

THESIS

OMEGA-3 FATTY ACIDS,
LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A₂ AND OXIDIZED LDL
IN GLUCOSE SENSITIVE AND GLUCOSE INTOLERANT MALES AND FEMALES

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JERUSA DHARA ENTITLED OMEGA-3 FATTY ACIDS, LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A₂ AND OXIDIZED LDL IN GLUCOSE SENSITIVE AND GLUCOSE INTOLERANT MALES AND FEMALES BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

OMEGA-3 FATTY ACIDS, LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A₂ AND OXIDIZED LDL IN GLUCOSE SENSITIVE AND GLUCOSE INTOLERANT MALES AND FEMALES

Lp-PLA₂ is a novel inflammatory marker that has been proposed as an independent risk marker for cardiovascular disease. Lp-PLA₂ in circulation is principally bound to low-density lipoprotein cholesterol (LDL-C) in humans and the substrate for the enzyme is provided by the oxidized phospholipid component of LDL-C. In the present study, plasma levels of Lp-PLA₂ concentration, activity and plasma levels of oxidized LDL were measured in fifty-nine weight stable adults, mean age 61 years, at baseline and following an 8-week supplementation of ALA, EPA+DHA or control (olive oil). Additionally, glucose, LDL-C, HDL-C, triglycerides and insulin levels were measured at baseline and following the intervention. Fasting blood glucose was measured for the purpose of assessing changes between glucose intolerant and glucose sensitive individuals.

Results showed that there was no significant change in the concentration or activity of Lp-PLA₂ either in low ox-LDL group or high ox-LDL group after ALA or EPA/DHA supplementation in diet. No particular trend was observed, however, a slight decrease in Lp-PLA₂ concentration in all the three groups (CON, ALA, and EPA/DHA) was seen in higher ox-LDL group. But Lp-PLA₂ activity only decreased in ALA group

(Baseline=163.42 mmol/min/mL + 27.29; week 8 =143.25 mmol/min/mL+14.52) in high ox-LDL group. A minor increase in Lp-PLA₂ concentration in the control group (baseline= 206 ng/mL +62.29; week 8= 215.01 ng/mL+73.26) in low ox-LDL group and a slight decrease in Lp-PLA₂ activity in control group was observed (baseline= 137.97 mmol/min/mL+ 27.50; week 8= 133.92 mmol/min/mL+28.56). There were no significant changes in fasting plasma ox-LDL after the diet supplementation in any of the groups, but a decreasing trend in LDL-C was observed. In glucose sensitive subjects, a positive correlation between Lp-PLA₂ activity and ox-LDL is seen at p<0.01 level. The results of this study also do not represent a baseline significant relation between Lp-PLA₂ concentration and activity and ox-LDL in glucose intolerant subjects. The results support the view that Lp-PLA₂ is associated with ox-LDL, but do not provide any evidence that omega-3 fats mediate the inflammatory process via Lp-PLA₂ or ox-LDL

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CHAPTER I

INTRODUCTION

Cardiovascular disease (CVD) is a significant cause of premature death not only in the western world, but according to WHO estimates, 16.7 million people around the globe die of it each year. Physical inactivity, poor dietary habits, increasing prevalence of obesity and diabetes with an underlying genetic component create an intricate etiology for the disease. Almost 65% of persons with diabetes have been shown to have CVD listed as the source of death. (Geiss, Herman, & Smith, 1995) Diabetes acts as an independent risk factor for several forms of CVD. Diabetics have been shown to have enhanced myocardial dysfunction which leads to accelerated heart failure called diabetic cardiomyopathy (Ettinger & Regan, 1989; Regan, 1983; Regan, et al., 1977; Rubler, et al., 1972; Spector, 1998). There are probably several factors that trigger diabetic cardiomyopathy: severe coronary atherosclerosis, prolonged hypertension, chronic hyperglycemia, microvascular disease, glycosylation of myocardial proteins, and autonomic neuropathy.

