The Effects of Ezetimibe on Omega-3 Fatty Acid Absorption in the Prevention of Cardiovascular Disease

By

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Abstract

Introduction: Elevated levels of circulating omega-3 polyunsaturated fatty acids like alpha-linolenic acid (ALA) may be beneficial for cardiovascular health. Circulating ALA concentrations are elevated dramatically by a cholesterol supplemented diet which increases ALA bioavailability through enhanced micelle formation in the intestines. Conversely, it is possible that drugs which inhibit intestinal cholesterol metabolism may also inhibit fatty acid absorption.

Objective: The purpose of this study is to determine if a cholesterol absorption inhibitor, ezetimibe, will decrease circulating levels of ALA in humans receiving statin therapy for hypercholesterolemia.

Design: Patients between 44-80 years old, requiring statin therapy to regulate blood cholesterol levels, were randomly assigned to one of four groups for a 6 week trial: 1) placebo; 2) ezetimibe therapy (10mg); 3) a supplement of flaxseed oil (containing 1.0g ALA); or 4) ezetimibe and flaxseed oil supplementation. All subjects were maintained on statin therapy.

Results: Ingestion of flaxseed oil resulted in a significant increase in circulating ALA levels in patients who were not given ezetimibe. However, in the presence of ezetimibe, circulating ALA levels did not increase significantly in the presence of flax oil supplementation. There were no significant differences amongst the groups in terms of circulating total cholesterol, LDL, HDL, triglyceride levels, or in the expression of hs-CRP, lipoprotein A, fibrinogen and fetuin-A in the blood.

Conclusion: Ezetimibe therapy inhibits the absorption of omega-3 fatty acids. Patients receiving ezetimibe therapy will not receive the expected cardiovascular benefits from dietary supplementation with omega-3 fatty acids.

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Introduction

Cardiovascular disease (CVD) remains a leading cause of mortality worldwide. It is now clear that a myriad of modifiable risk factors cause the majority of serious and chronic cardiovascular diseases. Risk factors are modified via changes in lifestyle and with pharmacotherapy. Over the past several years, advancements in medicine have lead to the enhancement of medical therapy for CVD and the exploration into new avenues for prevention and treatment. Currently, scientific investigations have expanded to foods and nutrients that provide the body with health benefits beyond normal nutrition. Many of these "functional foods" or "nutraceuticals" have demonstrated direct benefit for cardiovascular health– both for primary and secondary prevention of CVD. Flax seed is an example of a functional food which has demonstrated significant cardioprotective effects such as lowering risk of sudden cardiac death, or recurrent myocardial infarction. It has also demonstrated anti-arrhythmic, hypotensive, anti-oxidant and lipid lowering properties. Many of these beneficial effects have been attributed to the large amount of alpha-linolenic acid (ALA) found in flax seed.

One of the most prevalent causes of CVD in North America is hyperlipidemia, or high cholesterol levels within the body. While 3-hydroxy-3-methylgluterate-CoA reductase inhibitors (statin drugs) are well defined as the primary treatment for hyperlipidemia, additional pharmaceuticals have emerged as alternatives or add-on therapies for lipid control. One such drug is ezetimibe, which is an inhibitor of the absorption of dietary cholesterol. It acts by selectively inhibiting the Niemann Pick

C1-Like 1 (NPC1L1) transporter in the intestinal brush border which is responsible for sterol transport from the gut into circulation. A number of off-target pleiotropic effects are elicited by statins. As a result, statins demonstrate cardioprotective effects beyond lipid lowering. In light of this, several clinical trials have demonstrated the abilities of statin drugs to positively affect primary clinical cardiovascular outcomes such as myocardial infarction or death. Ezetimibe, however, has failed to elicit beneficial cardiovascular effects beyond its lipid lowering ability. As such, the overall role of ezetimibe for the treatment of cardiovascular disease remains questionable. Furthermore, while ezetimibe blocks cholesterol uptake from the gut, it may also interfere with the absorption of other contents from within the small intestine. Animal studies indicate that an increase in gut cholesterol will lead to an exponential increase in the absorption of fatty acids such as ALA. The mechanism is thought to be due to an overall increase in micelle formation and affinity of contents of the gut for the brush border of the intestine where absorption occurs. Thus, it is possible that a substance, such as ezetimibe, which decreases the absorption of dietary cholesterol may also indirectly decrease the absorption of cardioprotective omega-3 fatty acids, such as ALA, derived from functional foods like flax seed. We hypothesize that ezetimibe will inhibit the absorption of ALA when administered concomitantly.

Review of Literature

1. Cardiovascular Disease and the Development of Atherosclerosis

1.1. Incidence of Cardiovascular disease and Innovative Methods of Detection

Despite a myriad of advancements in biomedical research and intensive exploration of disease processes, cardiovascular disease (CVD) remains a leading cause of mortality in developed nations worldwide (1). In general terms, cardiovascular disease may be defined as any type of sustained injury to or deformation of the heart or vasculature. Currently, a large number of risk factors of cardiovascular disease are well described. Risk factors for CVD may be categorized as either non-modifiable or modifiable. Non-modifiable risk factors include age, gender and heredity. Modifiable risk factors are numerous. Currently, the most important modifiable CVD risk factors are obesity, dyslipidemia, hypertension, diabetes, level of exercise and diet (2-4).

Recent advancements in medical biotechnology have opened the door to a series of innovative tests and procedures for the detection and treatment of cardiovascular disease. Contemporary findings continue to isolate new targets, such as circulating biomarkers, for the detection and treatment of disease. Generally, a biomarker is a molecule, for example a protein or cytokine, which is strongly correlated (either positively or negatively) to a disease process in a concentration dependent manner. Often, it is a molecule which fluctuates in concentration within plasma. These changes in concentration directly correlate with the presence or level of disease.

Several current biomarkers are commercially available and are based on immunoassay detection platforms. Biomarker detection is regularly utilized both for experimental and clinical purposes.

A biomarker which has been utilized clinically for some time is troponin– and more specifically, cardiac troponin I– which is only found in cardiac muscle. An increase in serum cardiac troponin I levels has been associated with the presence of myocardial infarction and increased mortality risk (5, 6). Post myocardial infarction, troponin levels will become markedly elevated in the blood 2-4 hours following the acute coronary event and will remain elevated for up to seven days post-event (7, 8). Troponin is an objective tool used clinically to determine the presence and severity of an infarct via a simple and reliable immunoassay (7, 8). Biomarkers may also be used as a prospective screening tool and the use of biomarkers for disease risk assessment is a new and presently evolving practice. Commercially obtainable immunoassay screening kits are available which allow a multitude of parameters to be assessed using only a small sample of blood.

The present study utilizes immunoassay methods to analyze the prevalence and severity of disease in the population of study. The biomarkers of interest included high sensitivity C-Reactive Protein (hs-CRP), lipoprotein A (LpA), fetuin-A and fibrinogen. All of these markers have been closely associated with systemic CVD. High sensitivity C-Reactive Protein (hs-CRP), the marker of greatest interest in this clinical study, has been independently identified as a marker of cardiovascular risk. CRP is a downstream product of the inflammatory cytokine interleukin-6 and plays a prominent role in immune initiated inflammatory responses. An elevation of CRP

in circulation initiates the complement system which clears degraded LDL and apoptotic cells from within an atherosclerotic plaque or circulating within the vasculature (9). As such, CRP is considered a robust and accurate biomarker of systemic inflammation. As evidenced by the inherent mechanism of CRP action, it may become acutely elevated in response to infection. In terms of clinical biomarker detection, this may lead to an inaccurate value being reported at a certain time point. Chronic elevation of CRP, however, is independently associated with a significant increase in cardiovascular risk (10, 11). Furthermore, an acute rise in CRP following acute coronary syndrome has been identified as a predictor of mortality in combination with and independently of cardiac troponin (12). In light of this, the JUPITER study (13) identified the ability of Rosuvostatin to significantly lower circulating CRP levels and reduce cardiovascular risk in a population with elevated CRP and a normal lipid profile (13). The PRINCE trial demonstrated similar effects with pravastatin (14).

LpA is intrinsically involved with the function of LDL, while fetuin-A is a marker of systemic calcification in the vasculature and in the overall body. Fibrinogen is a molecule integral for blood coagulation. Elevated circulating levels of fibrinogen are considered to be pro-thrombotic (15). An elevation in the levels of any of these circulating biomarkers has been strongly correlated with an increase in cardiovascular risk (16, 17).

One of the leading causes of both chronic cardiovascular disease and acute cardiac events, is atherosclerosis (18). Atherosclerosis is a chronic inflammatory process which is best defined as a progressive narrowing of the arteries. Narrowing

of larger vessels is caused by fatty deposits and fibrous plaque formation along the vessel wall. Eventually, these fibrous plaques may occlude the vessel or negatively alter the dynamics of blood flow within the vasculature. This may lead to the formation of a thromboembolism, or blood clot, and will prevent the delivery of oxygen and nutrients to tissues. Eventually, the atherosclerotic plaque may also rupture and embolize causing a blockage in smaller downstream vessels in the heart, brain or lungs.

1.2. Response to Injury Hypothesis of Atherosclerosis

Resistance arteries are composed of a series of layers, mainly comprised of muscle and connective tissue, which provide structure– elasticity and tone– as well as a single cell endothelial layer which forms a continuous coating along the vessel lumen in healthy individuals. The continuous endothelial layer is important as it contributes significantly to laminar blood flow. The endothelial lining also has paracrine abilities to regulate local vascular tone in response to sheer stress or via interactions with circulating vasoconstrictive or vasodilatory factors (19-21).

The onset and progression of atherosclerosis is often explained by the response to injury hypothesis. As described by Ross *et al*. (22, 23), the response to injury hypothesis defines that an initial insult to the endothelium of the vessel wall causes a disruption of the continuous lining of the blood vessel. This injury will eventually lead to a cascade of deleterious events (24). This initial injury to the vessel may be caused by some kind of trauma or genetic vessel malformation but often arises from modifiable cardiovascular risk factors such as smoking or hypertension. Systemic hypertension is characterized by a chronic and uncontrolled elevation of mean arterial blood pressure. Hypertension has long been understood to play an important role in diseases of the heart and vasculature (25). Hypertension causes an increased load on the heart and can alter the velocity of blood flow within vessels. This increase in rate and/or force of myocardial contraction and subsequent disturbance of the velocity of flow can cause blood dynamics to change from laminar to turbulent flow within the vasculature over time. Turbulent blood flow will be exacerbated by changes in fluid flow dynamics such as at segments of arterial

branching within the vasculature and by an increase in blood viscosity caused by sources such as hyperlipidemia. Turbulent flow can also lead to an increase in sheer stress (ie. outward force) on the vessel walls and, eventually, will cause cellular injury to the endothelial lining of the vessel lumen. Over time, the site of injury will become aggravated and will serve as an area for attraction and infiltration of particles circulating in the blood.

