterol

and urinary thromboxane levels would be most pronounced at 40 g/d of administered flaxseed.

MATERIALS AND METHODS

Study participants

Healthy subjects, male and female, 18 - 49 years of age were recruited. Younger adults (< 50 years) were selected as a representative healthy population as they are less likely to be using prescription medications for chronic disease management, as are older adults (≥ 50 years). In addition, maintaining participant age within the indicated range reduced confounding factors from age-related conditions. All subjects provided written informed consent prior to recruitment including any study-related screening procedures, and without restriction to gender, race or socioeconomic status. During screening, interested individuals provided information related to age, height and weight and were asked to provide a complete medical history inclusive of current medications, vitamins or herbal remedies. Participants could be included in the study if they were deemed healthy by a physician, were willing to refrain from taking any vitamin, herbal or oil supplements for one month prior to and during the course of the study, were not eating fish more than one time per week, were not eating flaxseed fortified foods and were willing to comply with protocol requirements. Subjects were instructed by the study coordinator to continue with their normal dietary patterns throughout the course of the study bearing in mind the inclusion criteria. Individuals were required to store the flaxseed-enriched food products in a -20°C freezer for a 4-week period and be willing to consume one thawed food product daily for the one month duration of the study. A daily diary was also required to record muffin consumption dates and times as well as any comments or possible adverse events. Subjects were excluded from this study if any of the following criteria were met: cigarette smoking (tobacco products within the last 6 months), ingestion of supplementary vitamins or oils or more than 1 fish meal/week for one

month prior to the start of the study, planned use of any herbal/ antioxidant/ fatty acid/ nutritional supplements at any time in the duration of the study, were pregnant or planning to become pregnant, experienced diabetes, cardiovascular, renal or gastrointestinal problems, were using cholesterol-lowering drugs, hypertension drugs, anti-histamines, or hormone therapy, and experienced platelet abnormalities or abnormal blood clotting/ bleeding times. Similarly, subjects were excluded if they did not adhere to study diet requirements. Two visits to the clinic would be required during the course of the study. The baseline visit (visit 1) would involve the individuals providing a fasted blood sample after which time they were randomized using a computer generated randomization procedure into 1 of the 4 intervention groups and given a one-month supply of the food products. The final visit (week 4) also required a fasting blood sample. Individuals were asked to submit their record books as well as any remaining supply of the diet. Both visits also discussed any changes in individual medical histories. Blood samples were analyzed for any changes in lipids (cholesterol, triglycerides, fatty acids), lignan metabolites and platelet aggregation. Urine was analyzed for 11-dehydro-thromboxane B_2 concentrations.

Ethics and study design

The University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee approved the study design, which was done in accordance with the ethical standards established in the 1964 Declaration of Helsinki and its amendments thereafter. A total of 40 healthy, male and female volunteers were recruited. Ten subjects were randomized into one of four study groups. Subjects consumed one muffin per day containing a distinct dose of either 10 g, 20 g, 30 g or 40 g of milled flaxseed, respective of study group. Study participants were asked to thaw and consume one muffin per day for a

total of 4 weeks. Both the research team and the subject were blinded to the flaxseed dosage group from the time the subject was randomized until completion of the study, which included data analysis.

Experimental dietary intervention

The Canadian International Grains Institute, Winnipeg, Canada prepared the muffins for this study. Muffins were stored in sealed plastic bags at -20 °C. Organic milled flaxseed was supplied by Bioriginal Food and Science Corporation (Saskatoon, SK, Canada). The same batch was used to prepare all study muffins. Muffins were offered in 2 different flavors: banana chocolate chip and cranberry-orange. Formulation details and a sensory evaluation of the cranberry-orange muffin (30 g) has been reported (45). The calculated ALA and SDG contents for each 10 g addition of milled flaxseed are represented in **Table 9** as are the formulation, energy and nutrient compositions of the dietary interventions.

Compliancy

Only 1 subject in the 10 g group withdrew from the study and that was done so voluntarily prior to the first blood draw. Subjects were asked to maintain a dietary intervention record containing the dates that they consumed the muffin and the time of day at which it was consumed. These were submitted and collected at their final appointment. In addition, study foods that were not consumed were returned to the study coordinator who recorded this. Individuals were deemed compliant if at least one of ALA, END or ENL increased significantly in circulating plasma.

Sample collection and processing

Fasted venous blood samples (15 mL) were collected at both baseline and 4-weeks. Blood was collected in a total of three tubes. One tube contained 1 mg EDTA/mL which was

centrifuged at 1800 × g for 5 min at 4 °C after which the plasma was aliquoted into several cryovials (VWR International, Mississauga, ON) (P/N 479-0822). Samples were flash frozen in liquid nitrogen and stored at -80 °C until subsequent lipid and lignan metabolite analyses could be performed. Blood collected into the two sodium citrate tubes (10 mL) was gently inverted several times and maintained at room temperature for 30 minutes prior to immediate preparation for platelet aggregation measurements.

Urine was collected into certified urine sample containers (VWR International, Mississauga, ON) (P/N CA73240-106) at both 0- and 4-weeks at St. Boniface Hospital. The study coordinator then immediately transferred the samples on ice for storage at -80°C.

Table 9. Formulation, energy and nutrient composition of the study diets.

Parameter	Cranberry-Orange Muffins				Banana Chocolate Chip Muffins			
	10g Group	20g Group	30g Group	40g Group	10g Group	20g Group	30g Group	40g Group
Ingredients/muffin								
DRY								
Flour (g)	41.7	36.7	31.7	25.0	41.7	36.7	30.0	23.3
Milled flaxseed (g)	10.0	20.0	30.0	40.0	10.0	20.0	30.0	40.0
Baking soda (g)	0.4	0.4	0.4	0.4	0.7	0.7	0.7	0.7
Baking powder (g)	2.1	1.8	1.6	1.3	2.1	1.8	1.5	1.2
Salt (g)	0.6	0.6	0.5	0.4	0.6	0.6	0.5	0.4
WET								
Eggs (g)	16.7	16.7	16.7	20.8	16.7	16.7	16.7	16.7
Milk (g)	16.7	16.7	16.7	16.7	25.0	25.0	31.7	31.7
Granulated sugar (g)	8.3	8.3	16.7	16.7	0	0	0	0
Brown sugar (g)	0	0	0	0	15.0	15.0	15.0	15.0
Vanilla (g)	2.2	2.2	2.2	2.2	0	0	0	0
Frozen orange juice (g)	7.5	8.3	9.8	10.8	0	0	0	0
Bananas, mashed (g)	0	0	0	0	33.3	33.3	33.3	33.3
Water (g)	25.8	25.0	28.6	30.8	0	0	0	0
Orange rind (g)	1.7	1.7	1.7	1.7	0	0	0	0
Cranberries (g)	15.0	15.0	15.0	15.0	0	0	0	0
Chocolate chips (g)	0	0	0	0	11.7	11.7	11.7	11.7
Total ingredient weight (g)	148.6	153.2	171.3	181.7	156.8	161.4	171.0	173.9
Proximate Analysis								
Moisture (g)	69.0	68.8	73.4	79.2	66.8	67.0	73.0	72.9
Fat (g)	6.9	11.0	15.1	19.5	10.3	14.4	18.5	22.5
Σ SFA (g)	1.1	1.5	1.8	2.3	3.1	3.5	3.9	4.3
Σ MUFA (g)	2.3	3.0	3.7	4.8	3.4	4.1	4.8	5.6
Σ PUFAn-3(g)	2.3	4.5	6.8	9.0	2.3	4.5	6.8	9.0
C18:3n-3 (g)	2.2	4.4	6.6	8.8	2.2	4.4	6.6	8.8
Σ PUFAn-6 (g)	1.1	1.7	2.3	2.9	1.1	1.7	2.3	2.9
Σ PUFA (g)	3.4	6.3	9.1	11.9	3.4	6.3	9.1	11.9
Energy (kCal)	335.8	372.7	442.5	478.6	383.6	419.2	450.1	478.8
Carbohydrates (g)	58.9	58.4	66.6	64.9	64.9	64.1	62.4	60.3
Total dietary fiber (g)	9.8	11.2	12.6	14.2	10.7	12.1	13.5	14.6
Protein (g)	5.0	7.7	10.4	13.1	5.2	7.9	10.5	13.2
Flaxseed SDG Content								
SDG lignan[1] (mg)	38	75	113	150	38	75	113	150

Proximate analyses were calculated using the National Nutrient Database for standard reference, Agricultural Research Service, United States Department of Agriculture (70). Study muffins were formulated at the Canadian International Grains Institute (Winnipeg, MB)

[1]The SDG content was calculated based on published values of secoisolariciresinol, the aglycone of SDG, from a brown flaxseed strain typically consumed by Canadians and similar to that used in the present study (15).
MUFA monounsaturated fatty acid (12:1, 14:1, 15:1, 16:1, 16:1c, 16:1t, 17:1, 18:1, 18:1c, 18:1t, 20:1, 22:1, 22:1c, 22:1t, 24:1, 24:1c), *PUFA* polyunsaturated fatty acid (18:2n6cc, 18:2t,t, 18:2i, 18:3n3cccn-3, 18:4, 20:2cc, 20:3, 20:3n-3, 20:3n-6, 20:4n-6, 20:5n-3, 21:5, 22:2, 22:3, 22:4n-6, 22:5n-3, 22:6n-6), *SDG* secoisolariciresinol diglucoside, *SFA* saturated fatty acid (4:0, 6:0, 8:0, 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, 24:0).

Blood and Urine Measurements

Plasma fatty acids were extracted from thawed plasma aliquots and derivatized to their methyl esters using the method of Lepage and Roy (46). Plasma fatty acid methyl esters (FAME's) were quantified using an Agilent CP-3800 gas chromatograph (GC), equipped with a flame ionization detector (FID) and Agilent CP-Sil 88 capillary column (60m x 0.25mm x 0.20μm). The methyl esters, in toluene (1μl), were injected using a CP-8400 autosampler at a split ratio of 1:50. The flow rate of the helium carrier gas was 1.5 ml/min. The initial oven temperature was programmed at 80 °C and was held for 1 minute. It was then raised 30 °C /min to 140 °C, further increased by 5 °C /min to 225 °C and held there for 10 minutes. The total run time for each sample was 30 min. C17:0 was used as the internal standard and the fatty acid contents of the sample were identified by comparison with an authentic standard, GLC-462 (Nu-Chek Prep, Inc., Elysian, MN, USA).

A detailed description of plasma enterolignan extraction and measurement has been described previously using a method developed and validated within our laboratory (47). Briefly, 300 μL of human plasma, 300 μL of sodium acetate buffer (0.1M, pH 5.0) and 60 μL of 2600 units β-glucoronidase-sulfatase from *H. pomatia* (G1512) in 0.5M, pH 5.0 sodium

acetate solution were added to a silanized vial containing 1 μM hexadeuterated enterodiol ($^2H_6$-END) and hexadeuterated enterolactone ($^2H_6$-ENL) as the internal standards. The solution was incubated at 37°C for 4 hours and the resulting mixture separated using an Isolute SLE+ 1ml supported liquid extraction (SLE) column (Biotage, Charlotte, North Carolina, USA), contained on a 24-port vacuum manifold, using 70:30 diethyl ether:ethyl acetate (4 × 1.25ml). Following solvent evaporation under a steady stream of nitrogen gas with tube bases warmed to 37°C, the analytes were silanized to their trimethylsilyl derivatives using 120 μl of pyridine and 120 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The reaction proceeded at 90°C for 30 mins and the cooled samples were analyzed using gas chromatography/mass spectrometry in micro-selected ion storage mode (GC/MS-μSIS).

Total cholesterol (TC) and triglycerides (TG) were measured enzymatically using commercial kits from Point Scientific Inc. (Canton, MI, USA). High-density lipoprotein-cholesterol (HDL-C) measurements were performed enzymatically using a BioVision commercial kit (Milpitas, CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was calculated using the Friedewald equation (48).

Blood collected into sodium citrate tubes was gently inverted and then stored at room temperature for 30 min after which platelet aggregation studies were immediately performed. Platelet rich plasma (PRP) was obtained by a low speed centrifugation of the samples at 100 × g for 15 minutes. Removal of the upper PRP layer and further centrifugation of the remaining blood at 2400 × g for 15 minutes yielded the platelet poor plasma (PPP) which would behave as the blank for the aggregation experiments. Equal volumes of PRP and PPP were aliquoted into individual cuvettes. Collagen (4 μg/mL) or 0.5 units of thrombin was

then added to the PRP in separate controlled experiments. Both the PRP and PPP were stirred and spectrophotometric changes were monitored upon the addition of the aggregate using a Chrono-log Aggregometer, model 490 (Chrono-log Corp.). The Aggro/Link (vs 4.75) software was used to determine both percent aggregation and rate of aggregation.

Urine creatinine was measured using a modified Jaffe method based on the creatinine-picrate reaction (49). The thromboxane B_2 metabolite, 11-d-TxB_2 (11-dehydro-TxB_2,) was assayed using stable isotope dilution negative ion/chemical ionization gas chromatography mass spectrometry as previously described (50). Final results are presented as 11-d-TxB_2 in ng/g of creatinine.

Adverse effects monitoring

During the entire study, secondary adverse events associated with consuming dietary flaxseed (i.e. gastrointestinal discomfort, diarrhea, excessive gas, cramping, etc) were monitored. These were grouped and classified using Palmer's method of adverse event monitoring where symptom severity was graded from 0 to 4: 0 = none, 1 = mild, 2 = moderate, 3 = severe and 4 = death (51). An adverse event was considered mild if it was transient and inconsequential whereas it was considered moderate if it was persistent and/or systemic. A severe adverse event would be life-threatening or require hospitalization and death if an individual died from the direct consumption of flaxseed.

Outcomes and sample size calculations

The primary endpoint was to determine the change in plasma ALA concentration within- and-between groups after 4 weeks of consuming various doses of milled flaxseed. Secondary endpoints included changes in plasma enterolignan, cholesterol, triglyceride, fatty acid and urinary thromboxane concentrations as well as percent and maximum rate of

aggregation of platelets. Each individual at 0 week behaved as their own control for the study. Accounting for an estimated 20% sample loss, a sample size of 10 per group was assigned that would provide a minimum of 80% power to detect any significant differences in ALA between the groups. A two-tailed α of 0.5 was used with an estimated standard deviation of 35 μM based on published results (32).

Statistical analysis and efficacy

Results are represented as the means ± the standard error of the mean (SEM). All data was analyzed using IBM SPSS Statistics version 22.0 (International Business Machines, Armond, NY, USA). Categorical variables were compared with a χ^2 test. A repeated measure ANOVA, using the Greenhouse-Geisser test, was used to detect group effects and time and group by time interactions. Time was the repeated measure with two levels set at 0 and 4 weeks and group was the fixed factor. A Tukey multiple comparison post hoc test was used to detect significant differences between groups. All biomarkers were measured as a function of time and their interactions, or lack thereof, were determined using averaged values applied to bi-plots with visualization of interactions determined by intersecting lines. Paired t-tests were used to compare changes between baseline and end of treatment and a one-way ANOVA was used to compare mean values across the same time point. Differences were considered significant when *P*-values were ≤ 0.05. Trends were noted if $P \leq 0.1$. The efficacy of the data was included in the statistical analysis if subjects had consumed a minimum of 80% of the supplied muffins, donated blood samples at both time points and maintained study instructions as originally detailed during their screening visit.

RESULTS

Participant characteristics and anthropometric measurements at baseline

Ninety-eight percent of the enrolled subjects indicated that they had successfully completed the dietary study; however, only 85% (34 subjects) were included in the data analysis. One participant in the 10 g group dropped out of the study prior to starting the dietary intervention. Another five individuals had to be excluded from the study due to various reasons. Three individuals, one from each of the 20, 30 and 40 g groups, were removed as baseline plasma ALA concentrations were elevated (> 200 μM). Baseline concentrations of ALA are typically < 75 μM for healthy, age-matched adults in the Winnipeg vicinity (29, 32). Consumption of flaxseed based-supplements prior to study onset was previously established as part of the exclusion criteria. Two individuals were also excluded from the 40 g group as they had no detectable ALA (limit of detection, LOD = 1.1 μM) or enterolignans (LOD for END = 9.9 nM and ENL = 10.1 nM) in their plasma after 4 weeks strongly suggesting non-adherence. Therefore, a total of 34 individuals were included in the study (**Table 10**). Power calculations were performed again on each of the groups to ensure that 80% power was still being attained for ALA. Using the same criteria as described previously, a minimum of 80% power was achieved for ALA in each of the 4 groups. Patient characteristics at baseline, namely age, gender distribution, BMI, weight and height, were similar between all 4 of the intervention groups ($P > 0.05$) (**Table 10**).

Plasma fatty acid concentrations

Plasma ALA concentrations were measured as the primary end point in the present study and significantly increased over time in the 10, 30 and 40 g/d dosage groups after 4 weeks of consuming milled flaxseed ($P < 0.01$) (**Figure 14A**). A similar trend was observed

in the 20 g/d group (P = 0.096). Each 10 g increment of flaxseed led to a mild dose-dependent response as observed by the 1.5-, 1.6-, 1.7- and 1.9-fold increases in plasma ALA concentrations after 4 weeks on the respective dietary interventions. The remaining fatty acids, as percent total fatty acids, were analyzed using a repeated measures ANOVA (**Table 11**). No significant main effects for time or group were observed in total saturated fatty acids (SFAs), total monounsaturated fatty acids (MUFAs) or omega-6 polyunsaturated fatty acids (n-6 PUFAs). However, n-3 PUFAs increased after 4-weeks compared to baseline in all study groups, with significance achieved in the 30 g/d group (P = 0.007). This increase in n-3 PUFAs led to an attenuation in the n-6/n-3 ratio in the 10 g/d (P = 0.045), 30 g/d (P < 0.01) and 40 g/d (P = 0.008) groups. Eicosapentaenoic acid (EPA, C20:5n-3) significantly increased after 4-weeks of consuming 30 g/d of milled flaxseed (P = 0.047), but not with 40 g/d (P = 0.12). No significant differences between groups or changes over time were observed for either docosapentaenoic acid (DPA, C22:5n-3) or docosahexaenoic acid (DHA, C22:6n-3).

Table 10. Baseline characteristics of study participants.

Parameter	g of Flaxseed per Muffin by Group				All Participants (n = 34)	*P* - value
	10g Group (n = 9)	20g Group (n = 9)	30g Group (n = 9)	40g Group (n = 7)		
Age (years)	31.6 ± 2.8	32.4 ± 1.6	26.9 ± 2.1	30.3 ± 3.4	30.3 ± 1.2	0.28
Gender (M/F)	4/5	5/4	5/4	4/3	18/16	0.95
Weight (kg)	70.1 ± 2.5	77.9 ± 6.4	70.1 ± 4.3	70.5 ± 4.9	72.3 ± 2.3	0.57
Height (m)	1.68 ± 0.03	1.71 ± 0.03	1.70 ± 0.03	1.72 ± 0.02	1.70 ± 0.01	0.80
BMI (kg/m^2)	24.9 ± 0.8	26.5 ± 1.8	24.2 ± 1.1	23.6 ± 1.1	25.0 ± 0.6	0.43

Values are mean ± SE. Significant if $P < 0.05$.
M male, *F* female, *BMI* body mass index
Table reprinted with permission from Springer on April 21, 2015; © 2015 Edel, Patenaude, Richard, Dibrov, Austria, Aukema, Pierce and Aliani. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults (3).

Table 11. Repeated measures ANOVA for percent total fatty acids in plasma at 0 and 4 weeks of dietary supplementation with milled flaxseed at various dosages.

Fatty Acids	Time (wk)	Ground Flaxseed Groups 10 g/d FX (%)	20 g/d FX (%)	30 g/d FX (%)	40 g/d FX (%)	Effects (F-Value) (df=3) G	T	G×T
Σ SFA	0	30.9 ± 2.8	29.8 ± 2.5	30.3 ± 3.0	30.7 ± 2.5	0.10	0.09	0.03
	4	31.0 ± 2.4	29.8 ± 2.3	32.0 ± 2.8	30.9 ± 3.1			
Σ MUFA	0	27.4 ± 2.5	25.6 ± 2.3	27.5 ± 3.5	25.4 ± 2.1	0.06	0.08	0.23
	4	26.7 ± 2.1	26.5 ± 2.6	27.0 ± 2.2	26.6 ± 3.7			
Σ PUFAn-6	0	38.5 ± 1.7	40.9 ± 1.6	38.7 ± 1.2	40.7 ± 2.1	0.31	3.00	0.65
	4	38.8 ± 2.4	39.8 ± 2.1	37.6 ± 1.8	38.7 ± 2.5			
18:3n-3	0	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.33	34.93***	0.59
	4	0.9 ± 0.1‡	1.1 ± 0.2	1.0 ± 0.1‡	1.1 ± 0.2‡			
20:5n-3	0	0.6 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	3.02*	5.02*	0.59
	4	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.1†	0.9 ± 0.2			
22:5n-3	0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	1.21	9.17	0.77
	4	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1			
22:6n-3	0	1.7 ± 0.2	1.7 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	0.51	0.05	1.24
	4	1.7 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.2			
Σ PUFAn-3	0	3.2 ± 0.3	3.7 ± 0.3	2.7 ± 0.4	3.0 ± 0.4	1.15	14.00**	0.80
	4	3.6 ± 0.2	4.1 ± 0.5	3.6 ± 0.4‡	3.9 ± 0.4			
Σ PUFA	0	41.7 ± 1.8	44.6 ± 1.6	41.3 ± 1.3	43.7 ± 2.3	0.45	0.62	0.32
	4	42.4 ± 2.6	43.8 ± 2.4	41.1 ± 2.0	42.6 ± 2.8			
n-6/n-3	0	12.6 ± 0.8	17.3 ± 2.6	11.8 ± 1.2	14.4 ± 1.0	1.37	22.83***	3.06*
	4	10.9 ± 0.5†	10.8 ± 1.2	11.4 ± 1.3†	10.2 ± 0.9‡			
TFA	0	99.9	100.0	100.1	99.8			
	4	100.0	100.1	100.2	100.1			

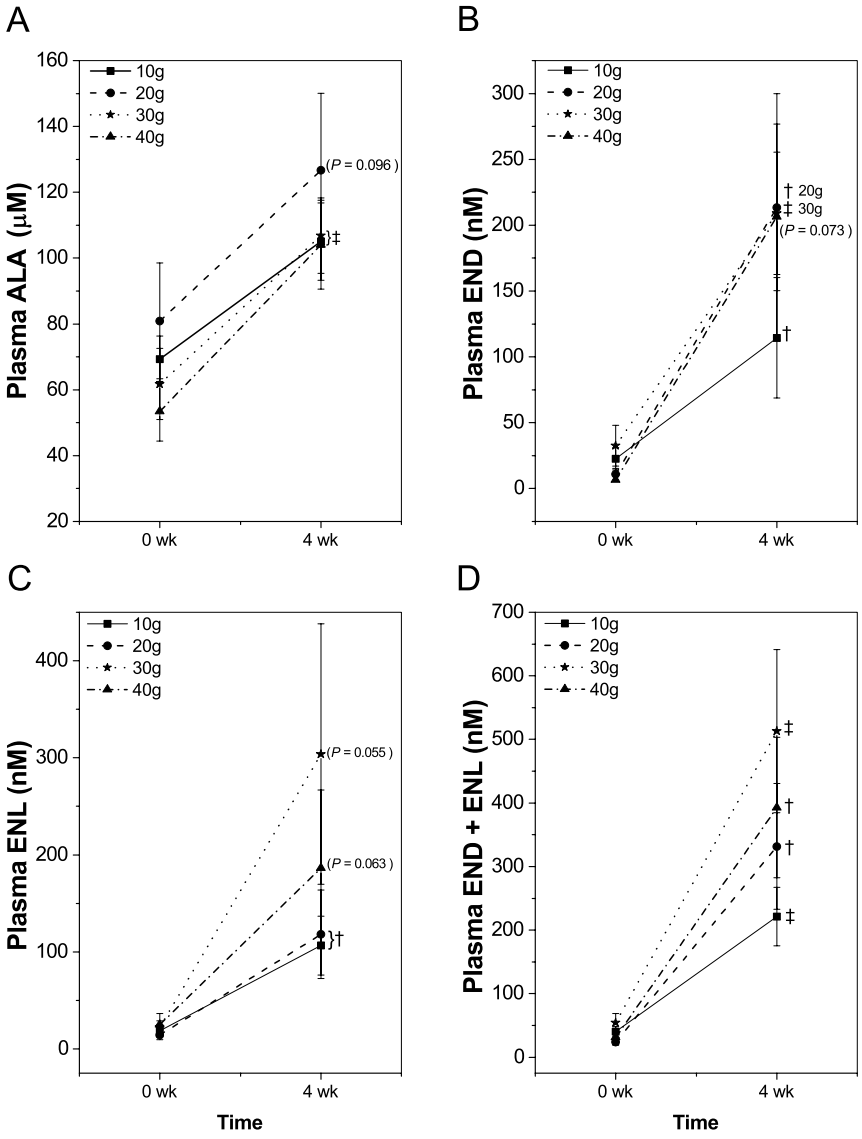
All values are mean ± SE
SFA's include 14:0, 16:0, 18:0 and 24:0; MUFAs include 14:1n-5, 16:1n-7, 18:1n-9, 18:1n-7 and 24:1n-9; PUFA n-6 include 18:2n-6, 18:3n-6, 20:3n-6 and 20:4n-6.
Between-group statistical significance, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Within-group statistical significance, † $P < 0.05$; ‡ $P < 0.01$
df degrees of freedom, *FX* flaxseed, *G* group, *G×T* group by time, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid, *SFA* saturated fatty acids, *T* time, *TFA* total fatty acid, *wk*, week.
Table reprinted with permission from Springer on April 21, 2015; © 2015 Edel, Patenaude, Richard, Dibrov, Austria, Aukema, Pierce and Aliani. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults (3).