A chronic inflammatory process stimulating emigration of macrophages and lymphocytes to the site of injury resulting in an atherosclerotic lesion has been recognized as an imperative course in the development of CVD. Along with the inflammatory process, an elevated concentration of serum low density lipoprotein (LDL) cholesterol is a major risk factor for CHD (Program, 1994) The cholesterol in a person's blood originates from

two major sources; dietary intake and liver production. The cholesterol that is secreted by the liver into the blood is combined either with very low-density lipoproteins (VLDL) or high-density lipoproteins (HDL). VLDL cholesterol is then metabolized in the bloodstream to produce LDL cholesterol. LDL lipoprotein deposits cholesterol on the artery walls, causing the formation of a plaque. The cells of the artery wall secrete oxidative products from multiple pathways that can seed the LDL trapped in the subendothelial space and initiate lipid oxidation (Parathasarathy, 1994; Witztum JL, 1991). The oxidative modification of the trapped LDL is thought to occur in two stages. The first stage occurs before monocytes are recruited and results in the oxidization of lipids in LDL. Ox-LDL recruits monocytes and T cells to the intima and promotes the transformation of monocytes into macrophages and the second stage begins when monocytes are recruited to the lesion (Weber, 1995). LDL lipids are further oxidized and there is a massive accumulation of cholesterol. Such cholesterol-loaded cells have a foamy cytoplasm and have been called foam cells; they are the hallmark of the arterial fatty streak and leads to the formation of a fibrous plaque which upon rupture hails thrombosis and the clinical event. Oxidized LDL is a marker of oxidative stress specific to LDL particles.

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is a novel inflammatory marker, an enzyme that may denote a mechanistic link to plaque vulnerability. It is being studied broadly for a possible association with cardiovascular events and could provide a crux for lifestyle and pharmacological interventions (Sudhir, 2005). Lp-PLA₂ is secreted by macrophages and circulated on LDL cholesterol in the plasma and within atherosclerotic plaque. Oxidative modification can be aggravated by high amounts of Lp-PLA₂ which is present in ruptured and high risk human coronary atheroma. While initial interest with

oxidized LDL stemmed from the foam cell formation, it is now considered a key factor in promoting atherosclerosis by different mechanisms (Holvoet, 2004). Therefore, reducing the concentration and activity of Lp-PLA₂ may attenuate the atherosclerotic process.

Diets high in polyunsaturated fatty acids (PUFA), in particular, omega 3 fatty acids have been associated with a lower risk for heart disease due in part to their anti-inflammatory characteristics (Mozaffarian, et al., 2005). The common PUFA include docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), linoleic acid (LA) and linolenic acid (LNA). AA is an omega 6 PUFA and consumed in high amounts in the western diet. The downstream products of AA metabolism are the pro-atherogenic 2-series prostaglandins and leukotrienes and 4-series thromboxanes. On the other hand, end products of alpha-linolenic acid (ALA), a plant based omega 3 PUFA commonly seen in flaxseed and canola oils are less atherogenic.