Low density lipoprotein (LDL) is an example of a cholesterol carrying molecule found in the blood. LDL is sticky in nature and will adhere to and infiltrate damaged areas of epithelium within the vasculature. LDL infiltration is often accompanied by immune molecules such as macrophages via an LDL driven stimulation of monocyte chemotaxis. This lipid raft infiltrate, which is composed largely of LDL particles and macrophages, will transcytose and migrate into the sub-endothelial layers of the vessel (26). Following transcytosis into the sub-endothelial space, LDL will become oxidized and trigger an inflammatory cascade leading to the migration of smooth muscle cells and macrophages into this sub-endothelial vessel compartment. At this stage, a necrotic core composed of macrophage-based foam cells will form. Finally, a fibrous cap will form over the injured segment of the vessel lining. An atherosclerotic plaque will progressively expand into the vessel lumen, driven by continuous migration of smooth muscle cells and fibrotic factors towards the luminal side of the blood vessel. Over time, blood flow through the vessel will become increasingly disturbed. At this advanced atherosclerotic stage, several negative events may occur: 1) the ebb of turbulent blood flow that is held behind the plaque may become thrombotic, and a blood clot may occlude the vessel or

embolize, occluding a smaller vessel downstream; 2) the vessel may become completely occluded by the atherosclerotic plaque, preventing blood flow; or 3) the fibrous cap of the atherosclerotic plaque may become vulnerable and rupture. This may cause deleterious blockages in downstream vascular beds, such as in the heart, brain or lungs.

Figure 1. Cross-sectional image of a normal human arterial vessel.

The *Tunica Externa* is composed of connective tissue and provides the vessel with structure. The *Tunica Media* is largely composed of smooth muscle cells and provides the vessel with tone. The *Tunica Intima* is composed of a continuous, single cell layer of endothelial cells which provide a smooth luminal surface, elicit paracrine effects on the vasculature and interact with circulating vasoconstrictive and vasodilatory factors.

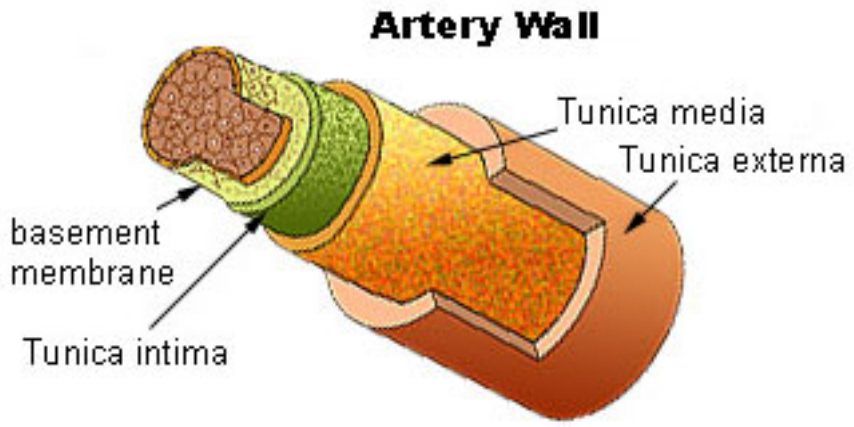

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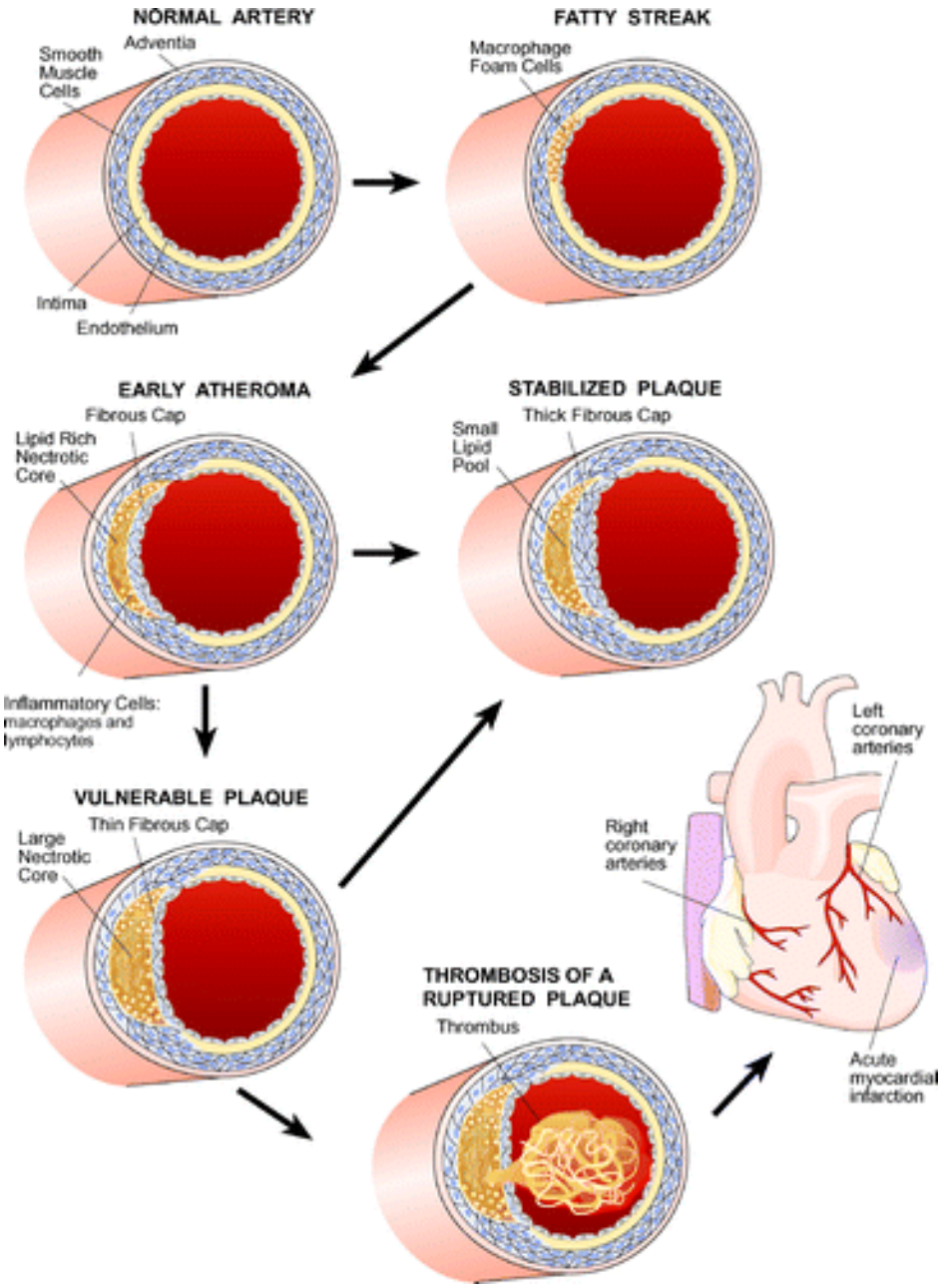
Figure 2. Progression of atherosclerosis within a human coronary vessel.



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1.3. Effects of Lipids and Hypercholesterolemia on CVD

In light of the above statements, LDL cholesterol clearly plays an important role in the development of atherosclerosis. LDL particles are the most abundant form of cholesterol found in circulation and may contribute to increased blood viscosity, cellular infiltration and initiation of inflammatory responses at sites of endothelial injury. Nonetheless, cholesterol is an integral molecule in the human body and not all cholesterol is carried in the same form. In fact, cholesterol exists in several forms ranging from the largest molecules called chylomicrons to the smallest, high density lipoprotein (HDL) molecules. Chylomicrons are mainly responsible for transporting absorbed lipid molecules from gut enterocytes to the liver for processing and transport throughout the body.

While larger, less dense cholesterol particles are implicated in the negative processes associated with CVD, HDL is essential for cellular membrane formation and fluidity and is also required for the synthesis of steroid hormones. Furthermore, HDL demonstrates a number of anti-atherogenic properties (27). Most significantly, while LDL contributes to the deposition of lipids at injured sites within the vasculature, HDL actually helps to prevent LDL initiated monocyte chemotaxis (28, 29) as well as aids in the extrusion of lipids from within developing atherosclerotic plaques, or atheroma, via reverse cholesterol transport. This process counteracts the formation of foam cells which, otherwise, eventually lead to the development of a necrotic atherosclerotic core. HDL in conjunction with apolipoprotein-A1 (Apo-A1) can also elicit a reverse cholesterol transport effect on macrophages within atherosclerotic deposits. These molecules act via ABCA1 transporters within the

phospholipid membrane to facilitate the extrusion of cholesterol from lipid rafts within the sub-endothelial vessel space in a “reverse transport” manner (30, 31). Recent findings, however, demonstrate HDL most aptly exudes these anti-inflammatory properties only under normal conditions. Under chronic inflammatory conditions, however, HDL appears to elicit a pro-inflammatory state stimulating LDL and monocyte vessel infiltration and increasing inflammatory immune responses (32, 33). Thus, HDL is an integral molecule that may play an influential role in the future treatment of atherosclerosis. Current research attempts to further identify the relationship between Apo-A1 and HDL, as well as to develop a novel therapy to harness the anti-atherosclerotic actions of HDL.

The fact remains however, that the bulk of circulating cholesterol in humans exists in the form of LDL. As a compelling amount of research argues increasingly for the negative role LDL plays in the development and progression of cardiovascular disease, it remains a pivotal target for lipid management therapy.