Plasma lignan metabolite content

One individual in the 20 g/d milled flaxseed group was taking antibiotics for the first 11 days of the study and was therefore excluded from all enterolignan measurements as antibiotics are known to interfere with the gut microflora required to produce these compounds (52). As expected, this individual had no measurable plasma ENL and only trace amounts of END. Baseline enterolignan concentrations were consistent between all study groups ($P > 0.05$). Each of the plasma enterolignans increased over time as represented by a significant main-effect for time determined using a repeated measures ANOVA: END ($F = 31.026$, $P < 0.001$), ENL ($F = 15.392$, $P < 0.001$) and END + ENL ($F = 47.738$, $P < 0.001$). Despite these increases over time, no group differences in plasma enterolignan concentrations were observed as a result of increased flaxseed consumption: END ($F = 0.58$, $P = 0.63$), ENL ($F = 1.32$, $P = 0.29$) and END + ENL ($F = 1.58$, $P = 0.22$). When mean 4-week values were compared to baseline concentrations in each of the flaxseed dosage groups, END increased significantly in the 10 g/d ($P = 0.041$), 20 g/d ($P = 0.014$) and 30 g/d ($P = 0.005$) groups with a trend in the 40 g/d group ($P = 0.073$) (**Figure 14B**). Final END concentrations in the 20 g/d (213.5 ± 63.2 nM), 30 g/d (209.1 ± 46.6 nM) and 40 g/d (206.4 ± 93.5 nM) groups were practically doubled compared to those in the 10 g/d (114.5 ± 45.7 nM) group (**Figure 14B**). ENL was significantly increased in the 10 g/d ($P = 0.018$) and 20 g/d ($P = 0.040$) groups with trends in the 30 g/d ($P = 0.055$) and 40 g/d ($P = 0.063$) groups (**Figure 14C**). Final ENL concentrations were highest in the 30 g/d group (303.8 ± 126.5 nM) and lowest in the 10 and 20 g/d groups (106 nM) (**Figure 14C**). Subsequently, total enterolignans (END + ENL) increased significantly in all four flaxseed groups ($P < 0.05$)

(**Figure 14D**). Overall, a 5- to 31-fold increase in plasma enterolignan concentration was detected following one month of milled flaxseed consumption.

Figure 14. Plasma ALA (A) and enterolignans (B, C and D) at baseline and following 4-weeks of consuming 10, 20, 30 and 40 g/d of milled flaxseed.



There were no between-group differences, $P \geq 0.05$
Within-group changes over time, † $P < 0.05$, ‡ $P < 0.01$
ALA alpha-linolenic acid, *END* enterodiol, *ENL* enterolactone, *END + ENL* total enterolignans, *wk* week. *Figure reprinted with permission from Springer on April 21, 2015; © 2015 Edel, Patenaude, Richard, Dibrov, Austria, Aukema, Pierce and Aliani.. The effect of flaxseed dose on circulating concentrations of alpha-linolenic acid and secoisolariciresinol diglucoside derived enterolignans in young, healthy adults (3).*

Plasma cholesterol and triglyceride concentrations

Healthy, younger adults consuming various doses of milled flaxseed did not exhibit any significant changes after one-month in TC, LDL-C, HDL-C, triglycerides or in the TC/HDL-C ratio (**Table 12**). However, a trend for a main effect over time was measured for TC concentrations ($P = 0.058$). Significant group differences were detected for both TC and LDL-C. This was attributed to significantly attenuated baseline values in the 40 g/d flaxseed group relative to the 20 g/d group for TC ($P = 0.030$) and LDL-C ($P = 0.047$).

Platelet aggregation

The maximum platelet aggregation potential of plasma from individuals who had consumed 10 to 40 g/d of milled flaxseed was examined (**Table 12**). No differences in percent aggregation or rate of aggregation were observed between groups when either collagen (4 μg/mL) or thrombin (0.5 units) were used as agonists. However, a main effect for time was detected in percent aggregation with collagen, which was attributed to the 40 g/d group as aggregation was inhibited after 4 weeks as displayed by a trend in our data ($P = 0.058$). Rate of aggregation over time was unaffected by collagen. No significant changes over time in platelet aggregation were observed when thrombin was used.

Urine 11-d-TxB_2

No changes over time or differences between groups were observed in 11-d-TxB_2 (ng/g of creatinine) concentrations ($P \geq 0.05$) (**Table 12**).

Adverse events

There were no significant differences between the four dosage groups in the number of reported adverse events as assessed by Chi-square tests (**Appendix 5**) or in measured body weight and BMI (**Appendix 6**).

Table 12. Repeated measures ANOVA of plasma lipids, platelet aggregation and urinary 11-dehydro-thromboxane B_2 at 0 and 4 weeks for each of the intervention groups.

Parameter	Time	Ground Flaxseed Groups				Effects (F-Value) (df = 3)		
		10 g/d FX	20g/d FX	30g/d FX	40g/d FX	G	T	G×T
Lipids								
TC,	0 week	144.5 ± 8.5^{ab}	153.6 ± 10.3^{a}	135.9 ± 6.1^{ab}	118.4 ± 5.8^{b}	3.41*	3.90	0.05
mg/dL	4 week	135.1 ± 6.7	146.4 ± 0.2	129.1 ± 6.3	112.8 ± 9.1			
LDL-C,	0 week	77.4 ± 6.35^{ab}	91.6 ± 0.3 ^{a}	74.0 ± 7.55^{ab}	59.8 ± 5.7^{b}	2.95*	2.43	0.41
mg/dL	4 week	66.2 ± 5.1	85.3 ± 9.9	71.3 ± 5.3	58.0 ± 7.7			
HDL-C,	0 week	47.2 ± 4.3	44.8 ± 7.1	43.3 ± 5.7	42.0 ± 3.0	0.50	1.18	1.20
mg/dL	4 week	50.3 ± 5.6	41.8 ± 5.2	40.0 ± 5.3	39.1 ± 2.8			
TC/HDL-C,	0 week	3.2 ± 0.2	4.2 ± 0.7	3.6 ± 0.5	2.9 ± 0.2	1.18	0.01	0.69
ratio	4 week	2.9 ± 0.3	4.2 ± 0.7	4.0 ± 0.8	2.9 ± 0.1			
TG,	0 week	99.6 ± 16.5	93.1 ± 15.5	93.1 ± 21.6	82.8 ± 13.8	0.19	0.11	0.38
mg/dL	4 week	93.1 ± 11.6	96.5 ± 13.0	88.8 ± 12.7	78.9 ± 15.6			
Platelet Aggregation								
Percent Aggregation								
collagen (4 μg/ml),	0 week	76.2 ± 2.3	73.8 ± 2.1	76.0 ± 2.9	77.0 ± 2.9	0.67	9.73**	1.90
%	4 week	73.4 ± 1.8	71.6 ± 3.3	72.0 ± 2.3	64.0 ± 4.0			

Parameter	Time	Ground Flaxseed Groups				Effects (F-Value) (df = 3)		
		10 g/d FX	20 g/d FX	30 g/d FX	40 g/d FX	G	T	G×T
thrombin (0.5 units),	0 week	92.2 ± 3.1	94.3 ± 2.5	91.6 ± 2.5	91.1 ± 3.2	1.22	0.54	0.89
%	4 week	87.3 ± 10.2	95.4 ± 1.6	93.2 ± 3.4	87.9 ± 5.1			
Max. Rate of Aggregation								
collagen (4 μg/ml),	0 week	140.4 ± 8.1	128.4 ± 8.4	124.8 ± 8.4	130.7 ± 11.6	0.35	0.03	1.68
% change/min	4 week	130.6 ± 9.3	140.2 ± 11.3	131.3 ± 6.8	120.4 ± 10.0			
thrombin (0.5 units),	0 week	240.7 ± 15.4	260.6 ± 18.9	262.9 ± 15.1	232.6 ± 16.4	1.16	0.02	0.12
% change/min	4 week	246.9 ± 28.0	255.7 ± 17.1	274.4 ± 13.8	228.0 ± 21.5			
Urine Analysis								
11-d-TxB_2,	0 week	189.8 ± 28.4	196.1 ± 26.2	182.9 ± 32.5	179.4 ± 38.5	0.22	0.07	0.26
ng/g of creatinine	4 week	185.4 ± 31.6	204.1 ± 20.5	161.9 ± 18.9	183.3 ± 36.5			

All values are means ± SE
df degree of freedom, *FX* flaxseed, *G* group, *G×T* group by time, *HDL-C* high density lipoprotein cholesterol, *LDL-C* low density lipoprotein cholesterol, *TC* total cholesterol, *TG* triglycerides, *wk* week
Difference over time, * $P < 0.05$. Within-group statistical significance, † $P < 0.05$
Means with the same letter at 0 weeks are not significantly different, ^{a,b} $P < 0.05$
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DISCUSSION

This study demonstrates that consuming doses of milled flaxseed higher than 10 to 20 g/d may be unnecessary for improving plasma ALA and enterolignan concentrations in healthy adults with no pre-existing clinical evidence of disease. It was also determined that milled flaxseed doses ranging from 10 to 40 g/d do not impact blood lipids, platelets, urinary thromboxane markers or adverse event reporting in individuals who are younger and already healthy.

ALA concentration in plasma is a measure of tissue availability. In the present study, significant increases in plasma ALA were observed after 4 weeks in all treatment groups but the 20 g/d group. This may be attributed to elevated baseline ALA concentrations in this group. A larger sample size would likely resolve this. Increased serum or plasma ALA concentrations, as a result of milled flaxseed consumption, have been reported in a number of human studies using doses as low as 13 g/d (41), 26 to 30 g/d (8, 29, 32, 41), 40 g/d (27, 33, 53) and as high as 50 g/d (36). Although a dose-dependent fold-increase in ALA was calculated with each 10 g increment of milled flaxseed, it was surprising that there were no significant differences between any of the treatment groups. In a crossover trial involving Lupus Nephritis patients, flaxseed was administered at doses of 15 g/d, 30 g/d or 45 g/d over a similar 4 week period (40). Unfortunately, these authors did not define the flaxseed form (i.e., milled or whole) used in the trial so it is difficult to fully compare these two studies. However, no differences in circulating ALA were detected between any of the flaxseed treatment groups, similar to our study. In another trial involving overweight, pre-diabetic individuals, plasma ALA concentrations significantly increased in a dose-dependent manner when a diet containing 0 g/d, 13 g/d and 26 g/d of milled flaxseed was ingested for 12 weeks

(41). This would suggest that the health status of the subject, and not the treatment duration (8, 29), may influence ALA concentration as a result of flaxseed dose. As doses ≥30 g/d were not examined (41), it is unknown if higher doses would elevate plasma ALA concentrations beyond those achieved with the 26 g/d dose.

Another interest of the present study was to determine if ALA metabolism, to the longer chain polyunsaturated fatty acids EPA, DPA and DHA, could be influenced in a dose-dependent manner as a result of milled flaxseed dose. It was surprising that only 30 g/d, and not 10, 20 or 40 g/d, significantly increased circulating EPA concentrations. Plasma EPA concentration, as a result of milled flaxseed dose, has only increased in one other human study to our knowledge at this same dose (8) yet not in others (29, 32). Other studies using both higher doses (40 g/d) (27, 53) and lower doses (13 and 26 g/d) (41) of milled flaxseed found no significant changes compared to placebo. However, when EPA was measured in plasma or serum phospholipids it increased significantly when the flaxseed dose was 30 g/d (40, 54) and 50 g/d (36), but not 15 g/d (40). These results, along with ours, suggest that milled flaxseed doses ≥ 40 g/d or < 30 g/d are insufficient to elevate plasma EPA concentrations, yet do appear more favorable in improving plasma phospholipid EPA concentrations. Interestingly, the same ALA doses administered as pure ALA or added to a variety of food products in the form of flax oil, all increased EPA concentrations in plasma (55-57), serum (58), plasma phospholipids (54) and erythrocyte phospholipids (59). This suggests that ALA taken as milled flaxseed is not an efficient approach to increase circulating EPA concentrations. Unfortunately, DPA was not reported in most of these studies, yet in both trials that used 40 g/d of milled flaxseed, despite no measurable changes in EPA, significant increases in plasma DPA were detected (27, 53). When plasma

phospholipids were measured, only the 50 g/d dose increased DPA (36). Future studies using dietary milled flaxseed should report plasma DPA concentrations as DPA can inhibit platelet aggregation (60) and angiogenesis (61) and can improve endothelial cell migration and proliferation (62). The null effect of flaxseed dose on DHA is consistent with other clinical trials noting the poor conversion of ALA to DHA in humans (8, 27, 29, 32, 36, 41, 53, 54). The benefits of reducing the n-6/n-3 ratio as in the present study, or conversely improving the n-3/n-6 ratio, have also been demonstrated as this ratio has been correlated with improved plaque composition, reduced progression and improved regression of plaques within the coronary arteries (63).

This is the first study to examine milled flaxseed dose on plasma concentrations of END and ENL. Similar to ALA, we were interested in the dose of milled flaxseed that would provide maximum concentrations of circulating enterolignans and whether increased doses would yield a dose-dependent response. Although dietary consumption of milled flaxseed increased plasma lignan metabolites in all 4 intervention groups, it was once again surprising that no between-group differences were observed. An apparent dose-dependent response in total enterolignan concentrations was observed when doses of 10 to 30 g/d were consumed (**Figure 14D**), yet not at 40 g/d. As no group differences existed, this would suggest that 10 g/d of milled flaxseed is sufficient to significantly increase all reported enterolignans in the plasma of healthy adults. Similar to ALA, higher doses of 40 g/d may be unnecessary to achieve maximum circulating plasma enterolignan concentrations. Flaxseed dose may, however, exhibit a dose-dependent response in urinary enterolignan excretion (43, 44). It would seem necessary that future studies include measurements for both urinary

enterolignan output and circulating plasma concentrations as both values would be useful in assessing milled flaxseed dose.

High serum ENL concentrations have been associated with reduced mortality due to cardiovascular related diseases (64). It was hypothesized that the increased bulk fiber coming from higher flaxseed dose would alter transit time thereby converting more END to ENL. The ratio of END to ENL as a factor of flaxseed dose remained relatively constant between all four groups despite the increased fiber content, which is consistent with similar reports at a 25 g/d dose (65).

BMI and self-reported incidences of adverse events were also analyzed in relation to milled flaxseed dose. Averaged BMIs did not change within- or between-groups after consuming any of the four flaxseed doses. As the total fat content of muffins increased with each 10 g addition of milled flaxseed, it was important to see this null effect on BMI. Furthermore, participants reported only mild to moderate adverse events when consuming milled flaxseed with no particular difference noted at any dose. It was hypothesized that most of the adverse events such as bloating, increased flatulence or cramping would be reported with the highest flaxseed dose due to its elevated fiber content. However, there were no statistical differences in adverse event reporting between-groups within this study of healthy, young adults.

Health Canada has a cholesterol-lowering health claim for 40 g/d of flaxseed (66). This was based on studies at this dose in postmenopausal (67, 68), menopausal (35) and hypercholesterolemic (27) adults. Other studies using 30 g/d of milled flaxseed report no effect in healthy adults (29, 32) and significant decreases in total- and LDL-cholesterol in patients with peripheral artery disease (11). As the participants in the present study were

healthy and younger, it was not surprising to see no changes in these two lipid variables at doses ≤ 30 g/d. However, 40 g/d was still too low, despite having the highest fiber load. Only higher doses of 50 g/d have been shown to provide cholesterol-lowering effects in healthy, young adults (37).

Platelet aggregation, a critical process in wound healing, was explored in relation to flaxseed dose. It was hypothesized that platelet aggregation would be inhibited with higher milled flaxseed dose as flaxseed oil at higher doses has been shown to dramatically reduce platelet activity (69). Percent aggregation was only mildly attenuated with 40 g/d of flaxseed when collagen ($P = 0.058$), not thrombin, was used as the agonist. This lack of change in rate and extent of platelet aggregation with increasing amounts of milled flaxseed is very important for individuals that desire the health benefits of flaxseed, yet have or anticipate problems with bleeding times. These results confirm that consuming increased doses of milled flaxseed (10 to 40 g/d) may not affect bleeding times in healthy individuals which is critical for patients requiring surgery or that may have a predisposition to cardiovascular or thrombotic diseases.

In conclusion, this is the first study to present the effects of milled flaxseed dose, ranging from 10 to 40 g/d, on circulating concentrations of n-3 fatty acids, enterolignans and blood lipids, in healthy, younger adults. Additionally, it provides insight into the safety of consuming such doses in relation to reported adverse events, platelet aggregation and urinary thromboxane concentrations. The results indicate that milled flaxseed doses as low as 10 g/d may be sufficient to significantly increase circulating ALA, END, ENL and total enterolignan concentrations and that higher doses may be unnecessary. However, only 30 g/d of milled flaxseed significantly improved plasma concentrations of EPA and total n-3

PUFA content. DPA and DHA were unaltered by any dose. Total- and LDL-cholesterol were unchanged at even the highest flaxseed dose suggesting that 50 g/d (36), not 40 g/d, is required to reduce cholesterol levels in this study population. However, in older adults presenting lipid abnormalities, lower flaxseed doses of 40 g/d (66) and 30 g/d (11) were successful in lowering cholesterol levels. Dosing studies in elderly, healthy adults and elderly adults with elevated cholesterol levels are warranted as they are most at risk for developing CV related diseases. With few reported adverse events, and minimal changes in platelet aggregation and urinary thromboxane markers, milled flaxseed at both lower (10 g/d) and higher doses (40 g/d) appears safe for consumption in healthy, younger adults.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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CHAPTER VII: Age-dependency in the metabolism of flaxseed lignans by healthy adults[1]

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ABSTRACT

Flaxseed lignan metabolites, enterodiol (END) and enterolactone (ENL), have biological activities that may have potential therapeutic benefit through their antioxidative and estrogenic properties. Circulating enterolignan concentrations are influenced by gut bacteria, which itself is subject to change with age. The plasma availability of END, ENL and total enterolignans (END + ENL) after 0 and 4-weeks of dietary milled flaxseed consumption (30 g/d) in healthy, younger (18-29 years) and older (45-69 years) adults was determined by using gas chromatography/mass spectrometry. Plasma total enterolignan concentrations increased in both younger and older adults; however, the distribution of END:ENL was lower in older adults (0.74) compared to younger adults (0.96). Post flaxseed treatment, ENL was detected in the plasma of 100% of the older adults, but only in 78% of the younger ones. The potential therapeutic benefit offered by enterolignans may be particularly important for aging populations that are prone to chronic diseases.

Keywords

enterolignans • flaxseed • phytoestrogens • gut bacteria • lignans • cardiovascular disease

Chemical compounds studied in this article

Enterodiol (PubChem CID: 123725); Enterolactone (PubChem CID: 114739);

Secoisolariciresinol Diglucoside (PubChem CID: 9917980)

Abbreviations

ALA, alpha-linolenic acid; BMI, body mass index; BSTFA, N,O–bis(trimethylsilyl)trifluoroacet-amide; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; END, enterodiol; ENL, enterolactone; GC/MS, gas chromatography mass spectrometry; LDL, low-density lipoprotein; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; μSIS, micro-selected ion storage.

INTRODUCTION

Epidemiological studies list cancer and cardiovascular disease as the two main causes of death in North America (1). Asian populations have traditionally had lower risks of mortality associated with these outlined chronic diseases. This has been in part attributed to a diet high in phytoestrogens (2, 3). Phytoestrogens are plant-based compounds that are classified as isoflavones, coumestans and lignans (4). Isoflavones are found primarily in soy products, coumestans in sprouts and lignans in oil seeds, vegetables, fruits, nuts, whole grains, tea and red wine (4). Consumption patterns of phytoestrogens in Asian populations are about 20-80 mg/d and are largely isoflavone-based (3, 5). This is in contrast to Western diets with phytoestrogen intakes typically less than 1 mg/d and this is largely lignan based (80%) with 20% coming from isoflavones and <0.1% coumestans (5). Lignans have both weak estrogenic and antiestrogenic activities, antioxidative potential (6) and may have a role in disease prevention (6), which warrants further investigation.

Dietary flaxseed has by far the highest content of the lignan secoisolariciresinol diglucoside (SDG), measured as secoisolariciresinol (SECO), in food (375 mg/100 g) (4). The next highest food sources of SECO include rye (360-fold less) and wheat (870-fold less) (7), asparagus (180-fold less) (8), chestnuts (2170-fold less) (4) and legumes (9). Other lignans in flaxseed include matairesinol, pinoresinol and lariciresinol, but in much lower quantities, namely 2448, 514 and 134 times lower, respectively (4). In rabbit models, SDG ingestion lowered circulating LDL-cholesterol and attenuated atherosclerosis (10), reduced glycated hemoglobin and the onset of diabetic symptoms (11). Mouse models of breast cancer have documented the antitumorigenic effects of dietary flaxseed and pure SDG (12). More recent clinical studies using SDG supplements or a lignan complex report attenuations

in glucose concentrations (13), metabolic syndrome composite scores in males (14), diastolic blood pressure (14) and the inflammatory marker, C-reactive protein (15). Flaxseed lignans can also reduce the occurrence of breast cancer by 33-70% through the attenuation of angiogenesis, cellular proliferation and apoptosis (16). Flaxseed is also a rich source of the omega-3 fatty acid alpha-linolenic acid (ALA) and fiber, both of which have documented benefits in reducing risk factors of CVD (17, 18); however, only lignans can be metabolized to enterolignans and will be the focus of the present discussion.