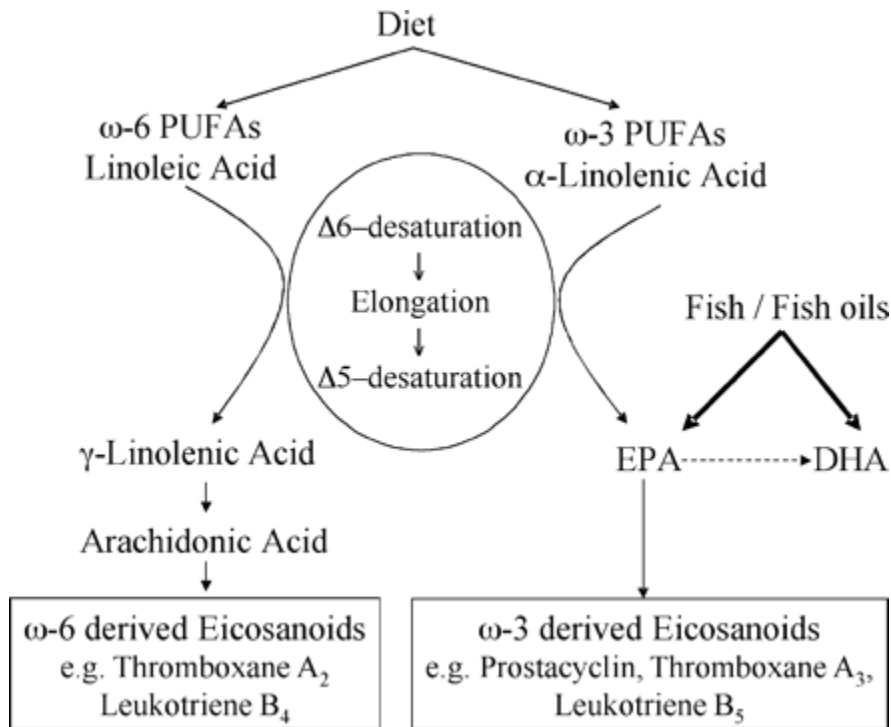


Figure 1: Desaturation and elongation pathways of omega 3 and 6 polyunsaturated fatty acids. Adapted from ‘the role of omega-3 fatty acids in the secondary prevention of cardiovascular disease’ (Lee & Lip, 2003)

ALA is desaturated and elongated to eicosapentaenoic acid whereas linoleic acid, the predominant omega-6 PUFA in humans can be desaturated and elongated to arachidonic acid. Eicosanoids derived from omega-3 PUFAs (ALA) tend to inhibit platelet aggregation and be anti-inflammatory whereas eicosanoids derived from arachidonic acid are pro-aggregatory and pro-inflammatory agonists (Simopoulos, 1991; Simopoulos, 1999). An increased intake of ALA may actually help in reducing the production of atherosclerotic mediators and attenuate the progression of CVD (James, Gibson, & Cleland, 2000). Browning, (2003) proposed an anti-inflammatory effect of EPA and DHA suggesting a reduction in pro-inflammatory blood plasma concentrations of eicosanoids, leukotrienes and cytokines mediated by omega 3 fatty acids. Diets high in PUFA are associated with

lower LDL levels; however the ability for ALA, EPA and DHA to directly alter LDL or Lp-PLA₂ levels has not been determined. Further research is necessary to determine the effects of ALA, EPA and DHA supplementation on the same.

STATEMENT OF THE PROBLEM

The purpose of this study was to determine the association of oxidized LDL with Lp-PLA₂ as well as the effects of EPA, DHA and ALA supplementation on ox-LDL concentration in glucose tolerant and intolerant male and female subjects.

HYPOTHESES

The following hypothesis will be tested ($p < 0.05$):

- 1) Baseline lipoprotein associated phospholipase- A₂ (Lp-PLA₂) concentration and activity will be positively correlated with baseline oxidized LDL (ox-LDL) in fasting plasma samples of both glucose sensitive and glucose intolerant subjects with the correlation being stronger in those who are glucose intolerant.
- 2) An isocaloric diet supplemented with α -linolenic acid (ALA, ~3% of total energy) or eicosapentaenoic acid/ docosahexaenoic acid (EPA/DHA, ~0.45% of total energy) will result in greater reductions of mean fasting plasma Lp-PLA₂ concentration and activity than the isocaloric diets not supplemented with ALA or EPA/DHA and these changes will be greater among those with higher oxidized LDL levels.
- 3) An isocaloric diet supplemented with ALA (~3% of total energy) or eicosapentaenoic acid/ docosahexaenoic acid (EPA/DHA, ~0.45% of total energy) will result in significant reductions in fasting plasma ox-LDL than the isocaloric diets not supplemented with ALA or EPA/DHA.
- 4) Changes in fasting plasma Lp-PLA₂ concentration and activity after isocaloric diet supplementation with ALA, EPA/DHA will be positively correlated with change in ox-LDL after the dietary

supplementation. This effect will be stronger among those participants who are glucose intolerant.