2. Pharmaceutical Therapeutics for the Treatment of Hypercholesterolemia

It is important to maintain normal circulating cholesterol levels in the body to sustain good health. However, a definitive consensus on the negative impact hypercholesterolemia exists, and it is well known that particularly high plasma concentrations of low density lipoprotein (LDL), has deleterious effects on overall health and long-term cardiovascular risk (34, 35). Clinically, hypercholesterolemia (high cholesterol in the blood) is typically categorized by an excess of either total cholesterol or LDL. Current Canadian lipid guidelines state that total circulating cholesterol concentrations of greater than 5.0mM is considered to be unacceptably high, and carries with it a significant increase for the risk of developing cardiovascular disease (CVD). Canadian guidelines further denote that a plasma concentration of circulating LDL of less than 2.0mM or a reduction of 50% should be targeted to avoid increased cardiovascular risk. For patients with hypercholesterolemia above the stated ranges, treatment is advised to achieve a reduction in plasma concentrations below these levels – specifically LDL (3, 36).

2.1. *Statin Pharmacotherapy*

Currently, 3-hydroxy-3-methylgluterate-CoA (HMG-CoA) reductase inhibitors remain the gold standard for primary treatment of hypercholesterolemia. The power of HMG-CoA reductase inhibition for lipid lowering in humans was first reported in the 1970's by the Endo group in Japan (37). Following this research, a number of pharmaceuticals (commonly categorized as statin drugs) evolved. The mechanism of action of statin drugs occurs via inhibition of the HMG-CoA reductase enzyme. This inhibitory action prevents the conversion of HMG-CoA to mevalonate within the endogenous cholesterol synthesis pathway in hepatic tissue. This causes a down regulation of the body's production of cholesterol, thus, leading to an overall decrease of circulating cholesterol within the body and increased reuptake of circulating LDL by the liver. Presently, statins remain the most effective pharmaceutical treatment for lowering circulating cholesterol levels. Statin therapy can lower total circulating cholesterol by up to 25% and may lower LDL cholesterol levels by up to 50% (38-40).

2.1.1. Statin Pharmacotherapy for the Prevention of CVD

Statins are also strongly indicated for the prevention of secondary CVD. The 4S Trial (Scandinavian Simvastatin Survival Study) demonstrated simvastatin's ability to lower LDL cholesterol levels by 35% over five years in patients with angina pectoris or a previous myocardial infarction and serum cholesterol levels above 5.0mM. The 4S study further demonstrated a significant reduction in long term cardiovascular risk, demonstrating a 37% decrease in myocardial revascularization procedures within the simvastatin treated group (41). Recent findings suggest that the ability of statins to elicit significant primary and secondary treatment effects may be attributed to a number of "off-target" pleiotropic effects.

Currently, there is little research to suggest that LDL lowering alone has significant effects on clinical cardiovascular endpoints such as prevention of myocardial infarction, revascularization procedures or cardiac death. Data suggests that the myriad of pleiotropic effects statins induce beyond cholesterol lowering may greatly contribute to the ability of statins to positively affect clinical cardiovascular endpoints. For example, a major side-effect of statins is their anti-inflammatory property. The JUPITER (2009) study, a large prospective clinical trial demonstrated resuvostatin's ability to prevent CVD in a healthy population with normal lipid profiles but elevated C-reactive protein (CRP) levels. Study participants who achieved a target lowering of hs-CRP to less than 2.0mg/L had a 62% reduction in negative cardiovascular events. The degree of hs-CRP lowering was directly correlated with the amount of clinical benefit and had little correlation with changes in LDL (13). The effect of statin therapy on hs-CRP lowering is also delineated in

other clinical studies (42, 43). Finally, statins also exhibit anti-atherogenic properties. These effects may be classified as positively influencing endothelial function (44, 45), aiding in atherosclerotic plaque stabilization (46, 47) and preventing thrombus formation(48).

2.1.2. Adverse Effects and Drug Interactions of Statins

Statins are relatively well tolerated by the majority of patients. Minor side effects associated with statin therapy are reported at a prevalence of approximately 5% and severe adverse drug reactions are reported at rate of <1% (49). Minor side effects experienced in people prescribed HMG-CoA inhibitors may include headache, abdominal pain, nausea, constipation and respiratory infection. Since statins are metabolized within the liver, however, some serious adverse effects may also occur in a smaller amount of the population. These negative effects include elevation of liver enzymes (specifically hepatic transaminase) and myopathy or rhabdomyolysis. Adverse drug reactions may arise from statin interactions with certain steroids, antibiotics, CYP450 inhibitors such as diltiazem or the ingestion of natural products such as high concentrations of grapefruit juice (49). These effects should be closely monitored and patients who experience these serious adverse effects should be removed from medication immediately.

The toxic effects stemming from statin-grapefruit interactions are well noted within the literature, and are an example of a negative food/drug interaction. The ingestion of grapefruit causes an increase in the bioavailability and subsequent accumulation of statins in the liver to concentrations which may result in elevated liver enzymes and severe damage to liver tissue (50, 51). Grapefruit inhibits cytochrome P-450 (CYP450), specifically the isoform CYP3A4 (52), within the enterocytes of the small intestine. As a result, pre-systemic metabolism of orally ingested drugs that would normally occur during absorption via CYP3A4 enzymes becomes bypassed. This leads to an increased bioavailability and consequent

metabolism within the body following absorption (53). Thus, this cumulative bioavailability will cause liver dysfunction.

2.2. Ezetimibe Pharmacotherapy

For patients who cannot tolerate statin therapy, or those who require additional or differential lipid management, three other drug classes presently exist. These include fibrates, niacin and dietary absorption inhibitors such as ezetimibe. While bile acid sequestrants were once utilized for lipid lowering, they have largely been replaced by the latter pharmaceuticals due to a low rate of compliance (54). Fibrates are a class of drugs typically utilized for the treatment of hypertriglyceridemia (55) while extended release niacin (vitamin B_{12}) has largely been indicated for use in raising HDL levels (56).

Ezetimibe is a relatively new pharmaceutical which has the ability to selectively lower circulating LDL cholesterol by up to 20%. Currently, it is prescribed concomitantly with statin drugs to enhance the LDL cholesterol lowering effect (57, 58). Ezetimibe is produced and marketed in Canada by Merck Canada Inc. as Ezetrol®, and is available in 10mg or 40mg doses (59). Ezetimibe monotherapy may serve a function in cholesterol lowering in patients who are intolerant to statins or have mild hepatic insufficiency. At present time, however, ezetimibe is not approved in Canada for monotherapy. Ezetimibe is also available as a combination pill containing ezetimibe and simvastatin which is marketed by Merck Canada Inc. as Zetia®. One of the purposes of this drug is to provide a similar cholesterol lowering effect as compared to large dose statin monotherapy but with a smaller statin dose. This may be a viable alternative for individuals who are intolerant to larger doses of statins.

2.2.1. Mechanism of Action and Adverse Effects of Ezetimibe

As described earlier, statins act mechanistically via the inhibition of the HMG-CoA reductase enzyme, leading to a down-regulation of endogenous cholesterol production and subsequent increase in hepatic re-uptake of circulating LDL. Ezetimibe acts differently, via direct inhibition of the Niemann-Pick C1-Like 1 (NPC1L1) transport protein mainly located in the intestinal brush border of the small intestine (59). The NPC1L1 is responsible for the absorption of sterol components of the diet from the gut into the circulation. Inhibition of the NPC1L1 with ezetimibe greatly down-regulates dietary cholesterol absoprtion. This action also leads to a decrease in circulating LDL levels. Thus, the combination of ezetimibe and statin pharmacotherapy lowers circulating lipid levels by inhibition of both endogenous cholesterol production and dietary cholesterol absorption. Due to ezetimibe's highly selective molecular target (NPC1L1), ezetimibe does not negatively interact with other lipid lowering medications such as fibrates and has a limited profile of negative side effects. The Ezetrol® product monograph (59) describes clinical trial data comparing adverse effects of ezetimibe monotherapy to placebo. Overall, ezetimibe demonstrated a similar safety profile to placebo. Discontinuation of treatment due to adverse drug events was reported at <3% in each treatment arm. The most common adverse side effect associated with ezetimibe therapy was gastrointestinal disturbances and was reported with a prevalence of <5%. More serious adverse drug related side effects included myalgia, respiratory agitation and infection reported at a rate of <5%. Similar drug safety

results were identified by clinical investigation of simultaneous ezetimibe/statin therapy.

2.2.2. Clinical Trial Data Addressing the Effectiveness of Ezetimibe

Current information indicates that the effectiveness of ezetimibe remains questionable. The results of several clinical trials and expert opinions have provided controversial evidence with regard to ezetimibe's use for primary prevention or secondary treatment of CVD (58, 60, 61). Three large clinical trials, the ENHANCE, ARBITER 6-HALTS and SEAS trials (all published within the last five years), have provided the majority of evidence describing the effectiveness of ezetimibe– and it is clear that significant controversy of its efficacy exists. The ENHANCE trial (63) compared standard doses of simvastatin monotherapy or simvastatin and ezetimibe in 720 patients with familial hypercholesterolemia. Based on surrogate cardiovascular endpoints such as arterial intima-media thickness, the study findings concluded that simvastatin with concomitant ezetimibe therapy did not offer significant reduction in intima-media thickness. This was despite obtaining a significant lowering of cholesterol compared to the simvastatin monotherapy treatment group (62, 63). In the ARBITER 6-HALTS trial (64), 315 patients undergoing statin therapy were allocated to either concomitant niacin or ezetimibe therapy. The primary endpoint attempted to identify the effects of either niacin or ezetimibe on atherosclerosis in a head-to-head trial format. The surrogate end point of carotid intima-media thickness (CIMT) was utilized as the primary end point. The investigators of this study concluded that niacin was able to significantly reduce CIMT and no significant changes were observed in the ezetimibe treatment group (64). In the SEAS trial (65), 1873 patients were enrolled from several different centres across Europe. This study utilized simvastatin-ezetimibe treatment versus

placebo to determine the ability of aggressive lipid therapy to decrease cardiovascular risk in a patient population with asymptomatic aortic stenosis and no indications for lipid lowering therapy. The primary endpoint utilized to assess the risk of CVD in the study was a multifactorial composition of major cardiac events. Again, the results were unable to provide clear evidence for the use of ezetimibe to improve patient health. While a significant reduction of 4.4% in the simvastatin-ezetimibe group was observed in the prevalence of ischemic events, the intervention failed to significantly reduce the number of patients requiring aortic-valve replacement. Also, there was a small, but significant increase in the occurrence of cancer during treatment in the simvastatin/ezetimibe group (65, 66). Furthermore, due to the simultaneous administration of statin and ezetimibe therapy, it is unclear as to whether the CVD benefit was due to the lipid lowering effects of ezetimibe or from the myriad of positive effects of statins.