Ingestion of SDG does not result in its entry into the circulation (19). SDG is metabolized by microflora in the gut to the mammalian enterolignans, enterodiol (END) and enterolactone (ENL), which are considered to be the two primary bioactive forms of SDG (19). The less abundant flaxseed lignans are also metabolized to one or both of these enterolignans (20). SDG metabolism involves bacterial hydrolysis to the aglycone secoisolariciresinol (SECO), subsequent demethylation and dehydroxylation to yield END, which may then undergo dehydrogenation within the intestinal tract to ENL (21, 22). Clavel has demonstrated in anaerobic models of the gut that specific strains of bacteria promote the formation of END and ENL (20). Once enterolignans are formed, they permeate the intestinal mucosa and are repackaged into sulphate- or glucuronide-conjugates in the liver (23). Following conjugation, they enter the enterohepatic circulation and may be eliminated in urine and bile, or they may circulate systemically (23) and/or be stored in a variety of tissues (24). A portion may also be deconjugated in the large intestine and eliminated in feces. Other factors that may influence enterolignan formation include intestinal pH and oxygen gradients (25), bile acids, transit time (26), diet (27), flaxseed cultivar (28), flaxseed

form (29) and antibiotic usage (30). An outcome of this is that there can be large inter-individual variations in enterolignan bioavailability in human plasma samples (29, 31).

Subject age may also influence lignan metabolism and enterolignan absorption. It is well established that gut microbiota composition changes throughout a person's lifespan (25). The three phases of change occur from birth to weaning, from weaning to a habitual diet and throughout old age. Generally, a decline in microbial variety has been observed in older populations (32), with increases in enterobacteriaceae, declines in bacteroides (33, 34), and reports of reduced (34) or stable numbers of bifidobacteria and lactobacilli (33). Individuals older than 65 years of age had greater bacteroidetes and less firmicutes compared to their younger counterparts (28-46 years) (35). The health effects of the microbial environment on lignan metabolism and enterolignan absorption are still largely unknown. Gender may also play a role in gut microbiota composition as significantly higher enterolignan producing organisms are present in females compared to males (36).

Chronic diseases are also influenced by increased age (37) and oxidative stress (38, 39). It is well established that blood antioxidative potential improves with consumption of foods rich in antioxidants, namely lycopene enriched tomato sauce (40) or golden flaxseed (41) as examples. Enterolignans exhibit free-radical scavenging capabilities *in vitro* (42) and *in vivo* (43). Therefore, flaxseed lignan therapy may be a potential solution for Western populations whose incidence of chronic disease in elderly subjects continues to rise. However, prior to using dietary flaxseed in a diseased population, lignan metabolism and enterolignan absorption needs to be studied in a healthy population to assess circulating concentrations. Therefore, the aim of this study was to examine lignan metabolism through

enterolignan plasma availability in healthy, younger and older populations consuming dietary milled flaxseed fortified in muffins as a source of lignans.

MATERIAL AND METHODS

Ethics and study design

This study design was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Twenty-eight healthy male and female adults were recruited and divided by age and gender into two separate groups. These individuals were considered healthy, as they did not have any chronic diseases. The two groups were differentiated by age and designated as a younger population (18-29 years of age) and an older population (45-69 years of age). Subjects were then randomized into one of two dietary interventions. Twenty participants were randomized to a milled flaxseed group with 10 subjects in each of the younger and older age categories. Two participants dropped out of the study during the first week, one from each of the two age groups, due to an inability to maintain study requirements. One sample from the younger group did not derivatize properly and therefore could not be analyzed. The remaining eight participants were allocated to a flax oil group with 4 subjects in each of the two age categories. Neither flax oil nor the remaining ingredients used to prepare the dietary interventions was expected to contain the bioactive of interest, but we included this smaller group as a control. Volunteers were included in the study if they provided written informed consent, could comply with the study schedule, were between 18 and 29 or 45 and 69 years of age, could store the food products in a home freezer (-20°C), were not using cholesterol-lowering drugs, hypertension drugs, antihistamines and did not ingest supplementary vitamins or oils one month prior to and during the study. Subjects were excluded if they had smoked tobacco products over the last 6 months, had taken supplements within one month of study onset, consumed more than one fish meal per week, had any medical history of

diabetes, renal or gastrointestinal problems or had platelet clotting abnormalities. The average age of subjects in the younger aged milled flaxseed group was 22.4 ± 1.2 years with an average BMI of 25.6 ± 1.4 kg/m^2. There were 6 males and 3 females in this group. The older participants consisted of 4 males and 5 females, an average age of 54.1 ± 2.1 years and a BMI of 23.9 ± 0.6 kg/m^2. The flax oil groups had an average age of 24.8 ± 2.2 years and 53.8 ± 2.8 years, for the younger and older groups, respectively, and BMI's of 24.0 ± 1.5 and 25.9 ± 2.6 kg/m^2. Each group had two females and two males.

In this randomized, parallel study design, subjects were blinded to the age component of the study and to their dietary group. Participants from both age groups ingested one muffin daily, containing either milled flaxseed or flax oil. Muffins were thawed daily and consumed for a total of 4 weeks. The research team was blinded to the treatment group from the initial randomization until study termination, inclusive of data analysis.

Experimental dietary intervention

Muffins, fortified with flaxseed, were prepared by the Canadian International Grains Institute, Winnipeg, Canada and were used as functional foods in this particular study. Milled flaxseed and flax oil were supplied from Bioriginal Food and Science Corporation (Saskatoon, SK, Canada). Muffins contained either 30 g of milled flaxseed or 12.05 g of flax oil, with an equivalent 6 g of ALA in each. The same batch of flax oil was used to prepare all flax oil muffins as was the batch of flaxseed for the milled flaxseed muffins. Fatty acid profiles, energy and nutrient compositions of the muffins have been previously reported (44). The lignan content of the flax oil used in the present study was negligible (45). In the milled flaxseed group, SDG, measured as the aglycone SECO, was approximately 113 mg in 30 g of flaxseed, based on published findings for Canadian brown flaxseed (4). Muffins were

prepared in two different flavours: cranberry orange and banana chocolate chip. Sensory evaluation and formulation details are available for the milled flaxseed cranberry-orange muffin (46). Formulation details for each of the milled flaxseed and flax oil muffins are available in **Table 13**.

Compliancy

Adherence to the dietary protocol was assessed using a combination of strategies. Subjects were asked to record their daily muffin intake in a food diary that was to be completed and returned at the final visit. They were also verbally asked about their ability to maintain the dietary regime throughout the 1-month period. Volunteers were requested to return any unused muffins at their final visit. All of this information was considered when assessing their compliancy to the dietary protocol. Confirmation of participant compliancy was also based on previously reported plasma ALA concentrations (44) and plasma total enterolignans as measured in the present study. Plasma ALA levels are typically doubled in plasma at this dose of 6 g of ALA (44, 47) whereas total enterolignans may increase anywhere from 10- to 50-fold (17, 48) when 30 g of milled flaxseed is ingested. Participants were included in the data analysis if one of these values increased (for subjects in the milled flaxseed group) or if ALA increased (for subjects in the flax oil group) following the 4-week dietary intervention.

Table 13. Formulation of milled flaxseed and flax oil muffins (recipe for one muffin).[1]

Ingredient	Cranberry Orange Muffins		Banana Chocolate Chip Muffins	
	Milled	Oil	Milled	Oil
DRY				
Flour (g)	31.67	53.33	30.00	53.33
Milled flaxseed (g)	30.00	0	30.00	0
Baking soda (g)	0.36	0.36	0.72	0.72
Baking powder (g)	1.58	1.60	1.50	1.87
Salt (g)	0.48	0.53	0.45	0.80
TOTAL DRY	64.08	55.83	62.67	56.72
WET				
Eggs (g)	16.67	13.33	16.67	16.67
Milk (g)	16.67	13.33	31.67	18.33
Flax oil (g)	0	12.05	0	12.05
Granulated sugar (g)	16.67	16.67	0	0
Brown sugar (g)	0	0	15.00	15.00
Vanilla (g)	2.17	2.17	0	0
Bananas, mashed (g)	0	0	33.33	33.33
Frozen orange juice (g)	9.77	9.77	0	0
Water (g)	28.57	28.57	0	0
Orange rind (g)	1.67	1.67	0	0
Chocolate chips (g)	0	0	7.25	7.25
Cranberries (g)	15.00	15.00	0	0
Total wet (g)*	92.17	97.55	96.67	95.38
Total dry and wet (g)	171.25	168.38	166.58	159.35
Dry:Wet	0.70	0.57	0.65	0.59
Muffin weight (g)	171.25	168.38	166.58	159.35

* excludes chocolate chips and cranberries.

[1] Muffins were prepared a dozen at a time.

Table reprinted with permission from Elsevier on June 26, 2015; © 2015 Edel, Pierce and Aliani. Age-dependency in the metabolism of flaxseed lignans by healthy adults (4).

Sample collection

Participants fasted 12-hours prior to providing blood. Venous blood samples were taken at study onset and after 4 weeks in the St. Boniface Hospital Satellite Laboratory. Blood for lignan metabolites (4 mL) was collected into one ethylenediaminetetraacetic acid (EDTA) tube (1 mg EDTA/mL) which was centrifuged at 1800 × g for 5 min at 4 °C. Plasma was then aliquoted into two cryovials (VWR International, Mississauga, ON) (P/N 479-0822), flash frozen in liquid nitrogen and placed in a -80 °C freezer. Analysis of lignan metabolites was conducted on thawed samples.

Sample extraction and analysis

Enterolignans were hydrolysed and extracted using a slightly modified method of Kuijsten *et al* (49). Briefly, one μM of a mixture of hexadeuterated enterodiol ($^2H_6$-END) and enterolactone ($^2H_6$-ENL) as internal standards (final concentration of 830 nM), were added to silanized vials. Three hundred microliter of plasma was combined with 300 μL of 0.1M sodium acetate buffer (pH 5.0) and 60 μL of 2600 units β-glucuronidase sulfatase from *H. Pomatia* (Sigma Aldrich, Oakville, ON, Canada) (G1512) made up in 0.5M sodium acetate solution (pH 5.0) and added to the internal standard mixture. The resulting mixture was incubated at 37°C for 4 hours and subsequently extracted twice with diethyl ether (1.5mL). The ether fractions were combined following centrifugation (2300 x g, 10°C, 10 min) and the solvent removed under a gentle stream of nitrogen to liberate the enterolignans. Silylation to their trimethylsilyl derivatives was then accomplished using 120 μL of pyridine and 120 μL of N,O–bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 90°C for 30 mins and cooling to room temperature overnight. Analytes were quantified using GC/MS in micro-selected ion storage (μSIS) mode as previously described (50).

Statistical analysis

Results are presented as average values ± standard error of the mean (SEM) and were analyzed using IBM SPSS Statistics version 22.0 (International Business Machines, Armond, NY, USA). A repeated measures 2-Way ANOVA, with sphericity assumed, was used to evaluate changes over time, by age and age × time interactions. Time was the repeated measure with two levels at 0 and 4 weeks. Age was represented as two levels with a younger age group (18-29 years of age) and an older age group (45-69 years of age). The data was further examined using gender as a covariate. Categorical variables were compared using a Fisher's Exact Test. Paired t-tests were used to compare changes between baseline and end of treatment and Independent Samples T-tests were used to compare changes between different age groups at the same time point. Spearman correlations were used to evaluate main associations between subject age and enterolignan concentration. Differences were considered significant when *P*-values were ≤ 0.05. Trends were noted if $P \leq 0.1$.

RESULTS

Plasma enterolignan concentrations in adults

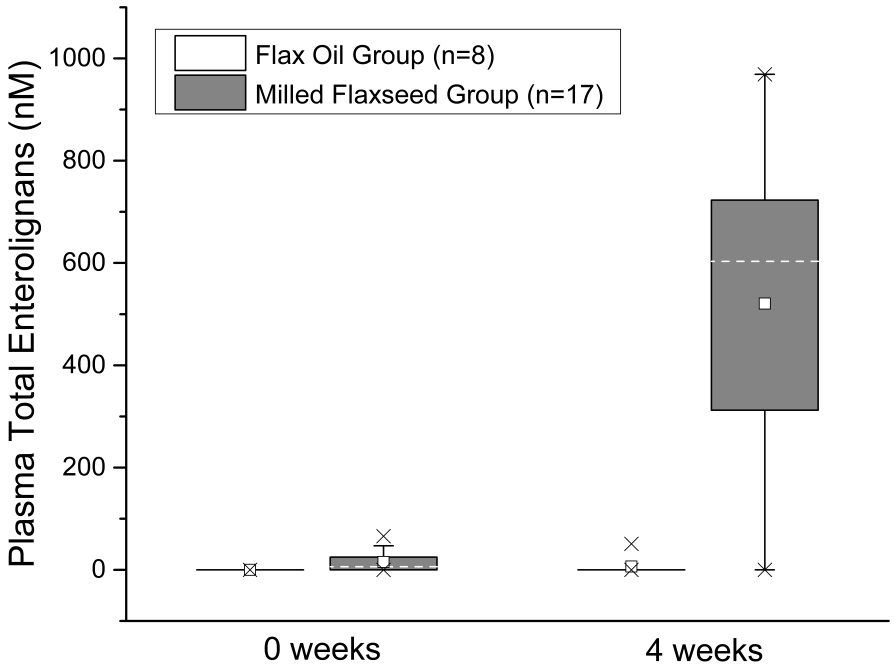
Combining all participants from both age groups (18-69 years of age) and intervention groups (flax oil and milled flaxseed), baseline enterolignan concentrations averaged 15.2 ± 4.8 nM for ENL and were below the limit of detection (LOD) for END, which was 6.6 nM (50). Therefore, total enterolignan (END + ENL) concentrations at baseline were 15.2 ± 4.8 nM. After 4-weeks of consuming muffins containing flax oil there were no changes in plasma total enterolignan concentrations (**Figure 15**). However, dietary consumption of muffins containing 30 g of milled flaxseed provided subjects with average plasma END and ENL concentrations of 233.4 ± 69.9 nM ($P = 0.004$) and 287.4 ± 60.6 nM ($P \leq 0.001$), respectively, compared to baseline values. The ratio of END:ENL was 0.81. Total enterolignans averaged 520.8 ± 78.0 nM after milled flaxseed consumption, which was significantly greater than the respective baseline concentrations and those at 4 weeks in subjects consuming flax oil ($P \leq 0.001$) (**Figure 15**).

Plasma enterolignan concentrations as a function of age

Plasma enterolignan profiles in adults grouped by age, younger (18-29 years of age) and older (45-69 years of age), at 0 and 4 weeks are described in **Table 14** and **Figure 16** for participants in the milled flaxseed group. Because the concentrations of lignan metabolites were negligible in participants consuming muffins made with flax oil, subsequent results only pertain to the milled flaxseed group. Baseline concentrations of END for both age groups were below the limit of detection. Baseline ENL concentrations averaged 9.8 ± 4.4 and 20.1 ± 8.5 nM for the younger and older groups, respectively ($P \geq 0.05$). After 4-weeks of consuming flaxseed, a significant main effect for time was observed for each of the

enterolignans ($P \leq 0.006$) (**Table 14**). Pairwise analysis revealed a significant increase for the older age group in measured plasma END ($P = 0.024$), ENL ($P = 0.001$) and END + ENL ($P < 0.001$) after 4-weeks compared to baseline values. This is in contrast to the younger group, which did not achieve significance for END ($P = 0.108$) or ENL ($P = 0.089$), but did for END + ENL ($P = 0.019$). Subject age did not result in any significant differences for measured enterolignan concentrations as suggested by the lack of main effects for age group in the repeated measures ANOVA analysis ($P \geq 0.05$).

Figure 15. Plasma total enterolignan [END + ENL] concentrations compared at baseline and 4 weeks for the flax oil (n=8) and milled flaxseed (n=17) treatment groups.



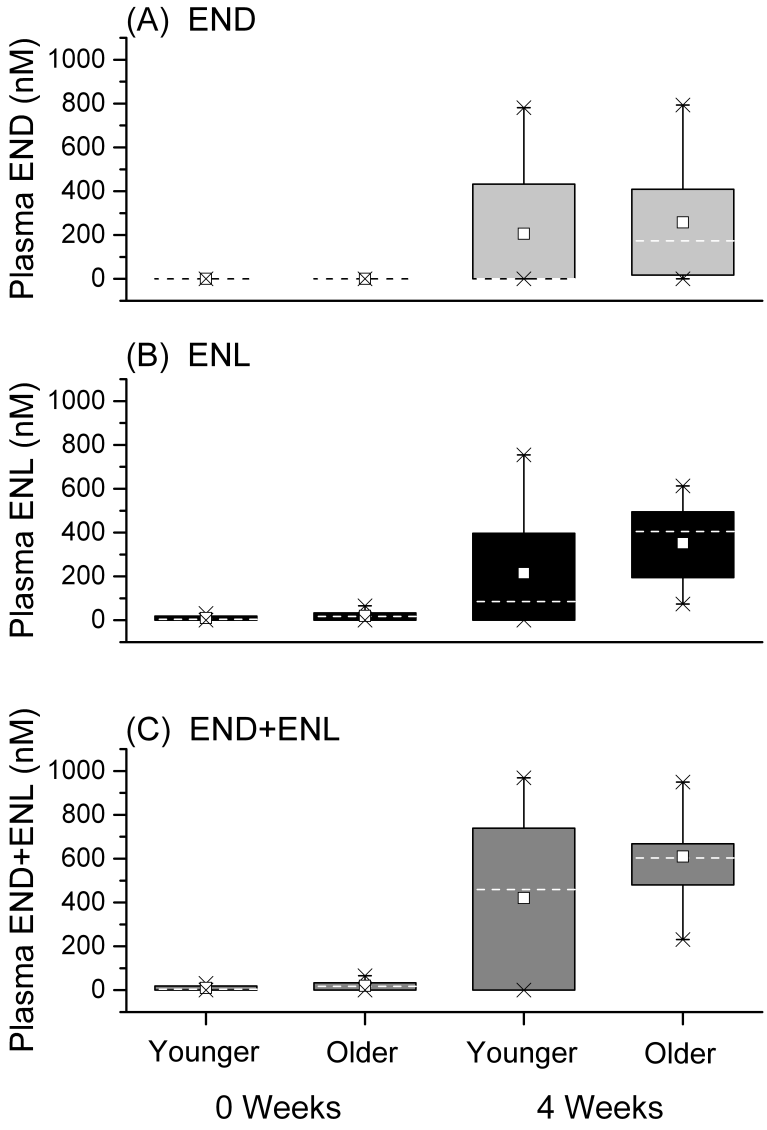
Individual variation is embodied in the rectangle of this statistical box plot with dashed lines representing the median value, the square representing the mean and × representing 1% and 99% outliers. *Image reprinted with permission from Elsevier on June 26, 2015; © 2015 Edel, Pierce and Aliani. Age-dependency in the metabolism of flaxseed lignans by healthy adults (4).*

Table 14. Plasma enterolignans measured at baseline and after 4-weeks of milled flaxseed intervention.

Enterolignans	18-29 Years of Age Group (n = 8)			45-69 Years of Age Group (n = 9)			Main Effects (*P*)		
	0 week	4 week	*P*-Value	0 week	4 week	*P*-Value	Age	Time	Age × Time
END									
Average, nM	< LOD	205.8 ± 110.9	0.108	< LOD	257.9 ± 98.1	0.024	0.72	0.006	0.72
Median, nM	< LOD	< LOD	-	< LOD	173.4	-	-	-	-
Range, nM	< LOD	< LOD – 781.5	-	< LOD	< LOD - 793.2	-	-	-	-
CV, %	-	152.4	-	-	107.6	-	-	-	-
ENL									
Average, nM	9.8 ± 4.4	214.9 ± 105.6	0.089	20.1 ± 8.5	351.9 ± 67.7	< 0.001	0.25	< 0.001	0.30
Median, nM	4.3	85.1	-	17.3	404.7	-	-	-	-
Range, nM	< LOD – 31.3	< LOD – 754.8	-	< LOD – 65.7	74.3 – 613.2	-	-	-	-
CV, %	125.8	139.0	-	119.5	54.4	-	-	-	-
END + ENL									
Average, nM	9.8 ± 4.4	420.7 ± 138.9	0.019	20.1 ± 8.5	609.8 ± 81.2	< 0.001	0.22	< 0.001	0.26
Median, nM	4.3	459.3	-	17.3	603.3	-	-	-	-
Range, nM	< LOD – 31.3	< LOD – 968.9	-	< LOD – 65.7	230.8 – 949.2	-	-	-	-
CV, %	125.8	93.4	-	119.5	37.7	-	-	-	-

Average data are expressed as mean ± SEM and were analyzed using a repeated measures ANOVA. CV: coefficient of variation, END: enterodiol, ENL: enterolactone, END + ENL: total enterolignans, LOD: limit of detection (< 6.6 nM for END; <6.7 nM for ENL). *Table reprinted with permission from Elsevier on June 26, 2015; © 2015 Edel, Pierce and Aliani. Age-dependency in the metabolism*

Figure 16. Plasma enterolignan concentrations compared between younger (n = 8) and older (n = 9) age groups at 0 weeks and after 4 weeks of consuming 30 g/d of milled flaxseed.