DELIMITATIONS, LIMITATIONS AND ASSUMPTIONS

This study required the participants to complete detailed dietary intake records every other week that were analyzed using a computer database. It was assumed that the detailed records and foods contained in the database accurately represent their dietary habits. Many efforts were taken to minimize any sort of error involved with these records including discussing the records every other week with the participants, obtaining nutritional information from packaging and having a single operator input the complete record for a subject. Compliance was assessed by counting the number of supplements returned at each scheduled weekly meeting. There was a possibility that the participants were not complying with the supplementation protocol or were consuming additional sources of omega-3 fats. We also assessed compliance by measuring plasma erythrocyte membrane concentrations for the various fatty acids.

The participants were asked to maintain their level of physical activity and to report any illness during the study because exercise and any acute illness could alter inflammatory markers. Non-compliance to this could have resulted in the elevation of inflammatory markers and potentially reduce the apparent effects of the intervention. Inflammation associated with Lp-PLA₂ is a process occurring within the intima of the arterial wall and it is difficult to obtain a sample of the actual atherosclerotic lesion where Lp-PLA₂ is directly involved. Venous blood samples were used and assumed to represent the concentration and activity of the inflammatory mediators.

CHAPTER II

LITERATURE REVIEW

Cardiovascular disease is an important cause of premature death in the western civilization. To better understand this, numerous studies have been done and ‘The National Health and Nutritional Examination Survey’ has estimated that 79 million American adults (1 in 3) had 1 or more types of CVD in 2004. Mortality data show that as the underlying cause of death, CVD accounted for 36.3% of all deaths in 2004, or 1 out of every 2.8 deaths in the United States (Rosamond, et al., 2007). It has also been shown that CVD claims more lives each year than cancer, chronic lower respiratory diseases, accidents and diabetes mellitus all put together (Minino, Heron, & Smith, 2006). This is a problem not only confined to the western world, as according to WHO estimates, 16.7 million people around the globe die of cardiovascular diseases each year. It is estimated that by 2010, CVD will be the leading cause of death in developing countries. It is estimated that by 2020, it will become the major cause of death in all regions of the world (Chockalingam, et al., 2000).

Traditional Risk Factors and Heart disease

Ancel Keyes, the physiologist nutritionist was without doubt the first one to provide the fundamentals of the relation between dietary fat and coronary heart disease. It was in the late 1940s that his search for risk factors began with the conviction that “the physio-chemical characteristics of the individual should have ‘predictive value’ for coronary

heart disease (Keys, 1953). And this was the beginning of the concept of ‘coronary risk factors’”.

Traditional risk factors

Cardiovascular disease has no geographic, gender or socioeconomic boundaries. Extensive clinical and statistical studies have identified various factors that increase the risk of CVD. Factors that have been described to significantly increase the risk of cardiovascular disease are the major risk factors or the non-modifiable factors like increasing age, male sex and heredity including race. Other factors associated with increased risk of cardiovascular disease, whose significance and prevalence have not yet been determined are called contributing or modifiable risk factors like high blood cholesterol, high blood pressure, physical inactivity, obesity, diabetes mellitus and smoking.

High blood Cholesterol

In order to travel in the bloodstream, cholesterol is carried in small packages called lipoproteins which are made up of lipid on the inside and protein on the outside. Examples include the low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Small, dense LDL particles are in particular prone to oxidation, providing a possible mechanism for their atherogenicity (Chancharne L, 1999). It has been demonstrated that elevated levels of circulating oxidized LDL is associated with coronary disease (Ehara, et al., 2001; Holvoet, et al., 1998; Toshima, et al., 2000) Total blood cholesterol is the most common measurement of blood cholesterol and the desirable level that puts a person at lower risk

for coronary heart disease is 200 mg/dl according to the National Cholesterol Education Program, with high density lipoprotein (HDL-C) concentrations above 60 mg/dl being found to have a protective effect against CVD (Expert Panel on Detection, 2001). A higher measure of total blood cholesterol is a major risk factor for coronary heart disease and stroke.