These studies have questioned the ability of ezetimibe to elicit a positive net effect on the prevention or treatment of CVD. A growing body of literature suggests that, while the current medical goal for LDL levels appears to be, "the lower, the better", there is no specific, well-identified target of strictly LDL lowering or therapy that has been definitively correlated to a significant reduction in cardiovascular events. In view of the statins' clearly identified range of positive cardiovascular effects, the effectiveness of ezetimibe remains uncertain. More comprehensive information about ezetimibe may be revealed when the IMPROVE-IT trial (67) (which is slated to be completed in 2013) is published. This will be the first multi-center clinical trial of its magnitude which will demonstrate the effects of

simvastatin monotherapy or simultaneous therapy of simvastatin and ezetimibe on primary cardiovascular endpoints such as cardiac death, as well as non-fatal MI or stroke (67).

Thus, the full spectrum of ezetimibe's capabilities and general effects on the body beyond lipid lowering remain largely unclear. As ezetimibe elicits a marked effect on the absorption of ingested contents from the gut, it may be postulated that ezetimibe may also have effects on absorption of substances other than cholesterol. This may have health implications if the absorption of important nutrients is inhibited. However, the current literature provides little information about the effects of ezetimibe on the absorption of other molecules, such as essential fatty acids. Just as statin/grapefruit interactions elicit deleterious effects, so too may ezetimibe possess negative interactions with certain nutrients. This thesis will investigate whether a drug-nutrient interaction between ezetimibe and the cardioprotective essential fatty acid, alpha-linolenic acid, exists.

3. ***Functional Foods for the Prevention and Treatment of CVD***

Many natural food products possess a significant source of health benefit beyond normal nutrition. In recent years, a field of study has emerged which focuses on elucidating the beneficial or deleterious effects that specific foods and diets may have on various disease processes. One area of investigation is the role that specific nutrients may play in the prevention of chronic cardiovascular disease (68). Large scale animal and human trials continue to identify the central importance of how different food sources affect modifiable cardiac risk factors such as diabetes, obesity and inflammation (69, 70)– diseases which now are reaching epidemic proportions.

One example of a diet-based clinical trial which has changed food intake guidelines to benefit cardiovascular health is the DASH trial. The DASH trial (71) compared a typical western diet to a diet rich in fruits, vegetables, low-fat dairy products and low in saturated fats. After two weeks of intervention, systolic blood pressure decreased significantly by 5.5 mmHg compared to the control western diet (71, 72). This study demonstrates how simple alterations to a typical western diet may result in a direct, positive net-effect on health and the reduction of cardiovascular risk factors. Diet may also have significant implications on both the costs and benefits of long-term human health. As a result, many natural products have been identified which contain a myriad of properties that may directly influence chronic disease processes. Natural products which have been proven to elicit positive health effects are termed either “nutraceuticals” or “functional foods”. As defined by Health Canada, a nutraceutical is “a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A

nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease", whereas a " functional food is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (73). Several nutraceuticals and functional foods have been shown to improve cardiovascular health (74, 75).

3.1. *The Role of Fibre and Omega-3 Fatty Acids*

A product which has been clearly implicated in the reduction of cardiovascular risk is dietary fibre. Fibre possesses lipid lowering, blood pressure lowering and anti-oxidant effects (76, 77). Fibre is ingested, but it is a non-absorbable substance. Fibre may be ingested in either a soluble or insoluble form. Soluble fibre absorbs water, gaining viscosity and becomes fermented in the distal colon, while insoluble fibre binds gut contents, preventing absorption and increasing fecal mass (78, 79). Overall, dietary fibre interferes with the absorption of gut contents and increases gastric emptying. For example, fibre exhibits a cholesterol-lowering effect via its action as a bile salt sequesterant (80). Soluble fibre binds bile salts in the gut preventing their re-entry into circulation and eventual hepatic reabsorption. These salts then become excreted. The subsequent decrease in circulating bile salts causes an increase in expression of the hepatic enzyme alpha-cholesterol hydroxylase which transforms cholesterol to cholic acid to replenish lost bile salts (77). This process further decreases the amount of endogenous cholesterol available for packaging and its entry into the circulation as a lipoprotein.

Recent findings suggest that the fibre viscosity may, in fact, be more closely linked with the lipid lowering effects of dietary fibre. Vuksan *et al.* (81) demonstrated that the lipid lowering action of dietary fibre was significantly increased when a highly viscous form of fibre was administered in conjunction with a typical North American diet. This cholesterol-lowering effect was significantly greater than two other treatment arms containing greater amounts but less viscous forms of dietary fibre (81). Modern guidelines suggest the ingestion of 30-40 grams

of dietary fibre daily for a positive cardiovascular effect to be observed. The best dietary sources of fibre include whole fruits, vegetables, seeds and pulse crop products such as beans (82).

An example of a seed which provides a significant source of fibre is flax seed (Linum usitatissimum). Flax seed is composed of three major components: seed, lignin and oil. The fibre components found within the seed and lignin have been attributed to the modest cholesterol lowering effects observed with the ingestion of flax seed (83). In total, dietary fibre accounts for 28% of flax seed by weight (84). Flax seed has been identified as a functional food, and has been implicated in the reduction of primary cardiovascular risk and possible secondary prevention of cardiovascular disease. Beyond flax seed's fibre component, it is believed to be beneficial for cardiovascular health due to its high concentration of the omega-3 polyunsaturated fatty acid (PUFA), alpha-linolenic acid (ALA). Flax seed is one of the richest dietary sources of ALA (85). Approximately 50% of the oil component of flax is ALA.

3.2. *Omega-3 Structure and Metabolism*

In terms of chemical structure, omega-3 fatty acids are long chain fatty acids chemically configured in a –*cis* conformation and contain two double bonds within the chain. The final double bond is located at the third-last carbon of the chain. Omega-3 fatty acids are complex polyunsaturated fatty acids which require more energy to make bioavailable than various other food molecules such as carbohydrates or saturated fats. As a result, omega-3 fatty acids have a much slower rate of metabolism. Omega-3 fatty acids are structurally similar to their omega-6 fatty acid counterparts. Omega-6 fatty acids, however, are easily converted to arachadonic acid (AA). From AA, a series of pro-inflammatory downstream products are synthesized such as prostaglandins and thromboxanes. Omega-3 and omega-6 fatty acids are not interconverted within humans because of the lack of the proper desaturase enzyme. As a result, omega-3 and omega-6 fatty acids compete for enzymatic metabolism to other PUFA's and eventually eicosinoids which may confer either pro- or anti-inflammatory downstream effects. Under normal circumstances, omega-3 fatty acids are actually preferentially bound by metabolizing enzymes.

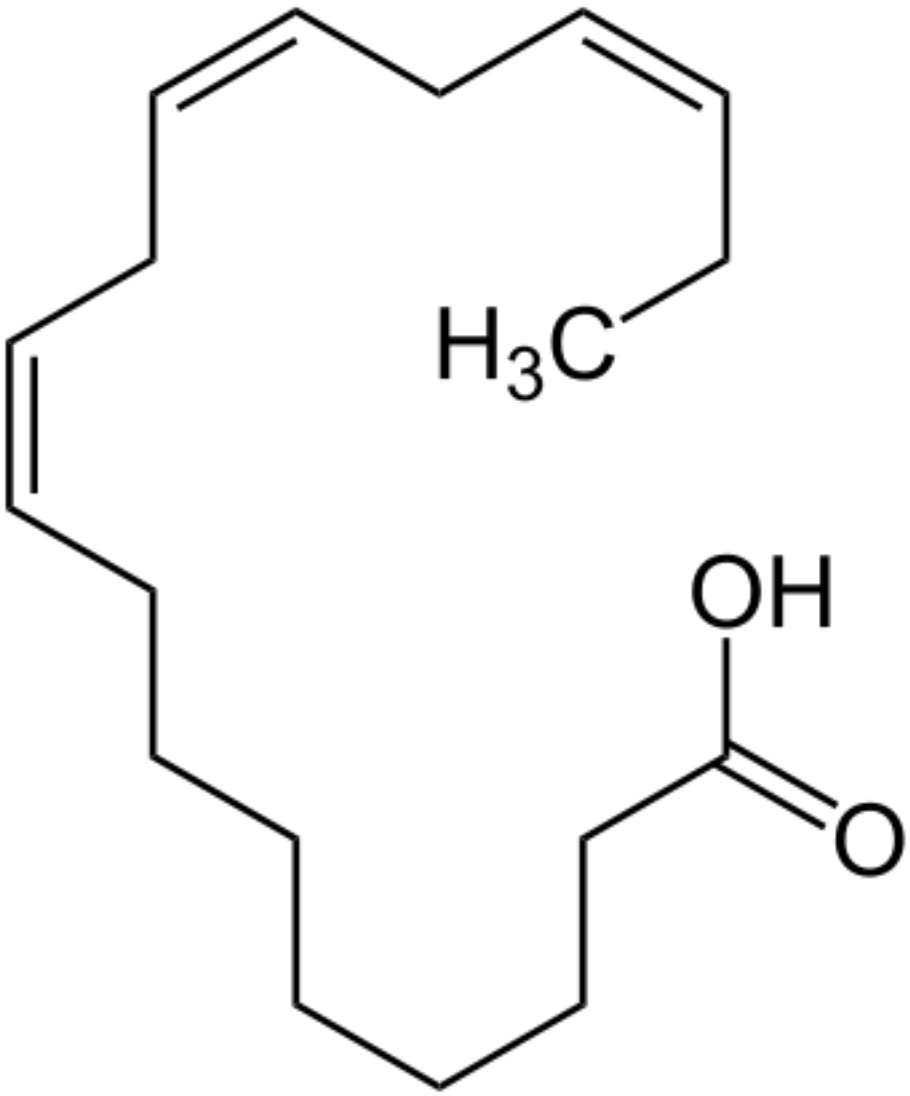
While ancient diets provided an omega-6 to omega-3 ratio of approximately one, today's guidelines suggest a ratio of 4:1 for maintenance of good cardiovascular health. Many estimates, however, place current ratios of omega-6 to omega-3 approaching 20:1 in a typical western diet (86). This provides the body with an exponentially larger amount of omega-6 fatty acids than omega-3's. As a result, the abundance of omega-6 fatty acids overcomes the preferential binding of omega-3 fatty acids by metabolizing enzymes. An increased amount of omega-6 fatty acids

become bound and form a myriad of pro-inflammatory eicosanoid molecules. Over time, this process may greatly increase an individual's overall inflammatory state which, in turn, has the potential to significantly accelerate chronic disease processes such as atherosclerosis. Omega-3 fatty acids actually provide an opposing effect when metabolized. While ALA possesses independent anti-inflammatory effects (87-89), it may also be metabolized to other PUFA's such as eicosapentanoic acid (EPA) and to a much lesser extent, docosahexaenoic acid (DHA). These PUFA's stimulate the synthesis of eicosanoids which decrease platelet adhesion and thrombosis (90, 91).