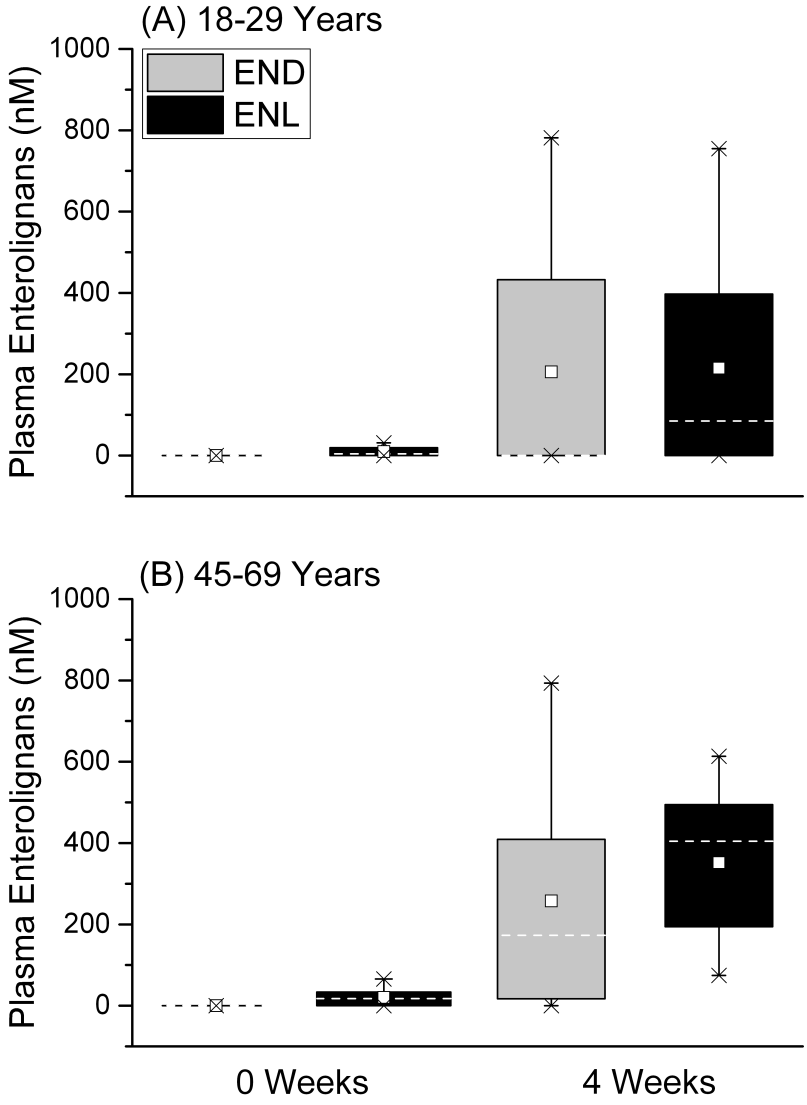


Plot (A) represents plasma END, (B) is ENL and (C) is END + ENL. Individual variation is embodied in the rectangle of this statistical box plot with dashed lines representing the median value, the square representing the mean and × representing 1% and 99% outliers. END: enterodiol, ENL: enterolactone, END + ENL: total enterolignans. *Image reprinted with permission from Elsevier on June 26, 2015; © 2015 Edel, Pierce and Aliani. Age-dependency in the metabolism of flaxseed lignans by healthy adults (4).*

Inter-subject variability of plasma enterolignan concentrations were noted as displayed by the extreme ranges measured within each group (**Table 14** and **Figure 16**). After 4 weeks of dietary flaxseed consumption in the younger group, END concentrations ranged from < LOD in some individuals to as high as 781.5 nM in one subject. The median concentration of END was below the LOD. Similarly, ENL varied from < LOD at the lowest extreme and 754.8 nM at the highest extreme. The median concentration in this group was 85.1 nM. A similar pattern was displayed in older individuals with END concentrations ranging from < LOD to 793.2 nM with a median of 173.4 nM and for ENL, 74.3 to 613.2 nM with a median of 404.7 nM. The coefficient of variation (CV) was over 100% for END in both age groups and for ENL in the younger group. However, ENL in the older group had a reduced CV of 54%. Total enterolignans were represented by a CV of 93.4% in the younger age group whereas only 37.7% for the older group. This attenuated between-subject variability for ENL and END + ENL in the older age group compared to the younger age group is visualized in the statistical box plot shown in **Figure 16**.

Of the individuals that responded to milled flaxseed, age may play a role in lignan metabolism as displayed by a trend in our data. In the younger group, 37.5% of the participants had measurable amounts of END in their plasma compared to 62.5% with ENL. In the older group, 77.8% of the participants had END and 100% had ENL. For both age groups, subjects that had measurable amounts of circulating END also had ENL, but not vice-versa. There were no significant differences in the number of individuals that produced END between the younger and older age groups; however, a trend was displayed for ENL ($P = 0.082$). The overall ratio of END:ENL for the younger subjects was 0.96 after 4 weeks of consuming dietary flaxseed and 0.74 for the older subjects (**Figure 17**).

Figure 17. Plasma END and ENL compared at baseline and following 4-weeks of consuming 30 g/d of milled flaxseed presented in younger (n = 8) and older (n = 9) age groups.



Plot (A) represents the younger age group (18-29 years of age) and plot (B) represents the older age group (45-69 years of age). Individual variation is embodied in the rectangle of this statistical box plot with dashed lines representing the median value, the square representing the mean and × representing 1% and 99% outliers. END: enterodiol, ENL: enterolactone. *Image reprinted with permission from Elsevier on June 26, 2015; © 2015 Edel, Pierce and Aliani. Age-dependency in the metabolism of flaxseed lignans by healthy adults (4).*

Plasma enterolignan concentrations as a function of gender

Combining all participants in the milled flaxseed group (n=17), plasma enterolignans were compared at the same time points between genders. There were 10 females and 7 males. No significant differences between END, ENL or END + ENL were found at either 0 weeks or 4 weeks between males or females (**Appendix 7**).

Enterolignan concentrations correlated with participant age

Plasma enterolignan concentrations after 4 weeks of dietary intervention were correlated with participant age. Neither END ($r_s = 0.13$, $P = 0.61$), ENL ($r_s = 0.40$, $P = 0.11$) nor total enterolignans ($r_s = 0.11$, $P = 0.67$) were positively correlated with participant age.

DISCUSSION

The findings of the present study demonstrate that all healthy, older adults, but not all healthy, younger adults, can metabolize milled flaxseed lignans to the enterolignan, ENL. Despite any statistically significant differences in plasma enterolignan concentrations between age groups after one month of milled flaxseed consumption, only the older adults, and not the younger adults, had significantly higher END and ENL concentrations compared to baseline values. However, both age groups had significantly greater circulating total enterolignan concentrations.

As previously mentioned, flaxseed is the richest source of SDG, and therefore a key precursor to the metabolites END and ENL. To ensure that participants were maintaining the study diet and not cheating by consuming high amounts of other lignan-containing foods that would contribute to similar concentrations of circulating END and ENL, we used flax oil as a control in a parallel arm of this study. Combining all participants at baseline allowed for the possible detection of lignan metabolites resulting from other lignan-containing foods. Since consuming flaxseed at least one month prior to study onset was an exclusion criterion of the study, only other food sources of lignans would be enterolignan contributors. Combined baseline data indicated that the typical consumption patterns of other lignan-containing foods (exclusive of flaxseed) in individuals 18-69 years of age, was minimal in Winnipeg, Canada. Although this is beneficial from the point of view of our study, this is a source of concern in light of the potential health benefits of these compounds on chronic disease prevention.

Plasma enterolignan concentrations resulting from consuming muffins containing either flax oil or milled flaxseed was then examined after 4 weeks in all participants. The lack of enterolignan metabolites in plasma of participants in the flax oil group confirmed that

no other types of lignan-containing foods that produce END and ENL as metabolites were being consumed alongside the study dietary intervention. Thus, our findings were not confounded by the ingestion of other lignan sources. Individuals in the present study that were consuming milled flaxseed for 4-weeks had an average 46-fold increase in circulating concentrations of total enterolignans, which is consistent with other studies at this dose (17, 29).

When participants were stratified by younger (18-29 years) and older (45-69 year) ages, average concentrations of END, ENL and END + ENL in each age group increased following 4-weeks of milled flaxseed consumption. However, only in the older age group were END and ENL significantly greater than baseline values, despite a trend in the younger group. This may be attributed to the reduced sample size in this group, to age-related differences in gut microflora (34) or to the lower liver volume of elderly people compared to younger people (51). This latter point may occur with drugs, like nutritional bioactives, which have increased presence in circulation of individuals ≥60 years of age compared to adults in their 20's, despite being administered equal doses of the same drug (52, 53). This highlights that age can be an important factor in nutritional dosing and must be considered when administering even flaxseed, as it has bioactive properties much like drugs.

As there were no main group effects for age, this suggests that 30 g/d of milled flaxseed can be absorbed and metabolized equally in both younger and older healthy adults. Consistent with this conclusion, there was no correlation of measured plasma enterolignans with age. This was surprising as increased age is often associated with changing gut microflora (25, 32-35) and reduced absorption and metabolism (51). The ability for both younger and older participants to process flaxseed lignans similarly is encouraging in light of

their suggested antioxidative and antitumorigenic benefits. As most chronic diseases occur more frequently in older populations, flaxseed as a source of lignans may represent a natural therapeutic strategy with potential advantages over more costly, prescription medications with associated negative side effects. Recently, this same flaxseed dose delivered to older (> 40 years old) patients with peripheral artery disease resulted in decreased blood pressure (17) and improved cholesterol lowering even in the presence of cholesterol-lowering medications without any increase in adverse events or alterations in platelet function in comparison to patients fed a whole wheat supplemented placebo diet (48). In these studies, blood pressure lowering was associated with elevations in plasma ALA concentrations and not with END or ENL amounts (17, 54) whereas cholesterol-lowering was related to the high fiber-content of flaxseed and not to any of the other bioactive ingredients (48).

High between-subject variability in measured plasma enterolignan concentrations is consistent with other reports at doses of 25-30 g/d in crushed (55), milled (56) or whole (57) flaxseed. Gut microflora is largely credited for the personalized differences in plasma END:ENL distributions (20). Reported plasma concentrations of ENL are typically 2-fold more prevalent than END in circulation following SDG (31) and flaxseed consumption (29, 58), with many reports noting the individualized differences in available enterolignans (55, 57). Increased age may reduce this variability as total enterolignans had a CV of 37.7% in the present study compared to the younger age group which was 93.4%. In general, the older group had the largest percent of "responders" or individuals that could metabolize lignans from ingested milled flaxseed than the younger population suggesting that older adults either contain the right distribution of colonic bacteria for this conversion or perhaps consume different foods than younger adults that would influence intestinal transit time. Average

plasma ENL concentrations were more prevalent than END in older subjects in the present study (**Figure 17**), albeit non-significantly, yet is in agreement with other reports for this age category (59). Serum ENL levels are positively associated with constipation (59), and older adults often suffer from this. However, increased dietary fiber tends to reduce constipation and both age groups received the same amount of daily dietary fiber from flaxseed, which is 28% fiber by weight (60). As final average END and ENL concentrations were similar in the younger age group and as no participants in the older group complained of constipation (44), something other than constipation was influencing lignan metabolism. Perhaps this can be attributed to an age-dependent gut microbial environment.

Several other possible contributing factors to enterolignan variability were considered. These included fasting time, the time of day the blood sample was collected, the elapsed time since the last flaxseed muffin dose was administered and enterolignan half-lives. Despite fasted blood samples being drawn approximately 12 h from the last consumed flaxseed dose, between-subject variability was still high in this study. The half-lives of these biologically active enterolignans have been reported as 4.4 h (31) or 9.4 h (19) for END and 13 h (19, 31) for ENL after a single oral dose of SDG. However, as both END and ENL achieve a steady state in circulation following as little as 3 (31) to 8 days (56) of daily flaxseed consumption, measurement time following the last flaxseed dose could not be a factor contributing to between-subject variability within the present study. All blood collections were conducted in the morning as time of day has been observed to alter enterolignan ratios (29).

In this particular study, we observed that after 4 weeks of dietary flaxseed, everyone with measured plasma END concentrations also had ENL in varying degrees, but not

everyone with measured ENL had END. As the metabolic conversion of SDG to enterolignans largely proceeds from SDG to SECO to END and finally to ENL (20), the individual variations in flaxseed-lignan metabolism are likely due to biochemical factors occurring along this pathway, which warrant further study. As a side note to this, since all participants had at least one enterolignan if not both, present in circulation, plasma total enterolignan (END + ENL) concentrations may be a suitable biomarker of flaxseed consumption and should be considered for monitoring compliancy in nutritional studies involving milled flaxseed.

A limitation of this study was the small sample size. Original power calculations for this study were based on expectations for changes in ALA concentrations (44). Recalculating the power for total enterolignans using a repeated measures ANOVA for within and between group interactions, and using the original sample size design of 20, the calculated power was 56%. Future studies measuring enterolignans as the primary outcome should use a total sample size of 42, estimating 20% dropout, to obtain 80% power.

CONCLUSIONS

Healthy younger (18-29 years) and older (45-69 years) adults can produce similar concentrations of enterolignans in circulation following daily milled flaxseed consumption, with variations only in the ratio of END:ENL. This variation in enterolignan distribution suggests an age-dependent response, perhaps related to one's gut microflora. This may particularly be important for aging populations that are prone to chronic diseases where enterolignans may be able to offer therapeutic benefit through their antioxidative and/or estrogenic interactions. Studies administering whole, milled flaxseed, and not just isolated

extracts, must acknowledge the individual contributions of ALA, enterolignans and fiber as well their potential synergism when reviewing health outcomes.

Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER VIII: Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol lowering medications alone in patients with peripheral artery disease[1-3]

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[3] Supplemental Tables 1, 2 and 3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

[9] Abbreviations used: ABI, ankle/brachial index; ALA, alpha-linolenic acid; C, cholesterol; CHO, carbohydrate; CLM, cholesterol lowering medication; CVD, cardiovascular disease; FX, flaxseed; G, group; G × T, group × time; PAD, peripheral artery disease; PL, placebo; PPP, platelet poor plasma; PRP, platelet rich plasma; RT, room temperature: T, time.

ABSTRACT

Background: Dietary flaxseed lowers cholesterol in healthy subjects with mild biomarkers of cardiovascular disease (CVD).

Objective: The aim was to investigate the effects of dietary flaxseed on plasma cholesterol in a patient population with clinically significant CVD and in those administered cholesterol-lowering medications (CLMs).

Methods: This double-blinded, randomized, placebo-controlled trial examined the effects of a diet supplemented for 12 mo with foods that contained either 30 g of milled flaxseed [milled flaxseed treatment (FX) group; n = 58] or 30 g of whole wheat [placebo (PL) group; n = 52) in a patient population with peripheral artery disease (PAD). Plasma lipids were measured at 0, 1, 6 and 12 mo.

Results: Dietary flaxseed in PAD patients resulted in a 15% reduction in circulating LDL cholesterol as early as 1 mo into the trial ($P = 0.05$). The concentration in the FX group (2.1 ± 0.10 mmol/L) tended to be less than in the PL group (2.5 ± 0.2 mmol/L) at 6 mo ($P = 0.12$), but not at 12 mo ($P = 0.33$). Total cholesterol also tended to be lower in the FX group than in the PL group at 1 mo (11%, $P = 0.05$) and 6 mo (11%, $P = 0.07$), but not at 12 mo ($P = 0.24$). In a subgroup of patients taking flaxseed and CLM (n = 36), LDL cholesterol concentrations were lowered by 8.5% ± 3.0% compared with baseline after 12 mo. This differed from the PL + CLM subgroup (n = 26), which increased by 3.0% ± 4.4% ($P = 0.030$) to a final concentration of 2.2 ± 0.1 mmol/L.

Conclusions: Milled flaxseed lowers total and LDL cholesterol in patients with PAD, and has additional LDL cholesterol-lowering capabilities when used in conjunction with CLMs. This trial was registered at clinicaltrials.gov as NCT00781950.

Keywords: flaxseed, cholesterol-lowering, peripheral artery disease

INTRODUCTION

Dietary flaxseed has provided beneficial cardiovascular effects in a number of animal studies. These include improving vascular reactivity (1), inhibiting the progression of atherosclerosis (2), promoting the regression of existing atherosclerotic plaques (3), inhibiting the incidence of arrhythmias during ischemia/reperfusion challenge (4) and lowering circulating concentrations of cholesterol (5). Several clinical trials have used flaxseed as a dietary supplement to investigate its efficacy on bone density (6), menopausal symptoms (6), blood glucose (7), lipid profile (8) and blood pressure (9, 10). The beneficial actions of dietary flaxseed have been attributed to its rich content of fiber, lignans and the omega-3 fatty acid, alpha linolenic acid (ALA) (1-5).

Although much is known of the effects of dietary flaxseed in both animal models and human clinical trials, several important parameters remain to be studied. For example, the effects of dietary flaxseed on circulating concentrations of total-cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides in patients with documented cardiovascular disease (CVD) may be quite different than the response exhibited by subjects without clinical symptoms of CVD or in those only presenting with risk factors (8, 11, 12). Furthermore, the effects of flaxseed in patients already administered cholesterol-lowering medication (CLM) are unknown. In view of a number of studies that have shown significant drug-food interactions (13, 14), this is an important parameter to consider. Finally, it is unclear if flaxseed may also inhibit platelet aggregation in a clinical population, which would be beneficial when blood clots may represent a serious health concern. The marine omega-3 fatty acids EPA and DHA can inhibit platelet aggregation (15). Dietary flaxseed with its rich content of ALA may have a

similar effect in cardiovascular patients at risk for clinical events. Therefore, understanding the effects of dietary flaxseed on a variety of clinical parameters in the circulation of a human population, particularly in those with pre-existing cardiovascular disease, becomes an important clinical topic to investigate.

Patients with peripheral artery disease (PAD) exhibit many characteristics of cardiovascular disease and because of this they are at high risk for myocardial infarctions and stroke (16, 17). They are frequently diabetic, hyperlipidemic, hypertensive, have atherosclerotic coronary artery disease and altered blood coagulation properties (9, 16-18). As a result, they are commonly on a combination of lipid-lowering, anti-thrombotic, anti-hypertensive and blood glucose lowering therapies (9, 17). In view of the effects of flaxseed on cardiovascular disease in animal models, it is possible that flaxseed may be beneficial in a patient population with PAD. The FLAX-PAD Trial was initiated to study the effects of consuming dietary flaxseed in a patient population with PAD (9). The significant anti-hypertensive action of flaxseed in this patient population has been recently reported (10, 19).

The aim of this part of the FLAX-PAD Trial, therefore, was two-fold: First, to determine if consumption of dietary flaxseed, in PAD patients and in a subgroup being administered cholesterol-lowering medications, would alter the cholesterol and triglyceride profile, and second, to determine if platelet aggregation would be altered. It was hypothesized that dietary flaxseed would lower circulating cholesterol concentrations in PAD patients. These effects on the PAD patients would be complicated by the on-going administration of cholesterol-lowering drugs (i.e. statins) and anti-thrombotic medication (i.e. clopidogrel). It is also possible that the dosage of medications required to achieve the desired effects on circulating cholesterol and platelet aggregation would be changed due to

consumption of flaxseed. For this reason, a double blinded, placebo-controlled, randomized trial (9) was initiated in a patient population with documented PAD to study the effects of milled flaxseed on a variety of blood characteristics over a 1-year ingestion period.

METHODS

The FLAX-PAD trial design

This was a single site, double-blinded, placebo-controlled study carried out in Winnipeg, Canada. Twelve months has been recommended as a duration ideal to study dietary effects (9, 20). The study was conducted after approval from the Natural Health Products Directorate of Health Canada, the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Each patient who participated provided written consent. A Safety Monitoring Committee was created and insured patient safety throughout the course of the study. Details concerning the FLAX-PAD experimental design are found elsewhere (9). Briefly, 347 patients with documented PAD were initially screened for entry into the study. Of these, 237 were excluded because they did not meet the study inclusion criteria, or there was an unwillingness to participate, or for other reasons (**Figure 18**) (10). The 110 patients that remained were randomized by a computer program into the flaxseed (FX) (n=58) and placebo (PL) (n=52) groups. Of the patients that were administered CLM at study onset, 40 patients (88.9%) in the FX+CLM subgroup (n=45) were on statins [Simvastatin (10-80 mg/d), Rosuvastatin (10-40 mg/d), Atorvastatin (10-80 mg/d) and Pravastatin (20 mg/d)], of which 4 of these were on a combination therapy merging a statin with either Ezetimibe or a fibrate. The remaining 5 patients (11.1%) were on other CLMs [Ezetimibe (10 mg/d), Bezofibrate (400 mg/d 3 × a week) and Fenofibrate (145 & 160 mg/d)]. In the PL+CLM subgroup (n=36), 35 patients (97.2%) were taking statins [Simvastatin (10-20 mg/d), Rosuvastatin (10-40 mg/d) and Atorvastatin (5-80 mg/d)] of which four patients were taking a statin in combination with either a fibrate (Gemfibrozil or Lipidil), Ezetimibe or with Fenofibrate and Ezetimibe. Only 1 patient (2.8%) was taking

Ezetimibe 10 mg/d. The number of patients in the FX Only and PL Only subgroups was 11 and 15, respectively. All patients were monitored by the attending physician and CLMs were adjusted as necessary in order to keep blood concentrations of lipids in normal ranges. All patients had an ankle/brachial index (ABI) of <0.9, which was the clinical criteria for the presence of PAD in this study.

The investigational products provided to the patients included buns, snack bars, muffins, bagels, pasta, and tea biscuits that each contained 30g of milled flaxseed or a placebo that contained milled wheat. Small bags of the milled product were also available. This allowed the subjects to mix the product into other foods like yoghurt, cereals or drinks to give them variety in their diet over the course of 12-months. In this case, the wheat was mixed with a very small amount of wheat germ, coconut oil and wheat bran to insure the colour and texture of the food product resembled the same food that contained flaxseed. The muffins, bagels and snack bars were also formulated with different flavorings to give the patients sufficient variety to insure compliancy over the time of the study. The placebo product contained the same flavorings but did not contain flaxseed. Food composition and sensory results are described for the muffins, bagels and snack bars in detail elsewhere (21, 22). The energy and nutrient composition of the available food products are presented in **Appendix 8**. One food item was ingested per day over the course of the study with subjects gradually introduced to the food products beginning with 10g in week 1, 20g in week 2 and then continuing with 30g for the duration of the study. Each test subject received a 1-month supply of food products. They decided what type of foods and which flavors they desired. The individualized monthly ration of food products was then delivered to each subject and was stored by the patient in their own freezer to insure freshness over the month. Although

the food products ingested varied from person to person, the flaxseed content of the diet was distinct in comparison to the placebo group. The individualized choice of flavors and food items was critical to maintain compliance throughout the 12-months. All personnel that collected or analyzed data were blinded as to the intervention. Only after all data were calculated was the un-blinding completed.

Body measurements and blood collection

Body weight, waist circumference and BMI were recorded at 0-, 6- and 12-month time points. Patients were requested to fast for 12 hours prior to the morning of their blood draw (35 mL). Ten mL were collected in citrate-containing vacutainer tubes for immediate platelet analysis and 5 mL into EDTA-containing tubes for fatty acid methyl ester and enterolignan analyses. Plasma was obtained by centrifugation as described previously (1, 2). Plasma was separated into small aliquots and stored immediately at -80^0C to be measured at a later date.

Plasma analyses

Plasma TC, LDL-C, HDL-C and triglycerides were measured by validated techniques in the St. Boniface Hospital Biochemistry Laboratory. Plasma fatty acids were measured by GC, as described in detail (1, 2). Plasma enterolignans were quantified by GC/MS as previously described (10, 23). Platelet aggregation studies were measured using freshly collected blood on a Chrono-Log 490-2D Platelet aggregometer (Chrono-Log Corp., Havertown, PA, USA) as previously reported (24). Collagen (5 μg/mL) (Number 385) and thrombin (0.3 units/mL) (Number 386, Chrono-Log Corp., Havertown, PA, USA) were the agonists used to perform platelet aggregation experiments.