Thus, in contrast to omega-6 fatty acids, omega-3 fatty acids confer anti-inflammatory characteristics and have been shown to clinically lower circulating markers of inflammation such as CRP and serum amyloid A (92, 93). The ill-proportioned ratio of omega-6 to omega-3's may also be responsible for the negative or null results obtained in recent clinical trials investigating possible effects on CVD. In fact, the omega 6:omega 3 ratio is a possible source of error in the results of the recent Alpha Omega trial (94). In this dietary study, a margarine supplement rich in various omega-3 fatty acids was administered to post-MI patients undergoing modern optimal medical therapy (94). No positive beneficial cardiovascular effects were observed, however, the omega-6:omega-3 ratio in the margarine supplement was very high, in fact it was 2.3 times higher than the ratio utilized in the Lyon Heart Diet study (94). In light of this fact, added to the possibility that the Alpha Omega trial may have been underpowered, the results may become less significant. It is quite possible that the high omega-6 component of

the dietary intervention may have masked the cardioprotective effects of the low-dose omega-3 fatty acid supplementation. Many of the large clinical trials which demonstrated a potential benefit of omega-3 fatty acids for secondary prevention of cardiovascular disease also achieved a concomitant lowering of the omega-6 to omega-3 ratio (95, 96). Thus, the ability of omega-3 fatty acids to play a significant role in the secondary prevention of cardiovascular disease remains a viable possibility.

Figure 3. Chemical structure of the omega-3 fatty acid, alpha-linolenic acid.



Public domain image, obtained from the Wikimedia Commons.

(http://commons.wikimedia.org/wiki/File:Alpha-

Linolenic_acid_Structural_Formulae_V.1.svg.)

3.3. *Omega-3 Fatty Acids and Cardiovascular Disease*

Omega-3 fatty acids may be obtained from several dietary sources. The most common source is fish and marine products. Fish provide an excellent source of the long chain omega-3 fatty acids EPA and DHA. Bang *et al.* (1973) first reported the beneficial effects of a marine based diet on cardiovascular health (97). EPA and DHA obtained from marine sources have been demonstrated to reduce the risk of MI, sudden cardiac death and all-cause mortality (98, 99). The positive effects on cardiovascular health from omega-3 fatty acids derived from fish has prompted investigators to explore the potential benefits of other omega-3 fatty acids. Omega-3 fatty acids, such as the short chain fatty acids derived from plant sources may elicit similar protective effects and serve as an available alternative for people with allergies or general intolerance to fish. Increasing concerns about the contamination of marine products with elevated levels of substances toxic to the humans, such as mercury (particularly in commercially concentrated fish oils), has made the identification of alternative sources of omega-3 acids even more important.

One example of a plant source rich in an omega-3 fatty acid is flax seed. Flax seed is also called linseed. Canada is one of the largest producers of flax seed. Flax seed is unique in that it is one of the richest sources of alpha-linolenic acid (ALA). ALA is another omega-3 PUFA, apart from EPA and DHA which has been independently identified as possessing several cardioprotective properties. Flax seed exhibits cholesterol lowering, anti-arrhythmic, anti-atherogenic and blood pressure lowering effects (85, 100). It is believed that many of these beneficial effects may be due to its high content of ALA. Many of these effects have been well-demonstrated in various

species of animals, as well as in healthy individuals. Current research is expanding the benefits of ALA to patients with pre-existing cardiovascular disease. This research will help to define the role of ALA beyond primary prevention of CVD and elucidate the possibility of ALA therapy for secondary treatment of CVD. The FlaxPAD study is a recent example of a local, large-scale clinical trial which is designed to elucidate the health effects of long-term dietary supplementation with flaxseed in patients with peripheral artery disease (101).

3.4. Absorption Characteristics and Bioavailability of Alpha-linolenic Acid (ALA)

Essential fatty acids such as ALA must be ingested and absorbed because the body does not have the capacity to synthesize them. Given the benefits of increasing circulating ALA levels in blood and tissues, it is important to identify mechanisms which may influence the absorption and overall bioavailability of ALA.

At the present time, there is limited information about the characteristics of ALA absorption. Our lab, however, has elucidated the dosage and form of flaxseed which provide the greatest bioavailability of ALA in humans. In one study (102), three types of flax muffin, containing either whole or milled flaxseed, or flax oil and each providing 6 grams of ALA were administered daily to a population aged 18-49 over a 12 week time period. It was determined that flax oil provided the greatest bioavailability of ALA over 12 weeks of supplementation as compared to whole or milled flax seed. Another study from our lab demonstrated a significant rise in plasma ALA concentrations following only 4 weeks of flax oil supplementation (103). Milled flax seed, however, provided a similar bioavailability of ALA and was deemed to be much more tolerable over the longer, 12 week supplementation period (102). Furthermore, milled flax seed does not expire as quickly as flax oil and appears to be more easily integrated into a person's daily diet. Our lab also demonstrated that bioavailability of ALA obtained from ground flax seed is unaffected by a subject's age. ALA plasma concentrations remained similar when comparing a young adult (aged 18-29) and older adult (aged 45-69) human population (104). It still remains largely unclear, however, what effect interactions with other substances within the gut or enterocytes may have on the absorption and

metabolism of ALA and other omega-3 fatty acids. Supplementation of the diet with cholesterol has led to a ~120 fold increase in ALA in animals (105, 106). This is thought to be due to an increase in intestinal cholesterol which may stimulate ALA absorption through the intestinal wall (107, 108).

Essential fatty acids are not absorbed directly with cholesterol, however. While sterol transport occurs via the NPC1L1 transporter in the intestinal brush border in the gut, fatty acids are primarily absorbed passively via fatty acid binding transport proteins along the intestinal brush border in the small intestine (109). Under normal conditions, essential fatty acids have a very high rate of absorption, exceeding 90% (110). A potential mechanism for the observed increase in fatty acid absorption in the presence of increased gut cholesterol may be based on an increase in micelle formation and overall affinity and/or interaction of the intestinal brush border and gut contents (107, 108).

Thus, conversely, molecules which interfere with the formation of micelles or the affinity of gut contents for the intestinal brush border may markedly affect the overall bioavailability of cardioprotective fatty acids.

3.5. *Animal Research Demonstrating the Cardioprotective Effects of ALA*

In recent years, a number of animal studies have demonstrated several cardioprotective effects of ALA (85, 91). These have included effects on the vasculature as well as directly on the heart. With respect to the former effects, Bassett *et al.* demonstrated in an LDL receptor -/- mouse model that the ingestion of flax seed significantly attenuated the development of atherosclerosis induced by a diet either rich in cholesterol or industrial trans-fats (111). Prasad *et al.* has also demonstrated the ability of flax seed to reduce the amount of atherosclerotic plaque formations in hypercholesterolemic rabbits by 46% when ingested over eight weeks despite no change in serum lipid levels over that time (112). Recently, Francis *et al.* (113) has demonstrated that flaxseed not only slowed the progression of atherosclerosis, but also induced its regression once the plaques were established.

Flax seed has also been associated with improved vascular function. The ability of an artery to contract or relax is mediated in part via systemic or paracrine signaling of the endothelial vessel lining. Dupasquier *et al.* (106) determined that following eight weeks of dietary supplementation with cholesterol and flax in rabbits, the presence of 10% flax in the diet significantly reduced the progression of atherosclerosis compared to 0.5% cholesterol supplementation alone. Even the combined treatment of a diet containing both 0.5% cholesterol and 10% flax demonstrated the same anti-atherogenic effect. This observation was attenuated at 16 weeks, however. In light of this fact, flax supplemented groups still elicited a normalizing effect of aortic band relaxation fully at 8 weeks and partially at 16 weeks. These findings suggest a protective effect on endothelial dependent vessel

relaxation by flax seed supplementation in hypercholesterolemic conditions (106). Talom and colleagues also demonstrated in hypertensive rats that a diet rich in flax seed improved acetylcholine-mediated endothelial relaxation (114). Disturbances in vascular function may be caused by insult to the endothelium at the beginning of the atherosclerosis or the exacerbation of pre-existing atherosclerotic disease. The ability to positively affect vascular function may be one mechanism responsible for the success of flaxseed in the primary prevention of CVD.

The direct effects of flaxseed on cardiac function have also been investigated. Ander *et al.* (115) reported that the ingestion of flax seed by rabbits over a sixteen week period elicited an anti-arrhythmic effect in hearts during an in vitro ischemia-reperfusion challenge. The presence of flax in the diet caused a decrease in ventricular fibrillation and a shortening of the QT interval either alone or in conjunction with cholesterol supplementation (115). Similar anti-arrhythmic effects have been reported for different species and with other omega-3 fatty acid based interventions (116, 117). ALA may achieve its positive effects on post-ischemic cardiac function through an inhibition of Na^{+}/Ca^{2+} exchange (118). It may also achieve its effects through an anti-oxidative mechanism. Oxygen derived free radicals are thought to participate in ischemic injury to the heart (119-121).

However, while the lignan component of flax seed (secoisolariciresinol diglucoside, SDG) has marked anti-oxidative effects (122, 123), this finding has not translated to clinical trials (85). Thus, the clinical relevance of SDG therapy remains unknown.

3.6. *Clinical Trials Demonstrating the Cardioprotective Effects of ALA*

A number of large-scale, clinical trials have supported the cardiovascular benefits of a diet rich in omega-3 fatty acids (84, 96, 100, 124). In fact, several studies have implicated the significant role ALA supplementation may play in the primary prevention of CVD.