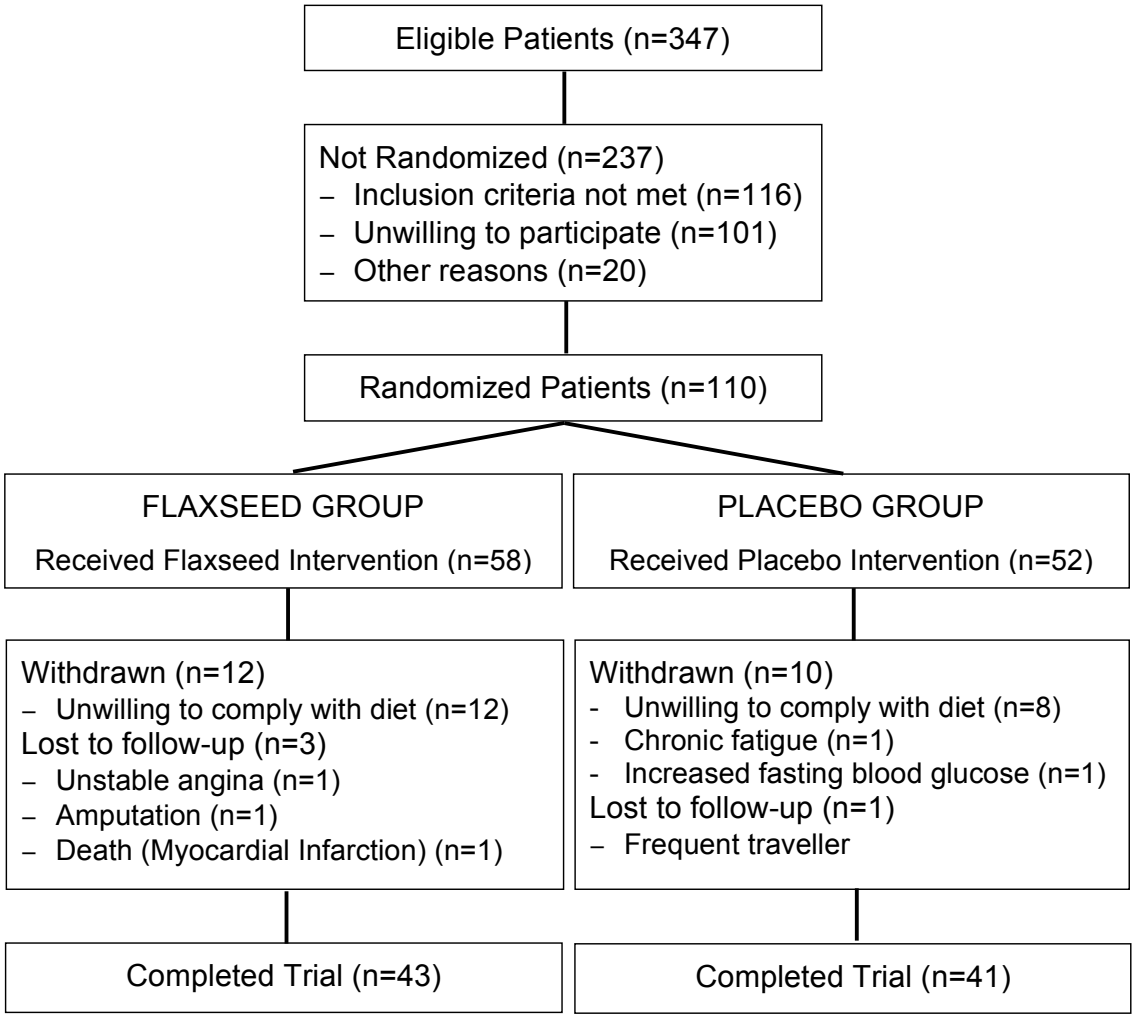
Statistical analyses

Continuous variables were expressed as mean ± standard deviation unless otherwise stated. Categorical variables were expressed as proportions. For comparing the two groups the following analyses were performed: A frequency test was used to determine if data was normally distributed. Categorical variables were compared with χ^2 test. Continuous variables were compared using an independent samples t-test. A repeated measures ANOVA was used to compare balanced data sets. The Greenhouse-Geisser model was used to measure main effects for time and group and group×time interactions. Pairwise comparisons over time were adjusted for multiple comparisons using least significant differences. Between-group comparisons in the present manuscript will be discussed as 'differences' where as time effects will be referred to as 'change.' Spearman correlations were used to evaluate main associations between flaxseed components and blood lipids. Differences were considered significant when *P*-values were <0.05. Trends were noted if $P \leq 0.1$. These analyses were carried out using SPSS 22.0 (International Business Machines, Armond, NY, USA).

For unbalanced data analysis, an advanced mixed 2-way repeated measures procedure was used to determine differences between groups and among time points. Group and time were between and within factors, respectively. Three models including compound symmetry, Huynh-Feldt, and unstructured were run. The best model was chosen based on the information criteria of Akaike (AIC) and Schwarz (BIC). If the mixed procedure provided a significant difference ($P<0.05$) for group, time, or group×time interaction, the least squared means were calculated and compared. To control for Type 1 errors for multiple comparisons, a Bonferroni correction was applied to the alpha level so that $P=0.008$ was the critical value of statistical significance for continuous variables and $P=0.017$ for values represented as

absolute or percent change. All statistical tests were determined using SAS statistical software version 9.2 (Statistical Analysis System, SAS Institute Inc., Cary, NC).

Figure 18. Participant eligibility, screening, randomization and follow-up of PAD patients for the 12-month FLAX-PAD Trial.



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RESULTS

Body morphometrics

Body weight, waist circumference and BMI measurements were measured at baseline, six and twelve months. There were no within-group changes over time for either of the study groups nor were there any between-group differences at any time point for any of the three measured parameters ($P \geq 0.05$) (**Appendix 9**).

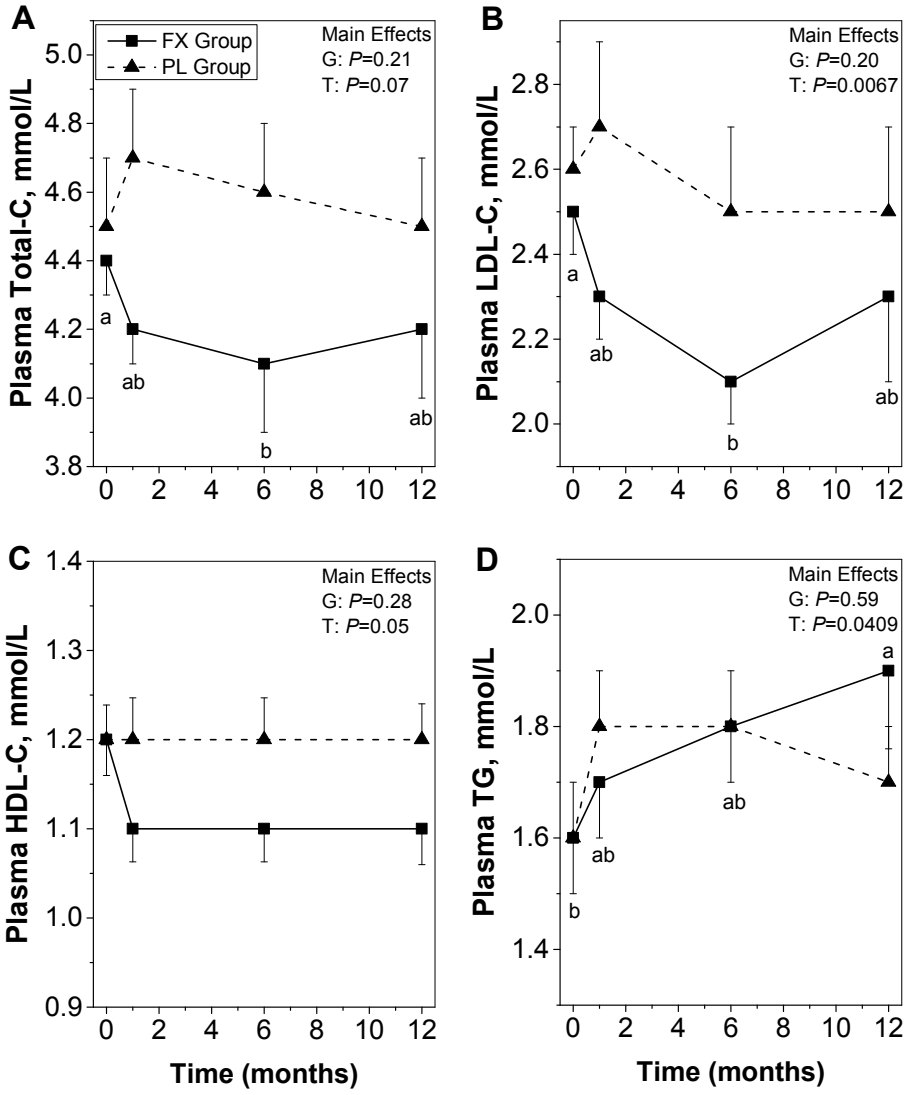
Lipid measurements

Baseline concentrations of all lipids were similar between PL and FX intervention groups. When all patients, including those with missing time points, were integrated into the analysis, flaxseed ingestion resulted in an 11-15% reduction of circulating concentrations of total- and LDL-C as early as 1-month into the trial ($P=0.05$) which was maintained for 6-months compared to placebo (**Figure 19A & B**). At 12-months, these differences were no longer observed. Plasma TC and LDL-C were significantly attenuated by flaxseed ingestion at 6-months compared to baseline values ($P=0.005$ and $P \leq 0.001$, respectively). Conversely, there were no changes in the PL group over time. Circulating HDL-C concentrations were unchanged within- or between-groups at any time point during the trial (**Figure 19C**). Circulating triglycerides were also not statistically different between groups throughout the study; however, the FX group was significantly increased at 12-months compared to its mean baseline value ($P=0.002$) (**Figure 19D**). When plasma lipids were compared using only patients that completed the 1-year trial (**Table 15**), LDL-C in patients consuming flaxseed decreased at 6-months compared to baseline values ($P=0.007$), as did HDL-C ($P=0.042$). A similar trend was observed for TC at these two time points (P=0.07). No other differences

between-groups or changes over time in plasma lipids including triglycerides, TC/HDL-C and LDL-C/HDL-C were observed.

A risk factor for developing PAD is increased circulating plasma cholesterol (25). Eighty % of the patients enrolled in the trial were hyperlipidemic (9). Therefore, it was not surprising that a majority of the patients (74%) were administered CLM prior to starting the trial. As expected, patients administered CLM (+CLM) exhibited lower basal concentrations of TC, LDL-C, TC/HDL-C and LDL-C/HDL-C than those not taking these medications ($P<0.01$) (**Table 16**). Baseline concentrations of all plasma lipids were similar when comparing FX+CLM and PL+CLM subgroups ($P\geq0.05$). Triglycerides were significantly lower in the FX Only subgroup than the PL Only subgroup ($P=0.030$), with all other lipids remaining similar between the two groups.

Figure 19. Mean plasma lipid concentrations in patients with PAD at baseline, 1-, 6- and 12-month time points for all patients in the flaxseed and placebo groups.



Values are represented as means (SEM). Plasma lipids include Total-C (*A*), LDL-C (*B*), HDL-C (*C*) and TG (*D*). [a,b] Mean values within the same group with a different letter are significantly different from each other, $P \leq 0.008$. Flaxseed: baseline n=58; 1 month n=52; 6 months n=45; 12 months n=43 and Placebo: baseline n=52; 1 month n=47; 6 months n=41; 12 months n=41. FX, flaxseed; G, group; PL, placebo; T, time; Total-C, total-cholesterol. *Image reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6)*.

Table 15 Plasma lipid concentrations measured at baseline, 1, 6 and 12 month time points for patients with PAD in both flaxseed and placebo treatment groups.[1]

Lipids	Baseline	1 Month	6 Months	12 Months	Effects (P-Value)		
					G	T	G×T
TG, *mmol/L*					0.57	0.10	0.07
FX (n=43)	1.6 ± 0.7	1.8 ± 0.8	1.8 ± 0.8	1.9 ± 0.9			
PL (n=41)	1.7 ± 0.9	1.7 ± 0.7	1.8 ± 0.9	1.7 ± 0.7			
TC, *mmol/L*					0.12	0.033	0.53
FX (n=43)	4.4 ± 1.2	4.2 ± 1.0	4.1 ± 1.1	4.2 ± 1.3			
PL (n=41)	4.7 ± 1.3	4.7 ± 1.4	4.6 ± 1.4	4.5 ± 1.3			
LDL-C, *mmol/L*					0.35	0.0022	0.19
FX (n=43)	2.5 ± 1.0[a]	2.3 ± 1.0[ab]	2.2 ± 1.0[b]	2.3 ± 1.1[ab]			
PL (n=41)	2.6 ± 1.0	2.6 ± 1.0	2.5 ± 1.0	2.4 ± 0.9			
HDL-C, *mmol/L*					0.22	0.051	0.13
FX (n=43)	1.20 ± 0.33[a]	1.12 ± 0.27[ab]	1.11 ± 0.25[b]	1.12 ± 0.25[ab]			
PL (n=41)	1.21 ± 0.29	1.22 ± 0.33	1.19 ± 0.30	1.22 ± 0.25			
TC/HDL-C, *ratio*					0.77	0.58	0.62
FX (n=43)	3.8 ± 1.2	3.9 ± 1.1	3.8 ± 1.3	3.8 ± 1.3			
PL (n=41)	4.0 ± 1.3	4.0 ± 1.3	3.9 ± 1.1	3.8 ± 1.1			
LDL-C/HDL-C, *ratio*					0.97	0.30	0.57
FX (n=43)	2.2 ± 1.0	2.1 ± 1.0	2.1 ± 1.1	2.1 ± 1.1			
PL (n=41)	2.2 ± 0.9	2.2 ± 0.9	2.1 ± 0.8	2.0 ± 0.8			

[1] Values are means ± SD. [a, b] Mean values within a row with a different letter are significantly changed over time, $P<0.05$. Differences between-groups were not statistically significant, $P\geq0.05$. C, cholesterol; FX, flaxseed group; G, group; G×T, group × time; PL, placebo group; T, time. *Table reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6)*.

Table 16. Subgroup analysis of plasma lipid concentrations at baseline represented by flaxseed or placebo group in PAD patients that were or were not taking cholesterol-lowering medications. [1]

Parameter	FX + CLM	PL + CLM	*P*-Value	FX Only	PL Only	*P*-Value
Sample size, *n*	37	26		7	15	
TG, *mmol/L*	1.7 ± 0.7	1.5 ± 0.7	0.42	1.2 ± 0.4	2.0 ± 1.1	0.030
TC, *mmol/L*	4.3 ± 1.1	4.1 ± 0.8†	0.54	5.1 ± 0.9	5.7 ± 1.5	0.33
LDL-C, *mmol/L*	2.3 ± 1.0†	2.1 ± 0.5‡	0.52	3.4 ± 0.8	3.6 ± 1.1	0.69
HDL-C, *mmol/L*	1.2 ± 0.3	1.3 ± 0.3	0.75	1.1 ± 0.2	1.1 ± 0.2	0.49
TC/HDL-C, *ratio*	3.6 ± 1.1†	3.4 ± 0.7‡	0.30	4.9 ± 1.0	5.1 ± 1.4	0.75
LDL-C/HDL-C, *ratio*	1.9 ± 1.0†	1.8 ± 0.5‡	0.31	3.3 ± 0.7	3.2 ± 1.0	0.86

[1] Values are means ± SD. Different from FX Only or PL Only subgroups within the same dietary group, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. C, cholesterol; CLM, cholesterol-lowering medication; FX, flaxseed; PL, placebo. *Table reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6).*

It was important to determine if the circulating concentrations of cholesterol that were controlled by the administration of CLM exhibited any additional cholesterol-lowering effects provided by the flaxseed (or, conversely, if the flaxseed would inhibit the cholesterol-lowering effects of the CLM). The data over time as percent change was examined in individuals that completed the 1-year trial to determine if TC and LDL-C concentrations could be further reduced by dietary flaxseed in patients using CLMs. A trend noting a group difference was observed for TC (P=0.06) (**Table 17**), but the most noticeable attenuation in blood lipids was observed with LDL-C in the FX+CLM subgroup (**Figure 20A**). After 12 months of consuming 30g/d of dietary flaxseed, LDL-C was attenuated by 8.5% in the FX+CLM subgroup compared to a 3.0% increase in the PL+CLM subgroup (P=0.030). This group difference for LDL-C was also evident by the significant main effect for group

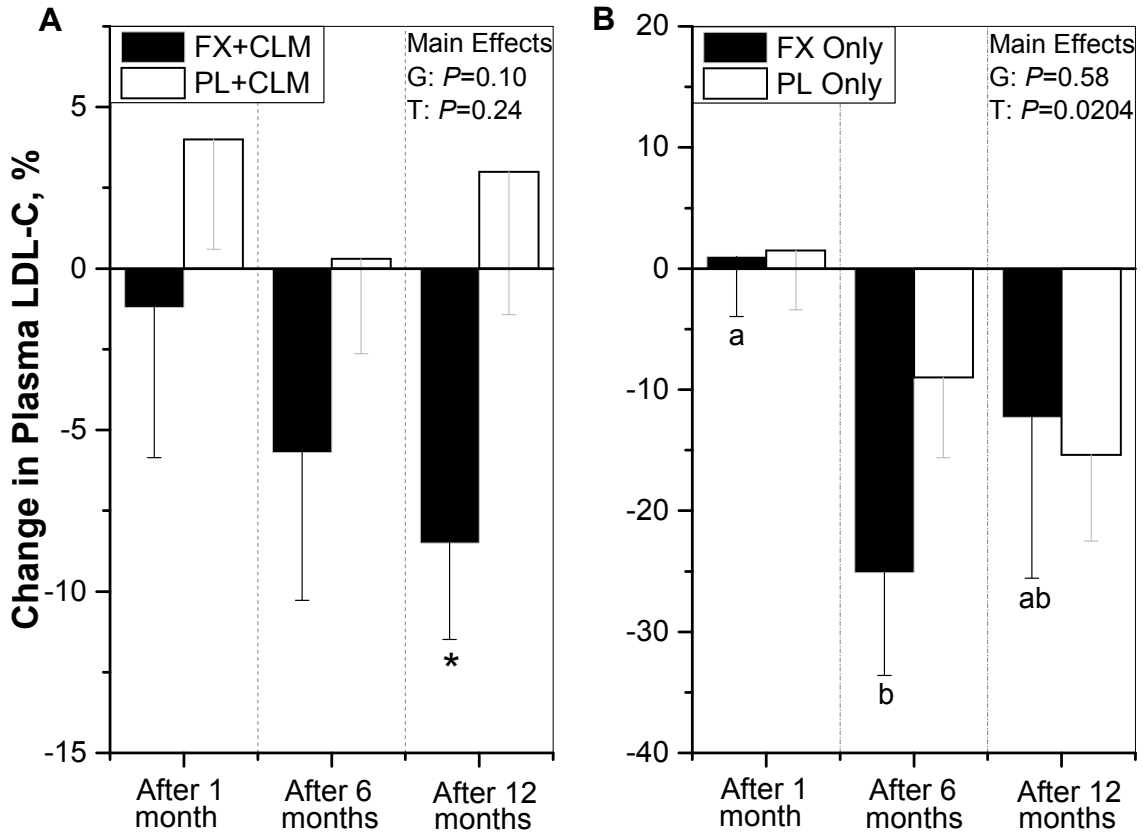
(*P*=0.020) when compared as absolute change (**Appendix 10**). To determine the effects of flaxseed alone in this patient population, we examined LDL-C in a subgroup of individuals not taking administered CLMs (**Figure 20B**). LDL-C was significantly attenuated after 6 months compared to initial values (*P*=0.009, **Appendix 10** and *P*=0.014, **Figure 20B**). No significant between-group differences were present at any time point (**Appendix 10**) (*P*=0.38). No other differences in plasma lipids including triglycerides, TC, HDL-C, TC/HDL-C ratio and LDL-C/HDL-C ratio were observed between any of the treatment groups after 1-, 6- or 12-months (**Table 17**). Despite a significant main effect for time in TC (*P*=0.020) (**Table 17**) for the FX and PL Only subgroups, the limited sample size prohibited detection of any changes over time in either of the treatment groups. Patient subgroups (FX+CLM and FX Only; PL+CLM and PL Only) were not compared due to the large differences in sample size.

Table 17. Percent change from baseline in plasma lipid concentrations in PAD patients by dietary flaxseed and placebo as a function of administered cholesterol-lowering medications or the standalone diet.[1]

Lipids	Percent Change over Time			Effects (*P*-Value)[2]		
	After 1 month	After 6 months	After 12 months	G	T	G×T
TG, *%*				0.43	0.63	0.45
FX + CLM	11.6 ± 5.4	16.4 ± 6.5	22.1 ± 6.3			
PL + CLM	11.0 ± 5.6	10.7 ± 6.2	7.9 ± 6.3			
				0.44	0.84	0.51
FX Only	11.3 ± 8.8	20.9 ± 14.7	10.4 ± 7.6			
PL Only	8.6 ± 6.6	-1.2 ± 9.4	4.3 ± 9.5			
TC, *%*				0.06	0.59	0.88
FX + CLM	-2.3 ± 1.6	-3.4 ± 2.2	-3.0 ± 2.5			
PL + CLM	2.8 ± 2.3	-0.3 ± 2.3	1.8 ± 2.8			
				0.42	0.02	0.44
FX Only	-0.7 ± 3.4	-17.0 ± 5.5	-9.1 ± 8.2			
PL Only	1.9 ± 3.9	-6.6 ± 5.1	-9.5 ± 4.6			
HDL-C, *%*				0.36	0.45	0.66
FX + CLM	-5.1 ± 1.8	-4.1 ± 2.9	-3.0 ± 3.4			
PL + CLM	-1.1 ± 2.2	-2.6 ± 2.3	1.4 ± 2.8			
				0.13	0.93	0.74
FX Only	-5.8 ± 3.2	-7.4 ± 8.4	-11.4 ± 3.8			
PL Only	1.7 ± 3.5	-0.4 ± 3.9	2.4 ± 3.5			
TC/HDL-C, *%*				0.68	0.79	1.00
FX + CLM	4.0 ± 2.2	3.3 ± 3.9	2.1 ± 3.7			
PL + CLM	5.5 ± 3.6	2.7 ± 2.0	1.8 ± 2.9			
				0.42	0.32	0.36
FX Only	6.2 ± 3.8	-4.3 ± 12.6	4.3 ± 11.5			
PL Only	1.3 ± 4.3	-5.4 ± 5.4	-10.4 ± 4.9			
LDL-C/HDL-C, *%*				0.52	0.52	0.85
FX + CLM	4.6 ± 5.0	2.5 ± 6.8	-2.4 ± 4.9			
PL + CLM	6.6 ± 5.1	2.4 ± 2.9	1.9 ± 4.1			
				0.53	0.09	0.39
FX Only	7.2 ± 5.2	-13.3 ± 14.9	0.3 ± 16.3			
PL Only	0.5 ± 5.3	-8.7 ± 6.8	-17.0 ± 6.5			

[1] All values are means ± SEM. The absence of letters within a row indicates no significant changes over time ($P>0.017$). [2] Significant if $P<0.05$. Flaxseed Groups: FX+CLM after 1 month n=41; after 6 months n=37; after 12 months n=36 and FX Only after 1 month n=11; after 6 months n=7; after 12 months n=6. Placebo Groups: PL+CLM after 1 month n=32; after 6 months n=26; after 12 months n=26 and PL Only after 1 month n=15; after 6 months n=13; after 12 months n=15. C, cholesterol; CLM, cholesterol-lowering medication; FX, flaxseed; G, group; G×T, group × time, PL, placebo, T, time. *Table reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6).*

Figure 20. Mean percent change from baseline in plasma LDL-C in patients with PAD in flaxseed or placebo subgroups consuming the diet plus cholesterol-lowering medications or just the diet after 1-, 6- and 12-months.