Ascherio *et al.* (1996) studied 43,757 men aged 40-75 years old and free of previous CVD or diabetes. After six years of follow-up, it was determined that following a multivariate analysis, ALA was inversely correlated with coronary disease. Furthermore, this effect was not witnessed when analyzing marine omega-3 fatty acids, such as EPA. Thus, ALA may function by an independent cardioprotective mechanism other than via conversion to long chain fatty acids such as EPA or DHA (125).

In a prospective, ten year study of 76283 women, Hu *et al.* (1999) demonstrated that a long-term ingestion of a diet rich in ALA provided a significant protective effect against fatal myocardial infarction in women who had no previous ischemic heart disease (126). Djoussé *et al.* (2001) investigated the relationship between ALA intake and coronary artery disease in 4584 male and female study participants. The study determined that ALA intake was significantly inversely correlated with the prevalence odds ratio (95% CIs) of coronary artery disease. Those subjects (male or female) with the highest ALA intake had the greatest reduction in risk (127).

As described in earlier text, ALA possesses anti-arrhythmic properties. Another large population, prospective study conducted by Albert *et al.* (2005) analyzed the ability of ALA to protect against sudden cardiac death in 76763 women. After 18

years of follow-up, the investigators determined that the quintiles of women with the two highest daily intakes of ALA had a 38% and 40% reduction in sudden cardiac death respectively (128).

There is also a growing body of evidence supporting the role of omega-3 fatty acids for the secondary prevention of CVD. For example, the DART trial (129) was one of the first large clinical trials to investigate the potential role omega-3 fatty acids may play in the secondary prevention of cardiovascular disease. The DART trial enrolled over 2000 men who had recently suffered a MI. Subjects were randomized to one of three dietary interventions for two years. One treatment arm ingested fatty fish two to three times per week. After two years of dietary intervention and consultation, this group had a 29% reduction in all-cause mortality. Although there was no significant change in the primary endpoint of re-infarction or ischemic heart death amongst any of the groups, the marked increase in survival in the fatty fish group lead to the conclusion that a diet rich in fatty fish that are rich in omega-3 fatty acids may decrease risk of mortality in men following a MI (129).

The GISSI-Prevenzione study (96) was another large-scale prospective clinical trial with over 11,000 study participants. Again, this trial enrolled patients who had recently suffered their first MI. The study demonstrated that over 3.5 years of intervention, a significant 10% decrease in relative risk of the primary endpoint of death, non-fatal MI or stroke was observed in the treatment group receiving regular omega-3 fatty acid supplementation. There was also a small, but significant reduction in circulating triglyceride levels in the omega-3 fatty acids group (96).

In terms of ALA, the Lyon Diet Heart Study (95) examined the cardioprotective role that a Mediterranean-based diet rich in omega-3 fatty acids, specifically alpha-linolenic acid (ALA), may exhibit on the secondary prevention of cardiovascular disease. Patients were randomized to either control or dietary intervention during their hospital stay following their first myocardial infarction. The study investigated the recurrence of secondary events such as cardiac death, recurrent MI, unstable angina and heart failure over 46 months. The Lyon Diet Heart Study demonstrated a protective effect of approximately 15% against a second event in patients receiving the dietary intervention for the duration of the study. Furthermore, ALA was the only fatty acid to be significantly associated with cumulative survival and the absence of a second MI at follow-up (95, 130).

Given the multitude of cardioprotective properties elicited by omega-3 fatty acids, and ALA specifically, it is increasingly important to understand the factors that influence its bioavailability.

Hypothesis

The cholesterol lowering drug, ezetimibe, will inhibit the absorption of alpha-linolenic acid (ALA) in cardiac patients receiving statin therapy.

Methods, Results and Discussion

The methods, results and discussion of our study are presented as an original manuscript currently submitted for review and publication. My contributions are as follows.

1) Subject recruitment and overall study coordination of the clinical trial, including consenting of patients, coordination and attendance of patient study visits, processing of data and samples obtained at each study visit.
2) Planning and execution of experimentation to analyze lipid and immunoassay parameters.
3) Statistical analysis of data.
4) Writing of manuscript including production of figures and tables.

The Effects of Ezetimibe on the Absorption of Omega-3 Fatty Acids in the Prevention of Cardiovascular Disease

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ABSTRACT

Introduction: Elevated levels of circulating omega-3 polyunsaturated fatty acids like alpha linolenic acid (ALA) may be beneficial for cardiovascular health. Circulating ALA concentrations are elevated dramatically by a cholesterol supplemented diet which increases ALA bioavailability through enhanced micelle formation in the intestines. Conversely, it is possible that drugs which inhibit cholesterol metabolism in the intestine may also inhibit fatty acid absorption.

Objective: The purpose of this study is to determine if a cholesterol absorption inhibitor, ezetimibe, will decrease circulating levels of ALA.

Design: Patients between 44-80 years old, requiring statin therapy to regulate blood cholesterol levels, were randomly assigned to one of four groups for a 6 week trial: 1) placebo; 2) ezetimibe therapy; 3) a supplement of flaxseed oil (containing 1.0g ALA); or 4) ezetimibe and flaxseed oil supplementation.

Results: Ingestion of flaxseed oil resulted in a significant increase in circulating ALA levels in patients who were not given ezetimibe. However, in the presence of ezetimibe, circulating ALA levels did not increase significantly even in the presence of flax oil supplementation. There were no significant differences amongst the groups in terms of circulating total cholesterol, LDL, HDL, triglyceride levels, or in the expression of hs-CRP, lipoprotein A, fibrinogen and fetuin-A in the blood.

Conclusion: Ezetimibe therapy inhibits the absorption of omega-3 fatty acids. Patients receiving ezetimibe therapy will not receive the expected cardiovascular benefits from dietary supplementation with omega-3 fatty acids.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide (131). While pharmacotherapy has become the leading primary therapy for the treatment of various cardiovascular diseases, there is a recent growing interest in elucidating the effects of natural health products for the prevention and treatment of CVD. One kind of natural health product which has demonstrated desirable effects for the treatment of CVD is omega-3 fatty acids. Alpha linolenic acid (ALA) obtained from flaxseed is an omega-3 fatty acid that possesses several cardioprotective properties in animal studies. These include anti-atherogenic, anti-inflammatory and anti-arrhythmic capabilities and it is protective against vascular dysfunction (100, 106, 115). Many of these cardioprotective effects have been demonstrated in human populations as well (132, 133). Furthermore, several human trials have shown a correlation of circulating ALA levels with a reduction in both fatal and non-fatal myocardial infarction (95, 128, 129). The Lyon heart study (95), for example, found that a diet rich in ALA was associated with a significantly lower rate of recurrence of the major adverse cardiac events of cardiac death or non-fatal myocardial infarction. If ALA is important to our cardiovascular health for primary and/or secondary prevention, then understanding factors that influence its bioavailability are critical. Previous studies have demonstrated that 2-4 weeks were required to observe an increase in ALA levels in the blood in response to a dietary supplementation with ALA (102, 103). ALA was best absorbed from the diet when ingested as flaxseed oil in comparison to whole or ground seed (9). The age of the person does not appear to influence ALA bioavailability (104). However, in animal studies, an increase in

dietary cholesterol facilitated a dramatic increase in circulating ALA concentrations. It has been postulated that this effect may be due to a cholesterol-mediated increase in micelle formation in the gut (2-4,11,12).

If increasing the levels of cholesterol in the gut will increase omega-3 fatty acid absorption, then it is possible that the opposite is also true: inhibiting dietary cholesterol absorption in the intestine will interfere with ALA absorption. Ezetimibe is a cholesterol-lowering drug that is commonly used to treat patients at risk for CVD. Ezetimibe inhibits cholesterol absorption in the human intestine. It selectively targets and inhibits the Niemann-Pick C1-like 1 (NPC1L1) receptor (59). The NPC1L1 is located in the jejunal intestinal brush border and is the primary regulator of sterol transport from the gut lumen to within enterocytes (134). As a result, ezetimibe can lower LDL cholesterol, either as a monotherapy or in conjunction with statin pharmacotherapy. Clinically, ezetimibe may be utilized as an add-on therapy to statin pharmacotherapy when additional lipid lowering or a lower statin dosing is required (135-137). While ezetimibe can reduce LDL cholesterol, several trials have demonstrated ezetimibe's failure to offer a superior reduction of clinical endpoints such as atherosclerotic lesions or mortality (58, 64, 138). It is possible that this failure is due to other negative side effects that ezetimibe may have on risk factors associated with CVD.

The purpose of our study was to determine if ezetimibe therapy will alter the bioavailability of the beneficial omega-3 fatty acid, ALA. It is hypothesized that ezetimibe administration to patients with CVD will attenuate the rise in circulating ALA levels in response to ALA supplementation with flax oil.

METHODS

Study Design

This was a four arm, parallel group, randomly controlled, open-label clinical trial. The study design was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. Study subjects were recruited from the Bergen Cardiac Centre at St. Boniface Hospital. Male and female subjects aged 18-80 years old were included in the study. Patients were further included/excluded from the study based on several criteria. For inclusion in this study, patients were required to be undergoing statin therapy at the time of initial screening. Furthermore, patients were required to attend two study visits (baseline and six weeks) and donate 10ml of blood. Patients were also required to adhere to dietary restrictions as described below. The majority of subjects enrolled in the study had experienced a prior acute cardiac or cerebrovascular event and were undergoing statin, aspirin, ACEi/ARB and beta-blocker therapy for the secondary prevention of CVD at time of enrollment.

Cardiac patients undergoing statin pharmacotherapy were randomized into one of four treatment groups (all patients were maintained on statin therapy and all other regular medications throughout the study): 1) control (statins alone), or 2) plus 10mg ezetimibe pharmacotherapy, or 3) plus 2 capsules of flax oil (containing a total of 1g ALA), or 4) plus ezetimibe and flax oil treatment for a total of 6 weeks. Each subject provided written informed consent prior to beginning the study. The study schedule included two blood draws, one at baseline and one at follow-up after six weeks of treatment. At each appointment, medical history, height, weight and

blood were collected from each patient (**Figure 1**). Subjects were fasted for twelve hours prior to their blood draw. During treatment, subjects were not allowed to ingest oils or salad dressings containing oils, as well as seafood. In addition, subjects were required to abstain from any additional source of omega-3 fatty acid supplementation. Subjects who had been ingesting omega-3 supplementation prior to beginning the study were required to undergo a one month period of abstinence from omega-3 ingestion prior to beginning treatment.