Values are represented as means (SEM). This subgroup of patients is consuming either a flaxseed or placebo dietary intervention in conjunction with administered CLMs (FX+CLM or PL+CLM) (*A*). This subgroup of patients is only consuming the study diet (FX Only or PL Only) (*B*). Different from placebo, $^{*}P<0.05$. ^{a,b} Mean values within the same group with a different letter are significantly different from each other, $P\leq0.017$. FX+CLM: after 1 month n=41; after 6 months n=37; after 12 months n=36; and PL+CLM: after 1 month n=32; after 6 months n=26; after 12 months n=26. FX Only: after 1 month n=11; after 6 months n=7; after 12 months n=6; and PL Only: after 1 month n=15; after 6 months n=13 (2 patients could not make their 6 month appointment); after 12 months n=15. CLM, cholesterol-lowering medication; FX, flaxseed group; G, Group; PL, placebo group; T, time. *Image reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6).*

The next step was to assess if basal concentrations of circulating cholesterol influenced the subsequent effects of flaxseed and CLM. Patients were organized by dietary treatment into subgroups where baseline concentrations of TC were borderline to high (≥5.3 mmol/L). Twelve patients in the FX group fit these criteria. Two of these patients discontinued the study prior to the first month so only ten patients were included in the analysis. Of these individuals, 6 were already taking CLM at the onset. In the PL group, 11 patients were hypercholesterolemic at the onset with only two patients taking CLM. Two patients were unavailable for their 6-month follow-up, yet were present for their 12-month appointment. Despite a main effect over time for LDL-C (P=0.010), no significant changes over time were detected in either group when post-hoc tests were applied nor were any between-group differences (P=0.67) (**Appendix 11**). A similar trend was observed for TC with P=0.05 as a main effect over time and P=0.10 for the group effect. These trends can be attributed to the drop in TC of 0.9 mmol/L in the flaxseed group compared with only 0.2 mmol/L in the placebo group after 6 months of intervention.

It is possible that the cholesterol-lowering action of dietary flaxseed may have induced a reduction in the prescribed dosage of CLMs used over the course of the trial. Recall that patients were monitored individually by the attending physician. As the health of our patients was our priority CLM doses could be adjusted at any time as warranted by the physician. These results are therefore suggested based on the placebo-controlled nature of our trial. No changes in administered medications were reported in the FX+CLM subgroup until the 1-year visit. At this visit, 1 person (2.7%) had stopped taking statin medication (Rosuvastatin 40 mg/d), 33 patients (89.2%) had their CLM dose unchanged and 3 patients (8.1%) increased their statin dose (Simvastatin 40 mg/d to Atorvastatin 80 mg/d and 2

patients increased their Atorvastatin 20 mg/d dose up to 40 and 80 mg/d). Each of these changes occurred at or following 7-months of flaxseed intervention. In the PL+CLM subgroup, 4 patients (15.4%) had their CLM dose increased, one at 1-month (Atorvastatin 40 to 60 mg/d), two at 6-months (Atorvastatin 5 to 10 mg/d and Ezetemibe 10 mg/d to Cholestryamine 2 packs/d) and one at 12-months (Rosuvastatin 10 to 20 mg/d). One patient stopped their statin use at 6-months, only to be restarted on the same dose 2-months later. Only one patient had their statin dose reduced at 12-months (Atorvastatin 80 to 40 mg/d). In the PL Only group (i.e., none of the patients were taking CLM at the start of the trial), 33.3% were being administered CLM by the end of the study-monitoring period. This is in contrast to the FX Only group in which only 1 patient (14.3%) began a low dose statin therapy at 6-months (Rosuvastatin 10 mg/d), which was quickly discontinued shortly thereafter.

Consumption of milled flaxseed induced a 1-fold increase in circulating ALA concentrations and a 10-50 fold increase in plasma total enterolignans or the enterolignans species enterodiol and enterolactone (10). Absolute changes in these components after 1-, 6- and 12-months are listed in **Appendix 12**. The association of plasma ALA or enterolignans with changes in total plasma cholesterol was examined in patients who ingested flaxseed. No inverse correlations were observed between plasma ALA, enterodiol or enterolactone with either total- and LDL-C or triglycerides as a result of consuming flaxseed ($P \geq 0.05$) (**Appendix 13**).

Platelet aggregation

Platelet aggregation was monitored using both collagen and thrombin as agonists. Using all patients enrolled in the trial, a significant main effect for time ($P<0.01$) was observed with each agonist for both % aggregation and rate of aggregation (**Table 18**).

When thrombin was used as the agonist, % aggregation was attenuated in the FX group at 6-months compared to baseline (P=0.005) and rate of aggregation changed comparably for both FX and PL groups. Collagen did not influence any changes in the FX group in either % or rate of aggregation. There were no differences between the FX and PL groups at any of the measured time points ($P \geq 0.05$) despite these changes over time (**Table 18**).

Anticoagulant medications were monitored over time between treatment groups to see if dietary flaxseed affected any differences in administration. Of the 110 patients enrolled in the trial, 54 of the 58 patients in the FX group (93%) were being administered anticoagulant medications prior to commencing the study. Only 3 patients in the FX group had their medications adjusted over the study. In the PL group, forty-seven patients (90%) were taking anti-platelet medications at the study onset. Only 4 patients in the PL group had their platelet medications adjusted. No differences in anti-platelet medication resulted from dietary supplementation with flaxseed compared to placebo.

Table 18. Platelet aggregation in platelet-rich plasma of PAD patients at 0, 1, 6 and 12 month time points for flaxseed and placebo groups using all study participants.[1]

Platelet Measurements	Baseline	1 Month	6 Months	12 Months	Effects (P-Value)[2]		
					G	T	G×T
Percent Aggregation, *%*					0.21	0.0019	0.60
Collagen (5 µg/mL) - FX	75.7 ±18.1	72.5 ± 21.7	81.3 ± 15.4	77.8 ± 19.5			
Collagen (5 µg/mL) - PL	72.3 ± 18.6^{ab}	67.3 ± 25.1^{b}	78.5 ± 19.5^{a}	79.3 ± 19.9^{a}			
					0.54	0.0042	0.52
Thrombin (0.3 units/mL) - FX	99.1 ± 8.8^{a}	95.4 ± 11.0^{ab}	93.6 ± 11.7^{b}	92.1 ± 13.1^{ab}			
Thrombin (0.3 units/mL) - PL	96.4 ± 6.8	94.6 ± 13.8	95.0 ± 12.1	90.5 ± 20.3			
Maximum rate of aggregation, *% change/min*					0.80	0.0064	0.30
Collagen (5 µg/mL) - FX	103 ± 34.6	97.9 ± 44.9	111 ± 34.1	106 ± 40.3			
Collagen (5 µg/mL) - PL	103 ± 39.2^{ab}	90.7 ± 45.3^{b}	111 ± 38.6^{a}	116 ± 40.1^{a}			
					0.32	<0.0001	0.59
Thrombin (0.3 units/mL) - FX	293 ± 58.0^{a}	299 ± 58.0^{a}	261 ± 55.6^{b}	251 ± 54.1^{b}			
Thrombin (0.3 units/mL) - PL	280 ± 42.1^{a}	280 ± 64.5^{a}	261 ± 65.2^{ab}	247 ± 61.9^{b}			

[1] All values are means ± SD. [a, b] Mean values within a row with a different letter are significantly different from each other, $P \leq 0.008$.
[2] Significant if $P < 0.05$. Flaxseed: baseline n=58; 1 month n=51; 6 months n=45; 12 months n=43 and Placebo: baseline n=52; 1 month n=45; 6 months n=41; 12 months n=41. FX, flaxseed group; G, group; G×T, group × time; PL, placebo group; T, time.
Table reprinted with permission from the American Society for Nutrition on April 13, 2015; © 2015 Edel, Rodriguez-Leyva, Maddaford, Caligiuri, Austria, Weighell, Guzman, Aliani and Pierce. Dietary flaxseed independently lowers circulating cholesterol and lowers it beyond the effects of cholesterol-lowering medications alone in patients with peripheral artery disease (6).

DISCUSSION

A secondary endpoint of the FLAX-PAD Trial was to examine the effects of flaxseed on blood lipids in a patient population with pre-existing cardiovascular disease (9). Unlike other studies to date, ours is inclusive of patients being administered CLM, which is an exclusion criterion in many other studies (12, 26-28). Of the 110 patients enrolled in the study, 74% were on CLM. LDL-C dropped by 15% and TC by 11% in the FX group compared to PL after just 1-month of consuming dietary flaxseed and was maintained at these concentrations for up to 6-months (**Figure 19**). However, only within the flaxseed group and not between-treatment groups were significant decreases detected. This is consistent with other reports of flaxseed reducing circulating cholesterol in hemodialysis patients (12), in patient populations with hyperlipidemia (8, 26, 29, 30) and postmenopausal women (27, 28) in short-term studies. In healthy control populations, the effects of flaxseed are less consistent with a modest cholesterol-lowering effect at higher flaxseed concentrations (40 to 50g/day) (5, 20, 31, 32) and no effect at doses used in the present study (30g/day) (24, 33). Health Canada has recently approved a cholesterol-lowering health claim for 40g of flaxseed (34). This was based upon several studies with this dosage (20, 26, 27, 35). Our data is consistent with these data and confirm that a 30g dosage of flaxseed is at the lower dosage range for inducing significant total- and LDL-C lowering effects. Following 12-months of flaxseed consumption in our FLAX-PAD Trial, lipid concentrations did not differ. One explanation for this may be that some of the patients were not being adherent to the dietary protocol towards the end of the 12-months. Dietary compliance was monitored through plasma markers of flaxseed consumption. SDG metabolites, known as enterolignans, and ALA are two very sensitive indicators of flaxseed ingestion (23, 24, 36).

As shown previously (10), patients in the FLAX-PAD Trial who ingested flaxseed had a 1-fold increase in circulating ALA concentrations and a 10-50 fold increase in plasma enterolignans compared to placebo ($P<0.05$). Despite the large fold increases in plasma enterolignans and ALA after 12-months in the FX compared to PL groups, changes from baseline were lower at this time point than values measured after 6-months (**Appendix 12**). In fact, plasma ALA was significantly reduced in the FX group after 12 months compared to 6-month values ($P=0.002$). Another possible explanation may be due to desensitization to flaxseed. HDL-C concentrations did not differ between-groups when all subjects were included in the analysis (**Figure 19**), which is in agreement with other studies (8, 27, 28), but did have a modest significant decrease at 6 months compared to baseline values in the flaxseed group when only patients that completed the trial were used (**Table 15**). Two reports involving hypercholesterolemic patients note lower HDL-C concentrations with a higher prevalence of adult males (26) and in a pediatric population (37). In the present study, males were most predominant comprising 73% (PL) and 74% (FX) of the patients within this particular cohort. It is difficult to measure this outcome as a result of gender influence in our study due to the skewed ratio of males to females; however, the prevalence of male gender may be a contributing factor towards the observed lower HDL-C concentrations at 6-months in these patients. The effect of flaxseed on triglycerides is less understood. Associated increases in triglycerides with flaxseed consumption have been noted in several clinical trials (37, 38), with no changes (8, 27, 28) and decreases (12, 35) in others.

A limitation of this study was the small sample size that may have concealed significant between-group differences for total- and LDL-C. Original calculations for achieving a minimum of 80% power at an alpha level of 0.05 were based on incidence of

myocardial infarctions and strokes (9). Recalculating the power of the study for LDL-C, a power of 80% was achieved at baseline with a sample size of 110 participants, but was only 74% at 1 month (n=99), 63% at 6 months (n=84) and 62% at 12 months (n=83) when using a repeated measures ANOVA design. It is estimated that a minimum sample size of 132 participants would be needed to achieve a minimum of 80% power accounting for 20% sample loss and should be used in future studies to detect significant differences between treatment groups. A standard deviation of 0.96 mmol/L was used for LDL-C as determined in the present study which is similar to published results in PAD patients (0.91 mmol/L) (39). Similar sample sizes and power would be expected for TC.

The vast majority (74%) of PAD patients enrolled in this study were administered cholesterol-lowering drugs to treat their existing hyperlipidemic condition (9) and of these patients, 90% were administered statins. This allowed us to examine drug-food interactions in the present study. Several basic questions, therefore, arose because of the potential interactions of flaxseed with CLMs. First, did flaxseed interfere (positively or negatively) with the cholesterol-lowering action of the drugs in the present study? As shown in **Figure 20A**, FX+CLM resulted in a significantly greater decrease in LDL-C beyond that achieved by PL+CLM. Thus, it clearly does not interfere with the cholesterol-lowering action of CLM. In a small subgroup of patients not administered CLMs at the onset (**Figure 20B**), FX independently lowered LDL-C, which is in agreement with previous data obtained in the absence of medication (26-28), thus suggesting that FX works independently of the CLM to lower LDL-C.

The lack of effect of dietary flaxseed on circulating TC and LDL-C in hypercholesterolemic patients was surprising as this is in contrast to previous reports (12, 27,

28, 30). However, a significant main effect of time in LDL-C, with a similar trend for TC, suggests that the small sample size within this subgroup may have influenced the analysis. The ineffectiveness of dietary flaxseed on circulating triglyceride and HDL-C concentrations is consistent with previous reports (28, 40) and a recent meta-analysis that reported that 70–90% of trials demonstrated neutral effects of flaxseed on these lipid variables (8).

Dietary flaxseed may suppress the immediate need for administered statins or other CLMs in newly diagnosed patients. In the subgroups where patients were not taking any CLM at the onset, those that were consuming milled flaxseed did not require any CLMs to control their cholesterol concentrations over the course of the 12-months. However, 33% of the individuals consuming the placebo-supplemented diet were prescribed CLMs by the end of the study period. Flaxseed may provide a possible strategy for individuals looking for natural ways to control their plasma cholesterol concentrations. This may reduce the immediate need for prescription medications and their potential negative side effects. Additional studies with larger sample sizes are needed to accurately answer this question.

The component within flaxseed that was responsible for the cholesterol-lowering effect of flaxseed has not been identified in the present study. However, we can rule out two candidate bioactives. Due to the lack of an inverse correlation of ALA and enterolignans with measured cholesterol, it would strongly suggest that the cholesterol lowering effect of flaxseed does not involve either of these two compounds. Most of the published evidence in humans does not support flax oil (and therefore ALA) as having a primary role in cholesterol lowering (8, 41). Alternatively, higher doses of flax lignans in healthy (42) or unhealthy (43) individuals or defatted flaxseed in hyperlipidemic patients (30) have been suggested to play a role in lowering plasma cholesterol. These authors did not correlate the lignan metabolites,

enterodiol or enterolactone, with LDL-C, so although "high lignan" (42) or "high SDG" (43) doses yielded decreases in LDL-C, these two key lignan metabolites were likely not involved as outlined in our study. It is more likely that soluble mucilage fiber, resulting from flaxseed lignan extracts (43), may have yielded the attenuated cholesterol values observed in these studies. The dietary fiber content of flaxseed is 28% by weight, of which 33% is soluble fiber (26). It is more probable that the high fiber content of flaxseed is responsible for the LDL-C-lowering actions of flaxseed. This is consistent with other conclusions in the literature (27, 41, 44).

PAD patients are at great risk for thrombosis and the subsequent complications of myocardial infarctions and stroke (45). Since the fish-derived omega-3 fatty acids EPA and DHA have antithrombotic effects on platelets (46-49) and flaxseed is rich in another omega-3 fatty acid, ALA, it is possible that flaxseed consumption in the PAD patients may be of anti-thrombotic benefit. ALA has inhibited both collagen and thrombin-induced platelet aggregation in animal studies (48). Clinical studies in healthy subjects have shown that dietary supplementation with flaxseed or ALA had no effect on platelet aggregation (11, 24, 32, 33). The FLAX-PAD Trial is the first to assess the aggregatory actions of dietary flaxseed in a population with clinical evidence of hypercoagulation. The significant inhibition in % aggregation induced by flaxseed in the present trial was restricted to the agonist thrombin. This would suggest that selected changes in G-protein coupled receptors or in the leucine-rich repeat family of receptors may have occurred in the flax group in comparison to placebo (50). However, no significant differences were calculated between treatment groups at any time with either collagen or thrombin.

In summary, dietary flaxseed has the capacity to lower total- and LDL-C even in the presence of cholesterol-lowering drugs like statins. It does not appear to interfere with the cholesterol-lowering capacity of statins. In view of these actions and its effect on platelet aggregation, dietary flaxseed can be recommended for patients at risk for cardiovascular disease. A 10% decrease in total- or LDL-C would be predicted to induce a clinically significant reduction in the incidence of myocardial infarctions and stroke over time (51). However, our conclusions are limited by the sample size, particularly when the analysis involved smaller subgroups. Larger trials may be recommended. Despite this, the potential for flaxseed to lower both plasma cholesterol concentrations and decrease blood pressure (10) simultaneously is noteworthy. More than two out of three patients in the United States do not have control of both their blood pressure and their cholesterol levels (52). Controlling both cholesterol and blood pressure can substantially reduce the risk of heart disease by half or more (52). In view of this need, the potential for a dietary supplement like flaxseed to achieve this dual action of lowering blood pressure (as previously shown) (10) and cholesterol (as shown here) is very appealing in a patient population with significant documented cardiovascular disease.

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The authors' responsibilities were as follows: A.L.E. extracted enterolignans, performed correlations with blood lipids, prepared all figures and tables, interpreted statistics and contributed towards writing and editing of the manuscript; D.R.L. analyzed and interpreted cholesterol and triglyceride data and contributed towards writing and editing of the manuscript; T.G.M. performed all platelet aggregation experiments; S.P.B.C. performed and analyzed all mixed repeated measures 2-Way model statistics; M.A. assisted in enterolignan extraction design and edited the manuscript; J.A.A. did food distribution; W.W. did patient recruitment, consent, monitored patient health and safety, captured morphometric data and obtained blood samples; R.G. provided oversight for patient recruitment, was responsible for ensuring patient health and safety throughout the clinical trial and participated in the organization of the trial; G.N.P. provided project conception and developed the research plan, acquired financial support, edited the manuscript and had primary responsibility for final content. Funding organizations provided financial support solely and did not contribute to the study design, implementation, analysis or interpretation of the data or writing of the manuscript. All authors read and approved the final version of the manuscript.

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CHAPTER IX: DISCUSSION

The present findings have provided evidence regarding circulating concentrations of MFX bioactives in the context of various human conditions related to factors such as subject age, MFX dose and CVD status. As scientific evidence related to plasma enterolignan concentrations within these conditions is especially lacking, the present studies provide insight that may be useful not only for future cardiovascular-related research but also for other pathologies. Enterolignans have demonstrated anticancer potential and their role in the prevention of hormone–sensitive cancers (200) warrant further study and may benefit as well from the present findings. Research from the combined published chapters not only evaluate MFX consumption on circulating enterolignan concentrations, but assist in defining the safety and efficacy of consuming MFX based on reported adverse events and specifically, their influence on cholesterol-lowering in both healthy and unhealthy adults. It should be clearly stated that in the context of this thesis, only data that are established as statistically significant ($P \leq 0.05$) should be considered as evidence that the null hypothesis does not hold (201), whereas trends ($P \leq 0.1$) are unproven, but are provided to draw attention to possible relationships that may warrant further study, especially with the large inter-subject variability that exists in enterolignan plasma concentrations (195-197). Prior to determining any associations with flaxseed bioactives and blood lipids, we needed to first develop and create a method to analyze plasma enterolignans, the primary metabolites of flaxseed lignans. Methods for quantifying plasma ALA and other fatty acids are well established and validated (202), and were therefore not discussed within this document.

The focus of the first part of our research was to develop a method capable of extracting and quantifying circulating concentrations of END and ENL (2). Traditional

extraction methods employ LLE or SPE techniques, which are lengthy and cumbersome. SLE offered benefits of high recoveries (96.4 % and 96.0 % for END and ENL, respectively), removal of matrix effects, reduced sample preparation times, loss of emulsions and reduced sample volumes. Most enterolignan analyses involve LC/MS; however, GC/MS is equally suitable. Often the choice of one instrument or the other depends upon instrument availability in consideration with instrument sensitivity. GC/MS-μSIS compared to GC/MS-SIS and GC/MS in full scan mode substantially improved compound sensitivity due to the targeted approach of this technique. Improved limits of detection (LOD) of μSIS-MS compared to SIS-MS mode have been demonstrated previously in a MS detection comparison study involving pesticides (203). Running the ion trap mass spectrometer in micro-selected-ion storage (μSIS) mode enabled extraction of ions of interest at specified time points removing interfering compounds or background noise that would normally hinder peak detection. Accurate quantitation was ensured using $^{2}H_{6}$-labeled isotopes of END and ENL, similar to the ^{13}C-labeled internal standards used in an LC/MS/MS analysis of these lignan metabolites (204), which protected against incongruences in sample preparation and/or GC/MS instrument batch-to-batch reproducibility. Using this validated method, we have applied it to approximately 600 human plasma samples, resulting in several published manuscripts (3, 4, 6).

Previous studies have demonstrated that milling flaxseed prior to ingestion improved plasma bioavailability of ALA (184) and enterolignans (195). However, to date, the amount of MFX required to produce maximum circulating concentrations of these bioactive compounds, at potentially maximum therapeutic benefit, has not been determined. MFX doses of 10 g/d, 20 g/d, 30 g/d and 40 g/d were consumed for 4 weeks, a time period

previously demonstrated to be suitable for significantly increasing plasma ALA (184) and enterolignan (204) concentrations. The findings of the present study suggest that maximum circulating concentrations of ALA and enterolignans are not obtained in a dose dependent manner despite modest fold increases with increasing MFX dose. Doses as low as 10 g/d were sufficient to significantly increase each of these bioactive compounds in the plasma of healthy adults, 18 to 49 years of age, from baseline values. This dose after 4 weeks did not differ significantly from any of the other administered doses following the same time period. The ability for low dose MFX to significantly increase circulating ALA concentrations has only been demonstrated in one other study using 13 g/d in obese, pre-diabetic individuals (194). Enterolignan concentrations were not reported in this study, unfortunately (194). This cross-over study also examined a higher dose of 26 g/d of MFX. In this study, plasma ALA concentrations increased significantly in a dose-dependent manner when comparing all 3 MFX doses: 0 g/d, 13 g/d or 26 g/d. This study lasted 12 weeks compared to ours, which was only 4 weeks; however, 4 weeks has been shown to be an effective time period to significantly increase ALA bioavailability (184). Two possible explanations for the dose dependency observed in this study and not in ours could be the differences in patient health status (ours were healthy), or due to the cross-over design, which may have reduced inter-subject variability. The low sample size in our study may have impacted this as well.

When considering the dose of MFX to recommend for consumption in human studies, it is important to also consider the percent change values from baseline for each of the bioactive compounds. Despite any between group differences after 4 weeks for each of the MFX doses, average percent changes from baseline were doubled in the 20 to 40 g groups, ranging from 123.0 to 124.4%, compared to only 55.6% in the 10 g group for ALA. A

similar pattern was observed for total enterolignan concentrations. The small sample sizes may have contributed to these non-significant findings and should be taken into consideration. Based on percent change calculations, this would suggest that consuming muffins with as little as 20 g/d of MFX should be the lowest dose consumed to provide the greatest percent change in plasma ALA and enterolignan concentrations. However, 10 g/d is still a sufficient dose to provide significant plasma bioactive concentrations, a potential benefit for individuals who cannot tolerate higher fiber loads associated with greater MFX doses. Alternatively, we can also conclude that higher doses of 40 g/d are unnecessary to achieve maximum circulating concentrations of ALA and enterolignans. However, they did not result in any significant reported adverse events, changes in body morphometrics, nor did they alter platelet aggregation, urinary thromboxane levels or attenuate LDL-C or TC levels in normocholesterolemic, younger individuals relative to any of the lower MFX doses. In consideration of this latter point, only doses of 50 g/d of MFX have been observed to attenuate LDL-C levels in healthy adults (182), suggesting that 40 g/d is too low to effect this change in normocholesterolemic, healthy adults. The safety of consuming higher doses, like 50 g/d, has also been previously established (182), agreeing with the lack of associated adverse events reported within our study. As such, this study further demonstrates that consuming doses as high as 40 g/d of MFX appears safe for daily consumption in healthy, younger adults.

A final consideration of MFX dose is not only with regards to the contributions of ALA and enterolignans, but that resulting from dietary fiber. Dietary flaxseed is a rich source of fiber, which has demonstrated cholesterol-lowering benefits in moderately hypercholesterolemic individuals (10). Increasing the amount of dietary flaxseed, therefore,

not only impacts plasma ALA and enterolignan concentrations, but also simultaneously increases daily total fiber content. Dietary recommendations of daily fiber intake for individuals 14 to 50 years of age are 38 g/d for males and 25-26 g/d for females (205). However, current intakes of total fiber are typically much lower at about 16-17 g/d for both males and females in Western populations (206, 207). Consuming one muffin daily in the present study would provide as low as 9.8 g of fiber/d from 10 g of flaxseed and up to 14.6 g of fiber/d from 40 g. Eating one muffin per day from any of these dosage groups would markedly increase fiber-intake to the daily-recommended value. Considering dietary fiber has benefits both in gastrointestinal health (208), in attenuating atherosclerosis (209) and in lowering cholesterol, insulin, oxLDL and SBP (210), achieving recommended intakes through increased flaxseed consumption may be an important consideration.

As most diseases occur within elderly populations, the effect of age on lignan metabolism and subsequent enterolignan bioavailability for potential therapeutic purposes was of interest. Previously it was observed that participant age did not influence ALA metabolism and plasma bioavailability (5). However, lignan metabolism proceeds via a different pathway mediated by gut microbiota, which directs the amount of END and/or ENL produced (9). Bacterial species present in the microbial environment are known to vary with age (211). Therefore, we hypothesized that differences in age-associated microbiota may influence the metabolism of flaxseed lignans to their enterolignans. Only the older age group could significantly produce greater END and ENL after 4 weeks, relative to baseline values compared to the younger group, but this may be attributed to the smaller sample size in the younger age group. However, the ratio of END:ENL was tipped in the direction of ENL in older adults, unlike the 1:1 ratio observed in the younger age group. All participants in the

older group could produce ENL relative to only 62.5% in the younger group. END was produced in 77.8% of the older adults relative to 37.7% in the younger group. Unfortunately, bacterial species that metabolize lignans to END or ENL are only loosely characterized, yet certain strains have been identified in the sequential deglycosylation, demethylation, dehydroxylation and dehydrogenation of SDG (7). The dehydroxylation step occurs in the conversion of SECO to END through an intermediate compound that is catalyzed by the primary species *Eggerthella lenta* (8), which comprises a dominant community of the microbiome in humans (212). The conversion of END to ENL involves a dehydrogenation step, which appears to be a limiting step. Only one species to date has been identified that converts END to ENL, namely *Lactonifactor longoviformis,* which belongs to a subdominant community (8, 9). This study also established that the molecular species *Ruminococcus bromii* is significantly associated with blood and fecal ENL concentrations (9). Future research should aim to identify the presence and abundance of these species in aging populations to determine if an age-dependency regarding these particular species exists.

The influence of MFX on the microbiome is generating significant attention, yet to date, has not been investigated as a function of subject age. It has recently been suggested that dietary consumption of 30 g/d of MFX does not disrupt the microbial environment after only one week of ingestion in which these authors imply that the habitual intake of flaxseed may not alter the microbiome (9). However, another recent study demonstrated that diets high in soluble fiber skew the ratio of Bacteroidetes:Firmicutes with greater production of Bacteroidetes (12 to 13%) as a result of consuming two different types of soluble fiber (polydextrose and soluble corn fiber) (208). As MFX contains both soluble and insoluble dietary fiber (48) the effect of dietary fiber on the microbiome clearly requires further study.

Intervention periods longer than 1 week are necessary to assess the long-term stability of the microbiome as a result of consuming MFX, and should also be considered within the context of subject age.

The overlying significance of more, equal or less END, ENL or END+ENL in systemic circulation does not fall within the scope of this thesis. However, ENL prevalence in plasma has been associated with reduced risk of acute cardiac events (213) and suggested anti-cancer benefits specifically in regards to hormone sensitive cancers like breast cancer (200). It is this latter pathology that has acquired significant attention as high serum ENL concentrations are associated with a decreased incidence of breast cancer and breast cancer mortality (214-216). With enterolignan bioavailability more clearly understood by factors such as age, dose and health status as described within this thesis, future studies must begin to uncover the relevance of the specific enterolignans, END and ENL, in CVD disease prevention.

Our final study involved the addition of MFX (30 g/d) to baked products added to the normal diet of patients with diagnosed PAD. Prior to assessing the cardiovascular health outcomes of MFX in this population, we firstly wanted to assess lignan metabolism, plasma bioavailability and circulating concentrations within these patients that were concurrently being administered antihypertensive, statin, anti-aggregatory and/or blood glucose normalizing medications (18). It is well established that compound absorption is influenced by a host of intrinsic and extrinsic variables (217). Two important physiological variables influencing compound absorption, relevant within the context of this thesis, include subject age and disease status (217). The findings of our study demonstrate that despite being severely ill and taking concomitant medications, PAD patients were able to metabolize

flaxseed lignans, such that plasma enterolignan concentrations were similar to those measured in healthy patients of equal age (4). Circulating concentrations of enterolignans in PAD patients were elevated 10 to 50 fold and those of ALA were doubled from baseline values (74).

The antihypertensive action of MFX in PAD patients has been demonstrated (74); however, the effect on cholesterol-lowering until now (6), has not. A risk factor of PAD is hypercholesterolemia (37). When all patients in the FLAX-PAD study were examined, TC and LDL-C were significantly reduced from baseline values (11 and 15 %, respectively) as early as 1 month into consuming the dietary intervention and were maintained at these conditions after 6 months. This represents the first RCT demonstrating the action of MFX on cholesterol lowering in patients with PAD. Cholesterol lowering by MFX is not surprising in patients with hypercholesterolemia for in 2014, Health Canada established a health claim documenting the cholesterol lowering abilities of dietary MFX in this patient population (10). What our research needed to address was if MFX could attenuate TC and LDL-C in the presence of CLMs or would it inhibit their action? The critical nature of this question stems from the above-mentioned health claim which was based solely on research studies that excluded individuals that were being administered CLMs (167, 173, 174, 180). Therefore, we examined cholesterol lowering in a subgroup of patients taking concurrent CLMs, the most prevalent being statins (90%). Our findings demonstrate that MFX improved LDL-C lowering in patients taking concurrent CLMs and most importantly, did not inhibit their action. LDL-C was reduced 8.5% from baseline values following 12 months of combined MFX and CLM treatment, which was significant when compared to the placebo group also taking concurrent CLMs. As drug-food interactions exist that can alter circulating

concentrations of prescribed medications (218), this was an important consideration. Therefore, these findings provide some valuable insight into the safety and efficacy of MFX with administered CLMs, in particular, statins. A drug-food interaction has been noted previously with statins and grapefruit juice such that a glass of grapefruit juice consumed with lovastatin (219) and simvastatin (220) significantly elevated circulating concentrations of both compounds. Naringinen, the bioactive compound in grapefruit juice is an antioxidant, and was proposed to inhibit the metabolism of statins through cytochrome P-450 3A4 within the small intestines (219) (220). A consequence of this is severe rhabdomyolysis and acute renal failure. Therefore, as scientific evidence concerning food-related interactions with statins is limited, the current thesis findings are important, specifically as enterolignans themselves have suggested antioxidative potential (95).

It was also important to determine if MFX alone, without the influence of CLMs, could lower cholesterol levels in patients with PAD. Therefore, a subgroup of patients consuming only the MFX-supplemented diet was studied. Unfortunately, the sample size of this subgroup was extremely small, yet the preliminary findings were intriguing. Following 6 months of dietary intervention, significant changes from baseline values were noted in participants consuming the flaxseed diet but not the placebo diet, suggesting that flaxseed can lower cholesterol independently of CLMs in PAD patients, a population that until now, has not been studied. Another interesting point from this subgroup analysis was that dietary MFX appeared to prevent the immediate need of CLMs compared to patients in the placebo group as 33% of the individuals consuming the placebo-supplemented diet were being prescribed CLMs at the end of the 12 month study, compared to none in the MFX group. No differences were noted in the patients being administered anti-platelet therapy. The

independent use of MFX as a therapy for cholesterol-lowering in hypercholesterolemic patients warrants further study as this is a target population for increased risk of atherosclerotic CVD (221), yet we recognize the difficulty in performing this study as a RCT due to the unethical nature of denying patients CLMs when their cholesterol levels are elevated beyond normal, healthy levels. A candidate population to study this would be individuals whose cholesterol levels are borderline-high, who may want to explore lifestyle management strategies prior to drug treatment regimes that may span throughout their life. Current lifestyle guidelines endorsed by the AHA/ACC to reduce cardiovascular risk through cholesterol lowering include the DASH and AHA diets, reduced calories from SFAs (total caloric intake should be ≤ 5 to 6%) and reduced intake of trans fats (16).

The bioactive responsible for cholesterol lowering was not identified in the present study; however, ALA and enterolignans could be excluded based on the lack of inverse correlations with blood cholesterol data. This was not surprising for ALA, as most evidence in humans does not support flax oil, therefore ALA, as having a primary role in cholesterol lowering (179). This was surprising, however, for enterolignans as high lignan diets in healthy (99) or unhealthy (100) individuals or defatted flaxseed in hyperlipidemic patients (178) have been suggested to play a role in lowering plasma cholesterol. Although lignan-fortified diets were provided in each of these particular studies, the lignan metabolites END and ENL were not directly measured and correlated with cholesterol concentrations suggesting that it could be the soluble mucilage fiber in flaxseed lignan extracts (43) that attenuated the cholesterol levels. The high fiber content of flaxseed was proposed to be the major contributing component responsible for cholesterol lowering, agreeing with published data on the health benefits of consuming dietary fiber (189). This emphasizes the need for

further studies to identify the minimum MFX dose that can effectively attenuate cholesterol in PAD patients.

As half of all major cardiovascular events occur in apparently healthy individuals with average or lower levels of LDL-C (222), it is important to recognize that hyperlipidemia is not the only contributing factor in CVD progression. The Framingham Study was the first prospective study to demonstrate that in addition to elevated cholesterol levels, cigarette smoking and uncontrolled hypertension were primary contributing factors leading to increased CVD risk (223). Inflammation is also regarded as a potent risk factor as elevated baseline plasma concentrations of CRP, from apparently healthy adults, have been associated with future risk of developing PAD in men (224) and CVD in women (225). Inflammation is currently being investigated as a therapeutic target, independent of cholesterol-lowering, to assess future risk of developing negative cardiac outcomes (226, 227). Clearly other pathways are implicated in the pathogenesis of CVD and must be considered in the overall therapy and disease prevention strategy.

In summary, this thesis has documented the presence of circulating concentrations of MFX bioactives, in particular enterolignans for which scientific evidence is lacking, in human populations as a function of age, MFX dose and CVD status. We have established that enterolignans are available in the plasma of both healthy younger and older adults, in younger healthy adults at doses ranging from 10 to 40 g/d and in older adults with PAD. Establishing their presence in the circulation, we could then examine their therapeutic potential, in conjunction with ALA and dietary fiber, on cholesterol lowering and adverse events. The antihypertensive benefit of consuming MFX has already been established in PAD patients (74) furthered now by the benefits on cholesterol lowering (6). Although

enterolignans or ALA do not impact cholesterol-lowering as determined in our study, the high dietary fiber content within MFX likely does. The simultaneous reduction of hypertension and cholesterol with 30 g/d of MFX is noteworthy and future studies are encouraged to consider the dual action of MFX on these two primary risk factors of CVD when considering cardiovascular outcomes and recommended doses.

CHAPTER X: CONCLUSIONS

1. The SDG derived enterolignans, END and ENL, can be successfully extracted from human plasma using supported liquid extraction (SLE) and quantified accurately using GC/MS – μSIS.

2. A MFX dose of 10 g/d for 4 weeks was sufficient to significantly increase circulating concentrations of ALA, END and ENL in healthy, younger adults.

3. Higher MFX doses of 20 g/d, 30 g/d and 40 g/d for 4 weeks did not increase circulating concentrations of ALA, END and ENL in a dose-dependent manner or significantly more than the 10 g/d dose in healthy, younger adults.

4. Significant increases in circulating EPA concentrations, as a result of ALA metabolism from milled flaxseed ingestion, resulted from the 30 g/d dose only and not from the 10 g/d, 20 g/d or 40 g/d doses.

5. Cholesterol lowering in normolipidemic, healthy adults was not observed with any of the four flaxseed doses (10 g/d, 20 g/d, 30 g/d and 40 g/d), nor were there any alterations in platelet aggregation, body morphometrics or increases in reported adverse events.

6. Younger and older healthy adults can each metabolize flaxseed lignans as there were no significant differences in averaged plasma total enterolignan (END + ENL)

concentrations following 4 weeks of dietary MFX intervention (30 g/d); however, median concentrations were elevated in older adults compared to younger adults for both enterolignans.

7. The ratio of END:ENL in plasma was not equivalent in both age groups as older adults had a higher prevalence of circulating ENL, yielding an END:ENL ratio of 0.74, whereas the ratio was 0.96 in the younger adults.

8. ENL was detected in the plasma of all older adults, but in only 78% of the younger participants. END was present in 63% and 38% of the older and younger adults, respectively.

9. As at least one enterolignan was present in all younger and older adults, total enterolignans may be a suitable biomarker of flaxseed consumption in nutritional studies involving MFX.

10. In patients with PAD, circulating concentrations of TC and LDL-C were attenuated after 6 months as a result of dietary MFX relative to baseline values. Cholesterol values were unchanged in the group receiving the placebo dietary intervention.

11. In a subgroup of PAD patients administered CLMs, LDL-C decreased significantly compared to placebo after 12 months.

12. In terms of CLMs, patients in the flaxseed only group (those that were not being administered CLMs at the onset) did not require CLMs at the end of the 1-year intervention period; however, 33% of the individuals that were just consuming the placebo-supplemented diet (without concurrent CLMs at the onset) were being administered CLMs by the end of the study.

13. Circulating concentrations of ALA and enterolignans did not correlate inversely with plasma TC or LDL-C suggesting that the cholesterol lowering effects of dietary flaxseed did not involve either of these two compounds.

CHAPTER XI: IMPLICATIONS OF RESEARCH

CVD is the leading cause of death worldwide accounting for ~17.5 million deaths in 2012 (11). This is expected to rise to an estimated 23.3 million by the year 2030 (11). In the United States alone, over 4 million citizens are inflicted with CVD-associated disabilities, with CVDs accounting for over one-third of all deaths (12). The estimated overall cost in the U.S. in 2010 was 444 billion dollars (13). Primary prevention is necessary to manage risk factors of CVD with first recommendations often involving prescription medications. However, with the failure of patients to adhere to drug regimes, alternative strategies must be investigated.

Lifestyle modification through improved nutrition may provide a valuable approach for reducing the global burden of CVD. Dietary MFX is just one of these foods as it contains a minimum of three bioactive ingredients, namely ALA, SDG and fiber, with potential to reduce several key risk factors of CVD. Evidence from a randomized clinical trial details the potential for milled flaxseed to simultaneously lower both systolic and diastolic blood pressure (74) and plasma total- and LDL-cholesterol levels (6). Considering that more than two out of three patients in the United States do not have control of their blood pressure and their cholesterol levels (228), this dual action behavior of MFX is significant. Epidemiological studies also note that controlling both cholesterol and blood pressure can substantially reduce the risk of heart disease by half or more (228).

These suggested health benefits of dietary MFX encourage its use in a daily dietary plan. Implementation of this dietary strategy would be expected to be quite simple. Flaxseed is produced in at least 51 countries in the World (47) making it widely available to consumers. In addition, it is relatively inexpensive to purchase costing about $4.50 to

$8.00/kg in Canadian dollars (229). MFX can be easily consumed in its natural form, sprinkled onto foods such as yoghurt, cereal or smoothies or can be consumed in functional foods like muffins, bread, bagels, snack bars, pizza crusts, buns or pasta, as examples. The sensory attributes of foods containing doses of 30 g of MFX, which are the doses observed to induce CVD benefits, have been explored as the first step in insuring their acceptability to the general public (230, 231).

The health benefits of the individual bioactive ingredients in flaxseed are being identified. The anti-hypertensive benefits of MFX may be largely due to ALA, as observed by the inhibition of soluble epoxide hydrolase (sEH) by ALA (168). END or ENL appear to have less of an influence on blood pressure regulation (74). The cholesterol-lowering benefits are suggested to be due to the high soluble fiber content of milled flaxseed and not due to the other bioactive compounds (6). Enterolignans are bioavailable in plasma following flaxseed ingestion in both healthy younger and older adults and in patients with PAD (2, 6, 195, 196). However, the cardiovascular health benefits of flaxseed-derived enterolignans are largely unknown (232). Equally true is their demonstrated antioxidative potential *in vitro* (95) and *in vivo* (97) and their mild estrogenic and antiestrogenic behavior (233). Although the role of metabolized lignans in CVD prevention is yet unclear, the evidence for the health benefits in cancer prevention is mounting (200, 214-216).

Limitations of research

The primary limitation of this thesis was the low sample sizes that were used in each of the human studies. Original calculations for achieving a minimum of 80% power at an alpha level of 0.05 were originally designed for outcomes related to ALA in the MFX dosage

and age-dependency studies, whereas it was based on incidence of myocardial infarctions and strokes in the study involving PAD patients. Unfortunately, these sample sizes were too low and did not achieve 80% power when secondary outcomes like plasma enterolignans were measured. This has been identified as a limitation within each of the various chapters and within the published manuscripts. Suggestions for proper sample sizes were included to prevent similar outcomes in the future. It is hoped that future studies using MFX as an intervention with outcomes related to enterolignan bioavailability will consider the substantial inter-subject variability that exists when calculating sample size.

An additional limitation of each of these studies would be in determining if previous antibiotics were administered that could alter the gut microbiome, and therefore, circulating enterolignan concentrations. It is well established that serum ENL concentrations are negligible following antibiotic usage as early as 12 to 16 months prior to blood sampling (234). Information concerning the early administration of antibiotics in enrolled patients or healthy subjects was not documented. Only medications administered throughout the course of the study period were recorded in subject diaries or patient charts. If individuals had been prescribed antibiotics 12 to 16 months prior to starting the dietary intervention, circulating concentrations of enterolignans would be impacted thereby implicating reported measured values presented within this document.

As human dietary intervention studies do not include fully regulated meals to control dietary intake, it is difficult to ensure that participants were consuming strict, lignan-free diets. Many other foods contain dietary lignans that can be converted to the mammalian lignans, END and ENL; however, none of these are as rich as those found in flaxseed (61).

Future directions

1. Circulating total enterolignan (END + ENL) and ALA concentrations have potential to be suitable biomarkers of flaxseed consumption which may be useful, and should be considered, in future studies when monitoring nutritional compliancy.

2. To synthesize isotopically labeled SDG that could behave as an internal standard to help accurately quantify SDG in flaxseed foods used in dietary interventions.

3. To study age-related differences in the microbiome that would impact conversion of flaxseed lignan metabolites.

4. To measure and compare urinary enterolignan concentrations with those found in plasma to have a more complete image of lignan metabolism resulting from MFX consumption in both healthy adults as a function of MFX dose, subject age and CVD status.

5. Studies using MFX as complimentary therapy for CVD should consider the outcome on oxidative parameters in the blood as ALA and enterolignans both have antioxidative potential. Therefore, future studies on CVD prevention should quantify plasma concentrations of markers of oxidative stress, including isoprostanes, nitrotyrosine, myeloperoxidase, malondialdehyde and oxLDL, as these have been linked with independent risk factors of CAD.

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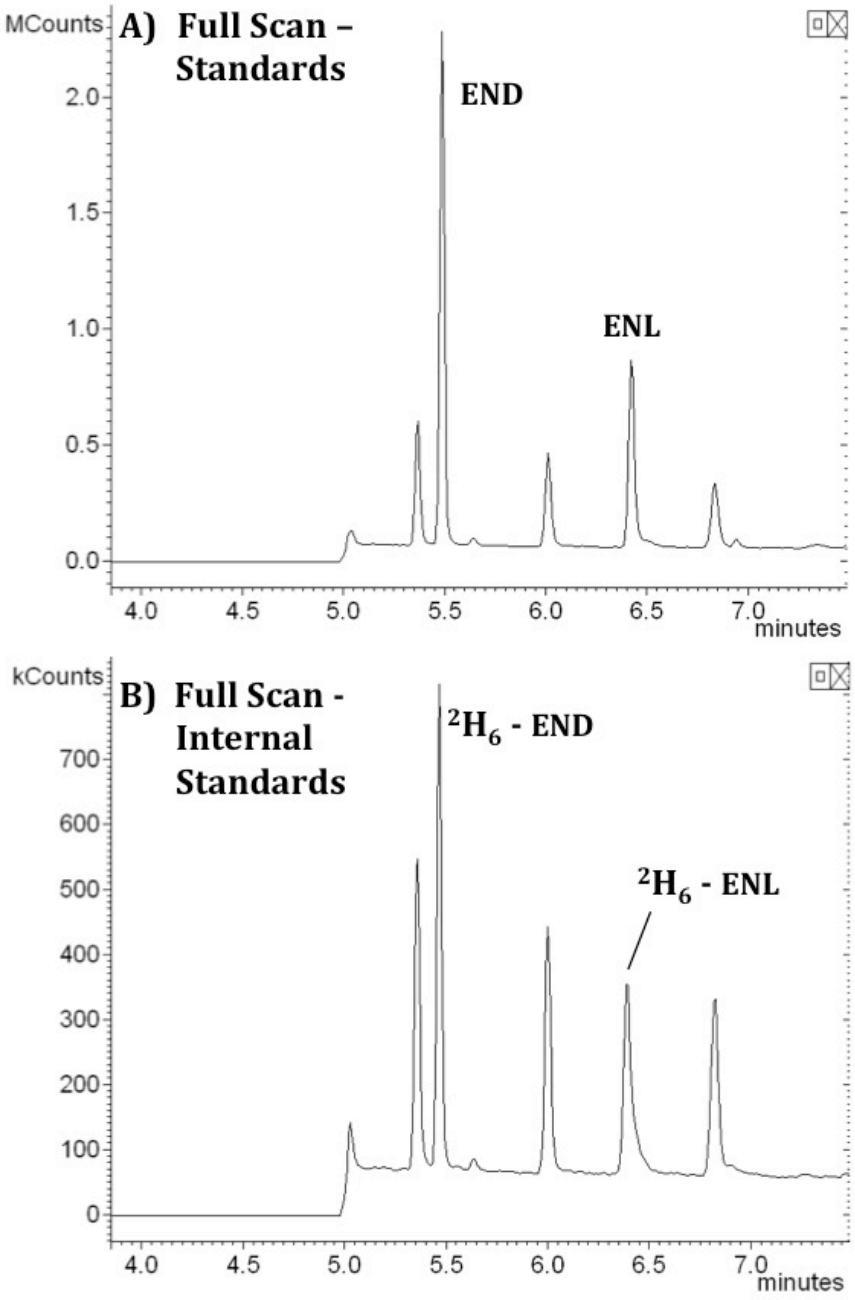
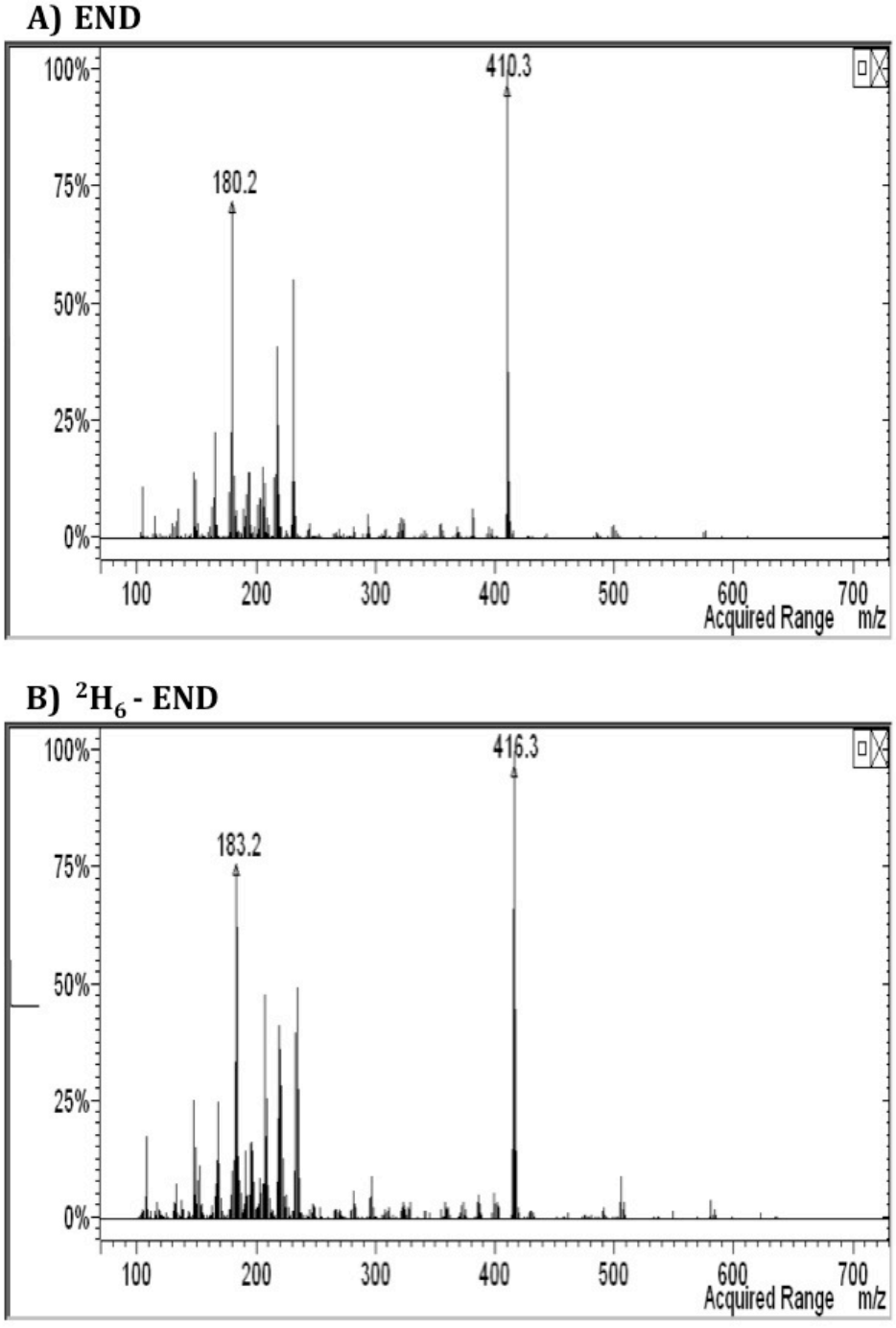
APPENDICES

Appendix 1. Full scan gas chromatograms of pure (A) END and ENL (33 µM) and (B) $^{2}H_{6}$ – END and $^{2}H_{6}$ – ENL (17 µM).