This study was conducted over a six week period and subjects were free to withdraw from participation at any time, for any reason, without penalty. This study was conducted on a volunteer basis. No reward (financial or otherwise) was given for participation.

Study Procedures

Blood Analysis: During each visit (week 0 and week 6), 10ml of blood was collected by venipuncture in tubes containing 1mg EDTA/ml from subjects who had fasted for 12 hours. Blood samples were centrifuged at 1000 x g at 4˚C for 10 minutes, and plasma was then stored at -80˚C until analysis at a later date.

Preparation of Plasma Fatty Acid Methyl Esters: Plasma fatty acids methyl esters were measured by gas chromatography coupled with flame ionization detection as described in detail elsewhere (2-4). In summary, plasma samples were derivatized directly to fatty acid methyl esters using 4:1 methanol:toluene as described by Lepage and Roy (139). A Varian CP-3800 GC w/ flame ionization detector and CP-Sil 88 capillary column 60m x 0.25 mm x 0.20 um GC apparatus was used for analysis. The oven temperature was maintained at 111^{o}C for 1 min, increased by 20^{o}C/min to

170°C, raised again by 5°C/min to 190°C and finally increased by 3°C/min to 225°C. It was maintained at that temperature for 10 min, for a total run time of 29.62 minutes. Equipment was standardized using GLC 462 (Nu-Chek Prep, Inc.). The internal standard used was C19:0 (Nu-Check Prep, Inc.).

Cholesterol and Triglyceride Assays: Commercial assay kits (Thermo Electron Corporation, Waltham, MA) were used to determine total cholesterol and triglyceride levels in plasma. A separate assay kit (Biovision Inc., Mountain View, CA) was used to analyze plasma HDL levels. LDL was calculated from total cholesterol and HDL measured values.

Immunoassay Screening: A commercial assay kit from Novagen RBM (Madison, WI) was used for screening cardiovascular biomarkers of interest including hs-CRP, lipoprotein A, fibrinogen and fetuin-A.

Adverse Effects Monitoring: During the six week follow-up visit, secondary effects associated with the treatments were monitored via questionnaire.

Statistical Analysis

Statistical significance was determined with one-way ANOVA using SAS® software (Sigma Stat, SPSS Science Inc., Chicago, IL, USA). Subjects were included in the statistical analysis for efficacy if they had donated two blood draw samples and were 100% compliant with the dietary restrictions as instructed.

RESULTS

In total, 78 subjects were screened for this trial. Those that did not meet the eligibility criteria or were unable to commit to the study schedule or dietary restrictions were excluded from analysis. The study was completed successfully by 42 of 44 subjects. Two patients were unable to comply with the study schedule and were removed on that basis. Eight additional subjects were removed from the study at the time of data analysis due to non-compliance to dietary restrictions as determined by his or her ALA and/or lipid profile. In total, data was analyzed from 34 patients. No major adverse effects were reported during the course of this study.

Subject Cohort Characteristics: Gender, Age and Body Mass Index

Out of the 34 subjects who successfully completed the study, 28 were male and 6 were female. Patients enrolled in the study ranged from 44-78 years old and the mean age of the overall subject cohort was 61 ± 11 years old. There were no statistically significant differences between the mean age or body mass index amongst any of the four treatment groups over the six week treatment period (**Table 1**).

Cholesterol and Triglyceride Levels

No statistically significant changes in plasma total cholesterol, low-density lipoprotein cholesterol or high-density lipoprotein cholesterol values between 0 and 6 weeks, in either individuals or between groups, were detected during the study duration (**Table 2**).

Plasma Fatty Acid Levels

After six weeks of treatment, subjects who received flaxseed oil supplementation alone showed a significant increase in plasma ALA concentrations (**Figure 2**). However, the administration of ezetimibe blocked this increase. Circulating ALA levels were not significantly different between control and ezetimibe, or between the control and the ezetimibe plus flax oil treatment. The presence of ezetimibe significantly attenuated the amount of circulating ALA compared to flax supplementation alone. This effect was selective for ALA and did not induce changes in other fatty acids (**Table 3**).

Immunoassay Analysis

Immunoassay techniques were utilized to screen for CVD biomarkers within the subjects' plasma. Baseline and six week samples were analyzed in parallel. No significance between treatment groups was observed for hs-CRP, lipoprotein A, fibrinogen and fetuin-A.

DISCUSSION

As expected, supplementing the subjects' diet with flaxseed oil alone provided a significant increase in circulating ALA levels. Previous trials in healthy volunteers have shown that this dosage of flax oil and the duration of the intervention used here would result in this increase in ALA bioavailability (102-104). This information, however, is now extended to a patient population with clinical symptoms of heart disease, and requiring statin pharmacotherapy for either primary or secondary prevention. The bioavailability of ALA from flax oil is also strongly influenced in animal studies by the presence of excess dietary cholesterol (105). Supplementation of the diet with cholesterol simultaneously with flaxseed can result in a marked 120 fold increase in ALA levels in the blood (105, 106). These studies have suggested that the enhanced micelle formation in the intestine induced by the additional dietary cholesterol can facilitate ALA transport across the intestinal wall and can result in elevated ALA levels in the blood (107). This would support the contention that intestinal cholesterol is critical to enhanced ALA bioavailability. If the intestinal cholesterol load is important in stimulating ALA entry, then it is possible that the corollary is true: disturbing intestinal cholesterol will inhibit ALA absorption.

Ezetimibe blocks the NPC1L1 transporter protein on the intestinal brush border preventing sterol transport from the gut lumen into circulation (59). It is currently the only clinically available synthetic dietary cholesterol absorption inhibitor (140). Although fatty acid absorption does not occur directly via the NPC1L1 transporter, there is a possibility that a decrease in cholesterol

absorption/micelle interaction with the intestinal brush border may lead to an overall decrease in ALA being absorbed from the gut. As hypothesized, ezetimibe therapy significantly lowered the absorption of ALA compared to flax oil supplementation alone. Flax oil supplementation was unable to significantly raise circulating ALA levels in the presence of ezetimibe. This drug-food interaction represents the primary finding of this study. Little is currently known about the effects of pharmaceuticals or other naturally occurring products on the absorption of ALA. This study provides the first clinical evidence of the interactions between ALA supplementation and ezetimibe. This finding further questions ezetimibe's overall efficacy for the prevention of cardiovascular disease. Not only may ezetimibe lack pleiotropic effects as compared to statins (141), but it may also elicit deleterious effects on the absorption of other cardioprotective substances lipid soluble vitamins whose absorption is facilitated by cholesterol uptake (142).

The conclusions obtained from this study are limited by sample size. Understandably, the small study population number renders the study unsuitable to extrapolate end points such as lipid lowering effects or overall cardiovascular risk of each treatment group to a larger population. There were no significant effects observed in the lipid profile or CVD biomarkers. Though there is a decreasing trend, no significant change in triglyceride or cholesterol levels were observed. This may be attributed to the relatively normal values obtained in these parameters from the patient cohort, indicating good control of circulating cholesterol with statin treatment. Indeed, the lack of fluctuation in these levels over the six week treatment span provides additional robustness to the hypothesis that changes in gut

cholesterol absorption are responsible for the observed changes in circulating ALA levels and they are not due to fluctuating levels of circulating cholesterol or triglycerides.

The present results have implications for the general public and for patients that are administered any agent that inhibits cholesterol levels in the gut. Patients administered ezetimibe may not receive the cardiovascular benefits that they expect when ingesting omega-3 fatty acid supplements. Our data would also suggest that bile acid sequestrants that bind bile acids in the intestinal tract and thereby enhance the excretion of cholesterol in stools may result in an attenuated capacity to absorb omega-3 fatty acids even when supplemented to the diet in higher levels. These effects are of importance to the CVD patient who may be spending money unnecessarily on these dietary supplements with the false assumption that they will receive some cardiovascular benefit.

Several important questions arise from our results that will require future investigation. We do not know if the effects observed in the present study extend to other forms of fatty acids supplements like fish oil or to their polyunsaturated fatty acid components like DHA and EPA. Based on the foregoing discussion of the mechanism of action involved in the inhibition of ALA bioavailability, it is entirely likely that the bioavailability of these fatty acids will also be inhibited by drugs that interfere with intestinal cholesterol. It is also unknown if other drugs (i.e. cholestyramine, colestipol, etc) or foods (i.e. phytosterols, dietary fiber, etc) that inhibit cholesterol absorption from the intestinal tract may also inhibit ALA absorption. With the growing use of plant sterols in our diet, this is an important

consideration. Our work, therefore, provides an important precedent for future studies in this direction. Further research elucidating the effects of ezetimibe (and other compounds that affect intestinal cholesterol) on the absorption of other healthy nutrients and, in general, the interaction between sterol and fatty acid intestinal absorption in humans is warranted.

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Table 1. Age and BMI characteristics of subjects enrolled in this study.

Treatment	Mean Age (yrs)	Baseline Mean BMI (kg/m^2)	6 Week Mean BMI (kg/m^2)
Control (n=8)	61±13	26±7	27±7
Ezetimibe (n=9)	64±11	30±6	31±6
Flax (n=9)	65±10	28±4	29±4
Ezetimibe + Flax (n=8)	58±11	30±6	30±6

Values represented as mean±SD. No statistically significant differences.

Table 2. Total serum cholesterol, HDL, LDL and triglyceride concentrations of each treatment group over six weeks of intervention.

Treatment	Total Cholesterol	HDL	LDL	Triglycerides
CONTROL				
Baseline	149±23	25±8	124±20	198±45
6 Weeks	117±10	29±5	89±11	232±44
EZETIMIBE				
Baseline	142±17	31±4	114±12	157±18
6 Weeks	123±19	35±6	79±13	150±27
FLAX				
Baseline	159±21	39±6	131±16	223±59
6 Weeks	152±19	36±6	119±16	275±28
EZETIMIBE + FLAX				
Baseline	122±18	35±9	88±11	252±82
6 Weeks	117±13	38±8	80±8	267±55

Values represented as mean±SE in mg/dL. No statistically significant differences.

Table 3. Plasma fatty acid concentrations of each treatment group over six weeks of intervention.