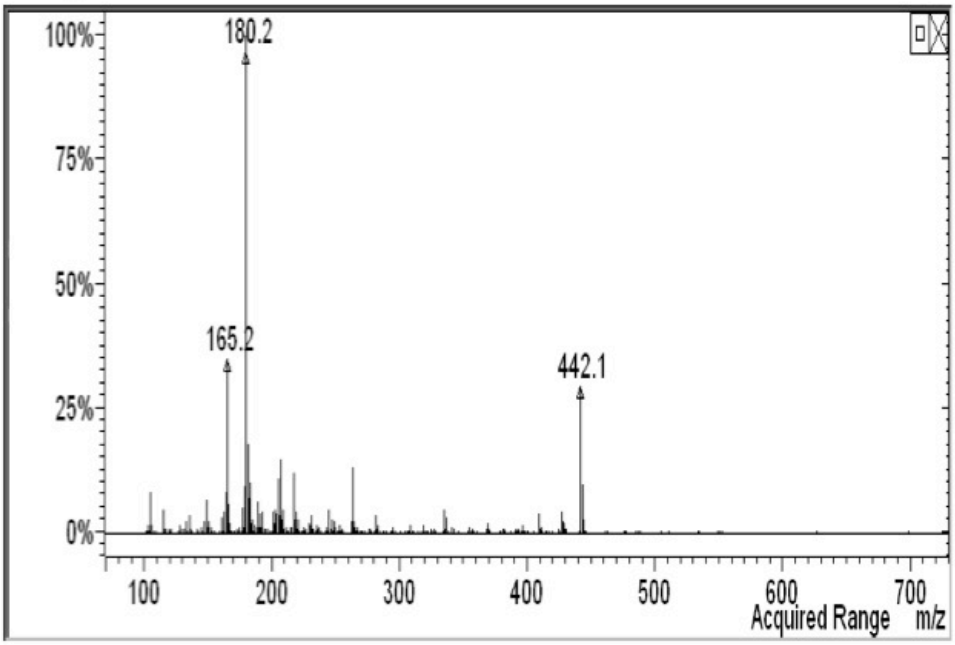


Appendix 2. Mass spectrometric images of pure (A) END (33 µM) and (B) $^{2}H_{6}$ – END (17 µM).

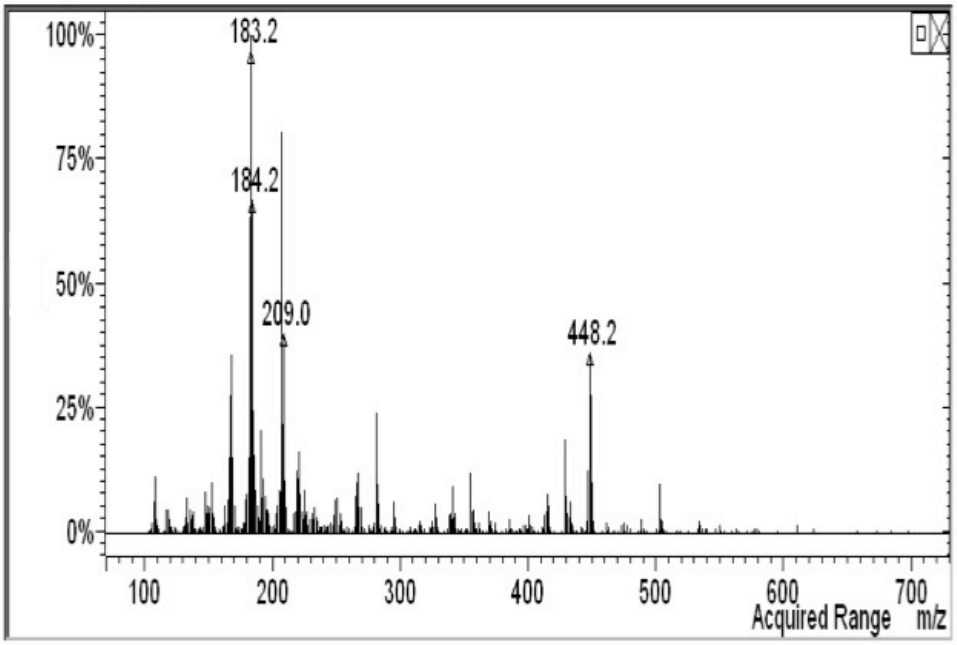


Appendix 3. Mass spectrometric images of pure (A) ENL (33 µM) and (B) $^{2}H_{6}$ – ENL (17 µM).

A) ENL



B) $^{2}H_{6}$ - ENL



Appendix 4. Variations in the amount of sodium acetate buffer (0.1 M) used in the initial SLE method development experiments.

Sodium Acetate Buffer	END		ENL	
(µL)	r^2	RSD	r^2	RSD
240	0.988	11.01	0.993	12.43
300	0.998	11.94	0.991	13.54

Abbreviations: END, enterodiol; ENL, enterolactone, RSD, relative standard deviation.

Appendix 5. Self reported incidences of adverse events with regards to consuming various dosages of ground flaxseed muffins.

Subject Reporting	10 g FX Group	20 g FX Group	30 g FX Group	40 g FX Group
Symptom Severity (n; %)				
None-0	5 (56)	5 (56)	8 (89)	4 (57)
Mild-1	2 (22)	3 (33)	0	2 (29)
Moderate-2	2 (22)	1 (11)	1 (11)	1 (14)
Severe-3	0	0	0	0
Death-4	0	0	0	0
Adverse Events				
Bloating/ Flatulence (n; %)	2 (22)	3 (33)	1 (11)	3 (43)
Gastrointestinal Discomfort (n; %)	1 (11)	1 (11)	0	0
Stomach Grumbling (n; %)	1 (11)	0	0	0
Headaches (n; %)	0	0	0	1 (1)
Gender Reported Incidences	3F/1M	2F/2M	0F/1M	2F/1M

Abbreviations: FX, flaxseed; n, number.

Appendix 6: Body weights and BMIs of participants consuming various doses of milled flaxseed daily for 4 weeks.

Body Morphometrics	Milled Flaxseed Groups				Effects (*F*-Value) (df = 3)		
	10 g/d FX	20 g/d FX	30 g/d FX	40 g/d FX	G	T	G×T
Body Weight, *kg*					0.75	1.81	1.27
0 week	70.1 ± 2.5	77.9 ± 6.4	70.1 ± 4.3	70.5 ± 4.9			
4 week	69.7 ± 2.5	78.2 ± 6.4	70.1 ± 4.3	69.6 ± 4.9			
BMI, kg/m^2					1.03	2.14	1.36
0 week	24.9 ± 0.8	26.5 ± 1.8	24.2 ± 1.1	23.6 ± 1.1			
4 week	24.7 ± 0.8	26.6 ± 1.8	24.2 ± 1.2	23.3 ± 1.1			

BMI body mass index*; df* degrees of freedom; *FX* flaxseed; *G* group; *G × T* group by time; *T* time.

Appendix 7. Enterolignan concentrations of combined age groups that were consuming milled flaxseed represented by gender in the age dependency of milled flaxseed study.

Enterolignan	Week	Enterolignan [C] in males (nM)	Enterolignan [C] in females (nM)	*P*
END	0	0.0 ± 0.0	0.0 ± 0.0	NS
	4	248.5 ± 93.8	222.8 ± 102.4	0.86
ENL	0	19.5 ± 8.9	12.3 ± 5.4	0.47
	4	210.7 ± 80.6	341.2 ± 85.3	0.30
END + ENL	0	19.5 ± 8.9	12.3 ± 5.4	0.47
	4	459.1 ± 117.4	563.9 ± 107.0	0.53

Values are represented as the mean ± SEM.
The number of females was 10 and males was 7.
Abbreviations: [C], concentration; END, enterodiol; ENL, enterolactone; NS, not significant.

Appendix 8. Nutrient composition and energy of the foods used in the flaxseed and placebo intervention groups (g/daily serving).[1]

Food Products	Moisture (g)	Ash (g)	Protein (g)	Fat (g)	CHO (g)	Fibre (g)	Energy (kcal)	Energy (kJ)	Weight (g)
Muffins									
Orange Cranberry-PL	63.5	2.5	7.7	9.8	66.1	2.3	383	1600	137
Orange Cranberry-FX	75.3	4.0	11.6	15.3	67.0	10.5	441	1850	153
Apple Spice-PL	75.9	2.8	7.8	9.7	60.4	3.0	360	1500	136
Apple Spice-FX	90.5	4.4	11.8	15.2	60.4	11.4	415	1740	149
Bagels									
Plain-PL	51.5	1.75	10.4	3.4	66.6	2.4	344	1440	98.0
Plain-FX	51.4	2.7	14.4	15	51.9	10.3	392	1640	98.8
Sunflower-PL	44.4	2.2	14.7	15.8	59.4	4.3	433	1810	108
Sunflower-FX	46.8	3.2	17.0	23.1	48.3	11.6	453	1900	102
Cinnamon Raison-PL	48.3	1.9	10.7	3.3	69.7	3.7	350	1460	112
Cinnamon Raison-FX	50.4	2.6	14.3	14.8	53.5	11.7	390	1630	102
Snackbars									
Orange Cranberry-PL	11.9	1.5	6.8	10.1	58.2	7.4	322	1350	91.2
Orange Cranberry-FX	12.6	1.4	7.4	15.2	53.3	9.9	359	1500	91.6
Cappuccino CC-PL	19.0	1.5	6.4	11.8	53.1	6.8	311	1300	97.0
Cappuccino CC-FX	18.5	1.5	7.2	16.9	49.1	9.5	353	1480	94.0
Gingerbread Raisin-PL	19.8	1.6	6.4	9.9	53.3	7.1	296	1240	92.3
Gingerbread Raisin-FX	19.2	1.6	7.1	15	49.3	9.8	338	1410	92.6
Other									
Biscuit-PL	51.5	3.1	11.1	17.7	68.4	2.3	480	2000	152
Biscuit-FX	52.5	4	13.4	22.5	58.7	9.2	484	2020	150
Roll-PL	59.6	2.7	12.3	8.9	58.2	5.6	363	1520	142
Roll-FX	61.5	3.8	17.1	12.9	49.6	13.2	373	1560	145

Pasta-PL[2]	NA	NA	8	0.5	50	2	240	NA	70
Pasta-FX[2]	NA	NA	11	11	38	10	280	NA	70
Milled Sprinkle-PL[2]	NA	NA	5	9	13	7	130	NA	30
Milled Sprinkle-FX	2.4	1.1	6	12.6	8.8	8.6	163	681	30

[1]Proximate analyses were calculated using the Canadian Nutrient File from Health Canada.
[2]Proximate analyses were obtained from nutrition labels. CC, chocolate chip; CHO, carbohydrate; FX, flaxseed group; NA, not available; PL, placebo group.

Appendix 9. Body weight, waist circumference and BMI measurements of patients in the Flax-PAD study at baseline, 6 and 12 month time points for flaxseed and placebo treatment groups.

Body morphometrics		Baseline	6 Months	12 Months	Effects (*P*-Value)		
					G	T	G×T
Body Weight, *kg*					0.53	0.35	0.79
	FX	81.0 ± 14.9	83.8 ± 14.8	83.6 ± 15.1			
	PL	82.4 ± 14.8	81.4 ± 14.5	80.8 ± 15.7			
Waist Circumference, *cm*					0.57	0.26	0.43
	FX	102 ± 11.7	104 ± 12.4	103 ± 12.5			
	PL	104 ± 13.5	101 ± 13.2	102 ± 13.7			
BMI, kg/m^2					0.45	0.07	0.42
	FX	27.4 ± 4.4	28.1 ± 4.2	29.2 ± 8.5			
	PL	28.2 ± 4.4	28.2 ± 5.0	27.9 ± 5.5			

Values are means ± SD. All differences between and within groups were not statistically significant, $P \geq 0.05$. Flaxseed: baseline n = 58; 6 months n = 45; 12 months n = 43 and Placebo: baseline n = 52; 6 months n = 41; 12 months n = 41.
Abbreviations: BMI, body mass index; FX, flaxseed group; G, group; G×T, group × time; PL, placebo group; T, time.

Appendix 10. Absolute changes in plasma LDL-C in PAD patients after 1, 6 and 12 months in the FX+CLM, PL+CLM, FX Only and PL Only subgroups.[1]

Cholesterol	Absolute Change in LDL-C over Time			Effects (*P*-Value)[2]		
	After 1 month	After 6 months	After 12 months	G	T	G×T
LDL-C, *mmol/L*				0.02	0.42	0.96
FX + CLM	-0.1 ± 0.1	-0.2 ± 0.1	-0.1 ± 0.1			
PL + CLM	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.1			
				0.38	0.02	0.15
FX Only	-0.1 ± 0.1[a]	-0.9 ± 0.3[b]	-0.4 ± 0.4[ab]			
PL Only	0.0 ± 0.2	-0.3 ± 0.2	-0.4 ± 0.2			

[1] Values are represented as means ± SEM. [a,b] Mean values within the same group with a different letter are significantly different from each other, $P \leq 0.017$.
[2] Significant if $P < 0.05$. Flaxseed Groups: FX+CLM after 1 month n=41; after 6 months n=37; after 12 months n=36 and FX Only after 1 month n=11; after 6 months n=7; after 12 months n=6. Placebo Groups: PL+CLM after 1 month n=32; after 6 months n=26; after 12 months n=26 and PL Only after 1 month n=15; after 6 months n=13; after 12 months n=15. CLM, cholesterol-lowering medication; FX, flaxseed; G, group; G×T, group × time; PL, placebo; T, time.

Appendix 11. Plasma lipid concentrations from hypercholesterolemic (≥5.3 mmol/L) patients in the Flax-PAD study compared over time and by flaxseed and placebo treatment group.

Lipids	Baseline	1 Month	6 Months	12 Months	Effects (P-Value)		
					G	T	G×T
TG, *mmol/L*					0.41	0.93	0.13
FX (n=10)	1.8 ± 0.7	2.0 ± 0.6	2.0 ± 0.6	2.2 ± 0.9			
PL (n=9-11)	2.4 ± 1.1	2.2 ± 0.8	2.5 ± 1.4	2.0 ± 0.8			
TC, *mmol/L*					0.10	0.0535	0.35
FX (n=10)	6.1 ± 0.7	5.7 ± 0.7	5.2 ± 1.1	5.6 ± 1.6			
PL (n=9-11)	6.5 ± 1.0	6.5 ± 0.8	6.3 ± 1.3	6.1 ± 1.3			
LDL-C, *mmol/L*					0.67	0.0100	0.15
FX (n=10)	3.9 ± 0.8	3.5 ± 0.8	3.3 ± 1.0	3.8 ± 0.8			
PL (n=9-11)	3.9 ± 0.8	4.0 ± 0.8	3.6 ± 0.8	3.5 ± 0.9			
HDL-C, *mmol/L*					0.39	0.21	0.22
FX (n=10)	1.4 ± 0.5	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.2			
PL (n=9-11)	1.3 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.3			
TC/HDL-C, *ratio*					0.61	0.98	0.32
FX (n=10)	4.9 ± 1.6	4.9 ± 1.3	5.2 ± 1.6	5.2 ± 1.8			
PL (n=9-11)	5.3 ± 1.7	5.3 ± 1.7	5.0 ± 1.4	4.8 ± 1.5			
LDL-C/HDL-C, *ratio*					0.54	0.92	0.10
FX (n=10)	3.2 ± 1.3	3.1 ± 1.1	3.3 ± 1.2	3.6 ± 1.1			
PL (n=9-11)	3.2 ± 1.1	3.1 ± 1.0	2.9 ± 0.9	2.8 ± 0.9			

All values are means ± SD. All differences between and within groups were not statistically significant, $P \geq 0.05$.
Abbreviations: C, cholesterol; FX, flaxseed group; G, group; G×T, group × time; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PL, placebo group; T, time; TG, triglycerides.

Appendix 12. Absolute change of ALA, END, ENL and END+ENL measured in plasma of patients with PAD after 1-, 6- and 12 months of consuming either a flaxseed or placebo diet.[1]

Flaxseed Component	Absolute Change From Baseline Measured in Plasma			Effects (*P*-Value)		
	After 1 Month	After 6 Months	After 12 Months	G	T	G×T
ALA, *μM*				<0.001	<0.001	0.29
FX (n=43)	42.7 ± 8.1^{***ab}	49.1 ± 9.5^{***a}	23.8 ± 7.7^{***b}			
PL (n=41)	-1.7 ± 3.9^{ab}	0.4 ± 5.8^{a}	-11.4 ± 6.0^{b}			
END, *nM*				<0.001	0.84	0.30
FX (n=42)	148 ± 34.2***	171 ± 46.6***	133 ± 31.7***			
PL (n=38)	9.6 ± 4.5^{ab}	-0.2 ± 1.2^{b}	18.5 ± 5.3^{a}			
ENL, *nM*				<0.001	0.23	0.19
FX (n=44)	319 ± 42.8***	274 ± 39.6***	226 ± 45.2***			
PL (n=38)	5.3 ± 7.8	-1.2 ± 7.1	7.6 ± 9.1			
END+ENL, *nM*				<0.001	0.50	0.22
FX (n=42)	457 ± 65.5***	449 ± 71.9***	375 ± 63.9***			
PL (n=38)	14.9 ± 9.4^{a}	-1.5 ± 7.6^{b}	26.2 ± 10.1^{a}			

[1] Values are represented as means (SEM). Different from placebo, $^{***}P<0.001$. ^{a,b} Mean values within the same group with a different letter are significantly different from each other, $P<0.05$. ALA, alpha-linolenic acid; END, enterodiol; END+ENL, total enterolignans; ENL, enterolactone; FX, flaxseed group; G, group; G×T, group × time; PL, placebo group; T, time.

Appendix 13. Absolute changes from baseline of plasma ALA and enterolignans correlated with absolute changes from baseline of plasma TC, LDL-C and TG levels in all patients that completed the Flax-PAD study.

Flaxseed Bioactive	Lipids	Group	Absolute Change after 1 month		Absolute Change after 6 months		Absolute Change after 12 months	
			ρ	*P*	ρ	*P*	ρ	*P*
ALA	TC	FX	0.30	0.04	0.18	0.26	0.19	0.23
		PL	0.30	0.04	0.58	<0.001	0.20	0.22
	LDL-C	FX	0.17	0.24	0.21	0.17	0.20	0.20
		PL	0.22	0.13	0.35	0.03	0.04	0.79
	HDL-C	FX	0.30	0.04	-0.21	0.17	-0.10	0.51
		PL	0.00	0.99	0.14	0.38	-0.10	0.54
	TG	FX	0.38	<0.01	0.38	0.01	0.31	0.04
		PL	0.30	0.04	0.63	<0.001	0.54	<0.01
END	TC	FX	0.10	0.48	0.06	0.69	-0.10	0.53
		PL	0.10	0.52	0.14	0.39	0.13	0.42
	LDL-C	FX	0.19	0.18	0.27	0.08	0.10	0.53
		PL	0.10	0.52	0.12	0.49	0.16	0.34
	HDL-C	FX	0.17	0.23	-0.72	0.65	-0.23	0.14
		PL	0.13	0.39	-0.07	0.68	-0.04	0.83
	TG	FX	-0.24	0.09	-0.13	0.41	-0.11	-0.51
		PL	-0.18	0.22	0.06	0.74	-0.04	0.82
ENL	TC	FX	0.09	0.52	-0.04	0.78	-0.18	0.27
		PL	-0.01	0.93	0.29	0.08	-0.12	0.47
	LDL-C	FX	0.13	0.37	0.10	0.52	0.09	0.56
		PL	0.07	0.65	0.08	0.65	-0.20	0.23
	HDL-C	FX	0.28	0.05	-0.21	0.18	-0.18	0.25
		PL	-0.31	0.03	0.02	0.91	0.04	0.79
	TG	FX	-0.18	0.21	0.09	0.56	-0.15	0.33
		PL	-0.01	0.98	0.35	0.03	0.10	0.55

Rho (ρ) values represent the Spearman correlation coefficient.
Abbreviations: ALA, alpha-linolenic acid; C, cholesterol; END, enterodiol; ENL, enterolactone; C, cholesterol; FX, flaxseed group; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; PL, placebo group; TG, triglycerides.