FATTY ACID	16:0	18:0	16:1	18:1	18:2*n*-6	20:4*n*-6	20:5*n*-3	22:5	22:6
CONTROL									
Baseline	634.1±103.4	200.3±23.3	62.4±22.5	798.0±116.5	607.7±72.7	220.0±27.4	15.6±3.9	11.2±1.9	38.7±4.5
6 Week	644.7±88.7	199.2±18.2	54.1±16.0	743.2±95.5	606.4±41.3	216±31.0	14.3±4.1	18.6±7.3	39.4±3.9
EZETIMIBE									
Baseline	543.4±52.5	182.1±18.3	46.5±10.5	642.4±63.2	682.2±47.8	207.9±19.8	17.6±3.0	9.9±1.5	41.8±3.9
6 Week	480.0±49.4	169.3±14.5	43.1±12.8	598.4±68.3	545.1±32.6	198.7±15.8	13.7±2.3	8.1±1.5	33.6±2.7
FLAX									
Baseline	596.2±88.5	199.3±28.0	51.6±15.8	751.9±88.4	647.8±59.1	211.9±26.7	18.4±4.0	12.2±1.9	45.4±4.7
6 Week	644.3±69.2	213.9±23.6	55.8±14.7	812.0±85.7	705.0±60.5	232.8±26.5	20.5±4.6	13.7±2.3	42.5±4.6
EZETIMIBE+FLAX									
Baseline	620.7±52.3	200.9±16.1	56.8±9.6	783.1±70.5	759.1±80.0	231.4±28.3	14.5±4.1	13.0±2.0	43.4±5.6
6 Week	577.0±68.5	188.8±18.9	61.3±15.0	706.1±84.6	571.2±70.8	236.7±26.6	23.0±4.3	14.6±2.5	36.9±5.3

Values represented as mean±SE, in µg/dL. No statistically significant differences.

Figure 1. Subject treatment schedule.

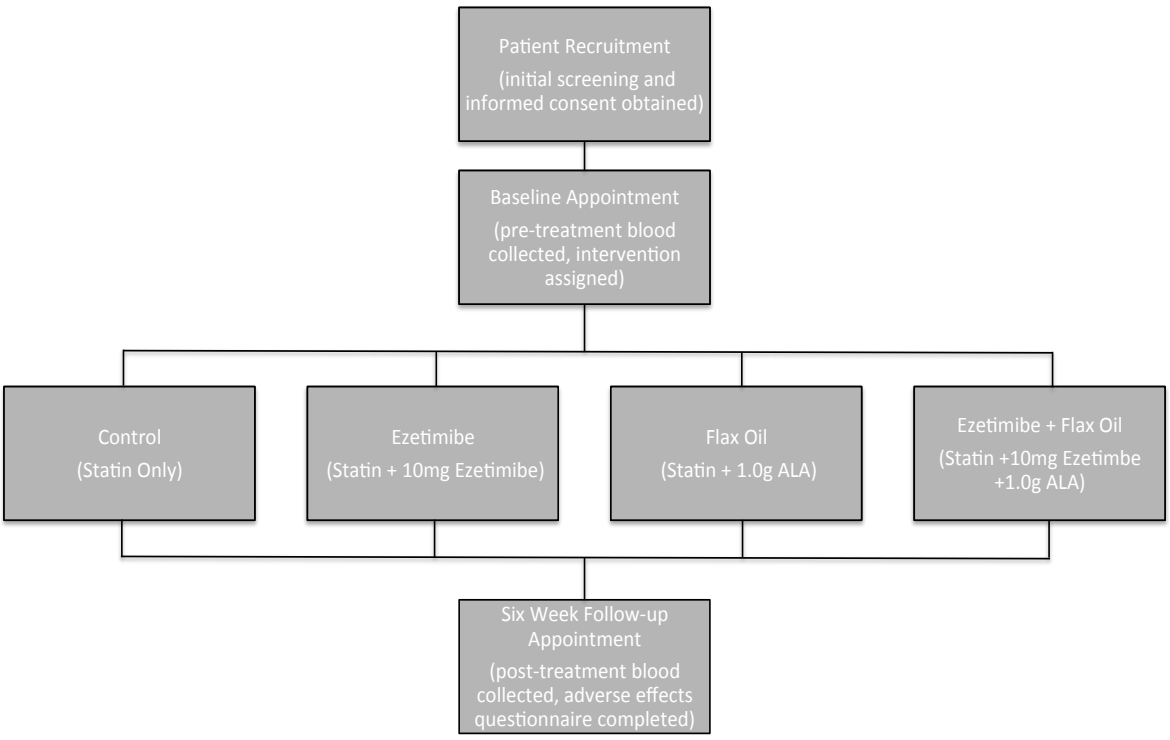
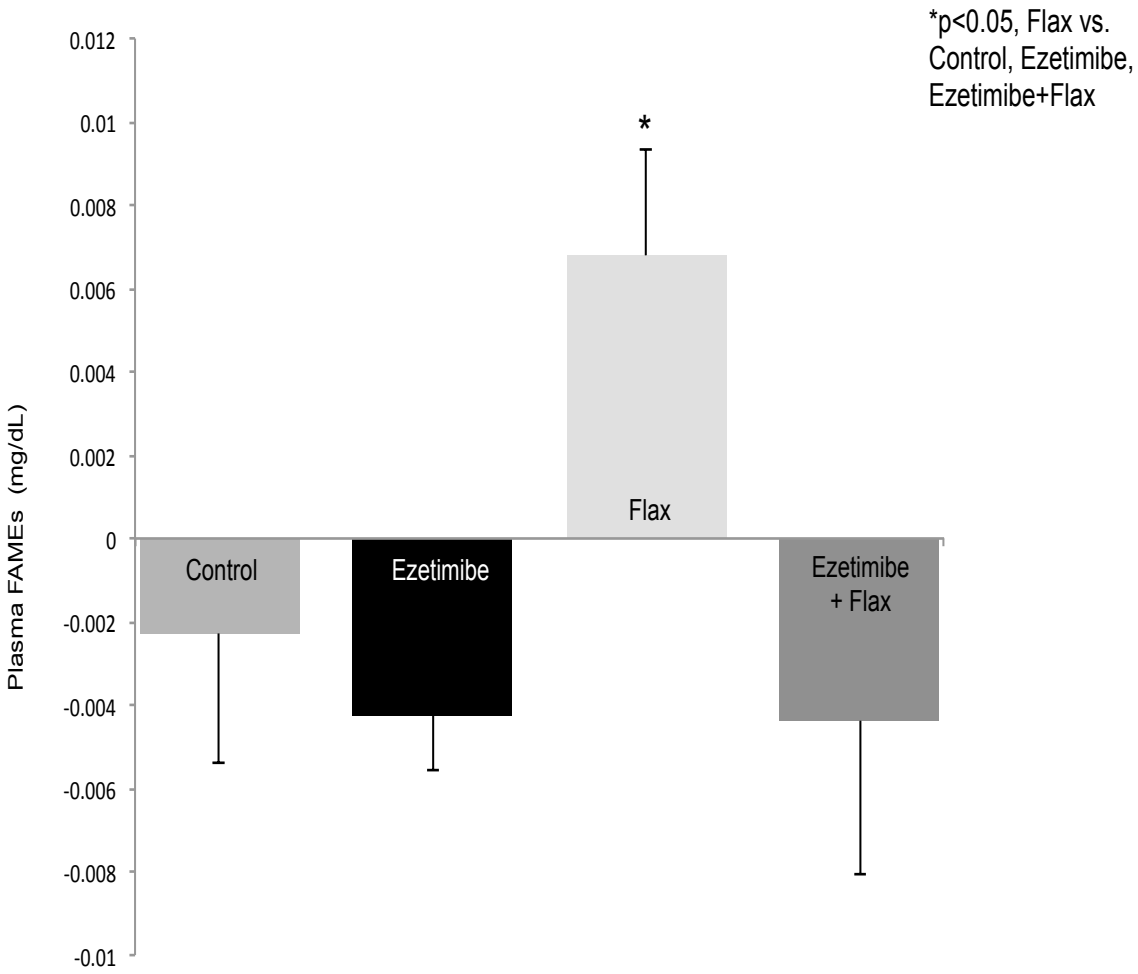


Figure 2. Change in subjects' plasma concentration of ALA following six weeks of treatment.



Appendix i

Immunoassay data was not included in the manuscript for publication. This data is presented below. A commercial assay kit from Novagen RBM (Madison, WI) was used for screening cardiovascular biomarkers of interest including hs-CRP, lipoprotein A, fibrinogen and fetuin-A. Concentrations of circulating biomarkers were analyzed from subjects' plasma at baseline and six weeks. No significance was found between the groups. A larger subject population is required to identify possible effects that ezetimibe and/ or flax supplementation may have on these circulating biomarkers in this study population. Please see following page for data **(Table Ai).**

Table Ai. Circulating concentrations of biomarkers analyzed at baseline and six weeks.

	hs-CRP		FETUIN		LpA		FIBRINOGEN	
TREATMENT	**BASELINE**	**6 WEEK**	**BASELINE**	**6 WEEK**	**BASELINE**	**6 WEEK**	**BASELINE**	**6 WEEK**
Control	1838±887	1713±509	521767±104553	501196±100905	60696±31155	116625±78151	450±98	1112±345
Ezetimibe	1923±632	2378±1134	498429±69201	495134±104228	76510±26421	42941±15600	921±156	802±109
Flax	10253±6502	3059±1034	574605±62426	657326±85497	106314±52093	116129±56954	730±95	920±169
Ezetimibe + Flax	1565±610	1911±1173	469848±85080	461744±77726	77162±66361	57936±37793	652±108	1076±179

Values represented as mean±SE in ng/ml

Conclusions and Future Directions

- **Circulating ALA levels were significantly increased in the plasma of patients who were receiving statin therapy following six weeks of flax supplementation.**
- **Ezetimibe significantly inhibited the absorption of ALA in patients receiving concurrent statin therapy.**
- **Simultaneous ALA supplementation from flax seed oil and ezetimibe treatment was unable to significantly increase ALA bioavailability in these patients.**
- **Lipid and immunoassay parameters were not significantly different between each treatment group.**
- **This study demonstrates a negative drug-diet interaction of ezetimibe and the cardioprotective fatty acid, ALA.**
- **A future study with a larger study population may elucidate significant positive changes in immunoassay parameters, such as hs-CRP, in patients receiving ALA supplementation.**
- **A future study may wish to determine other negative absorptive effects of ezetimibe. The bioavailability of other healthy substances which are linked to cholesterol uptake, such as phytosterols or vitamins, may also be negatively effected.**

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