High blood pressure

Increased blood pressure is a major risk factor for coronary artery disease, with a 15% increased risk for CVD with each increase of 10 mm Hg in systolic blood pressure (MacMahon, et al., 1990). It has been suggested that the pulsatile component of blood pressure is the main mechanism leading to plaque rupture and, consequently, to acute coronary syndromes and other vascular complications (Safar, 2009). Pulse pressure (PP) is the difference between the systolic and diastolic pressures and is believed to increase the cardiovascular risk because of an increase in after load leading to left ventricular hypertrophy. It has also been shown that low diastolic blood pressure, being in part responsible for high pulse pressure, leads to an impairment of myocardial perfusion with all its adverse consequences. (Jankowski & Kawecka-Jaszcz, 2007)

Physical inactivity

Physical activity reduces the risk of cardiovascular disease and also helps prevent the development of diabetes. It helps one maintain weight loss and reduce hypertension, all independent risk factors in cardiovascular disease. There are various studies that suggest an inverse association between physical activity and cardiovascular disease among men. (Berlin JA, 1990; Bouchard C, 1995) There also have been some studies among women-one particular study was done in diabetic women and it shows that

increased physical activity , including regular walking, is associated with substantially reduced risk for cardiovascular events (Hu, et al., 2001)

Obesity

A mounting concern throughout the world is the increasing prevalence of obesity and there is growing evidence that obesity is associated with a chronic system low-grade inflammatory state and that inflammation is one of the potential mechanisms of obesity-related morbidity (Clement & Langin, 2007). The anatomical distribution of adipose tissue is a key indicator of metabolic alterations in cardiovascular diseases. Obesity has emerged as an independent risk factor and the excess of fat mass in the upper part of the body constitutes a classical risk factor for diabetes and cardiovascular diseases (Clement & Langin, 2007). It has also been confirmed to correlate with a heightened risk for: 1) insulin resistance, 2) hypertension, 3) hypertriglyceridemia, 4) prothrombotic state and 5) proinflammatory state (Smith, 2007).

Diabetes Mellitus

It has been shown that coronary heart disease is common in people with Type 2 diabetes mellitus (T2DM) (Laakso M, 1997) T2DM increases the risk of coronary artery disease, stroke, peripheral vascular disease and heart failure by 2-4 fold. (Kannel & McGee, 1979) The acceleration of atherosclerosis and atherothrombosis in diabetic patients has been related to endothelial dysfunction, dyslipidemia, insulin resistance and chronic hyperglycaemia.

Type 2 Diabetes Mellitus and Reactive oxygen species

There are various explanations for the pathogenic mechanisms in T2DM. One pathology of interest is the process of oxidation of biological macromolecules (Ceriello A, 2004).

Many studies have proposed that β -cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acids (FFA) levels, or even a combination of both (Evans, Goldfine, Maddux, & Grodsky, 2003). β Cells have shown to be particularly sensitive to Reactive Oxygen Species (ROS) because they are low in certain antioxidant enzymes like catalase, glutathione peroxidase, and superoxide dismutase (Tiedge, Lortz, Drinkgern, & Lenzen, 1997) And hence, it was suggested that oxidative stress had the ability to damage mitochondria and markedly blunt insulin secretion (Robertson, Harmon, Tran, Tanaka, & Takahashi, 2003).

Type 2 Diabetes Mellitus and Dyslipidemia

T2DM is characterized by higher low-density lipoprotein (LDL) cholesterol and triglycerides but lower HDL. In T2DM, LDL particles tend to be smaller and denser, and this likely increases their risk for atherosclerosis. (Feingold, Grunfeld, Pang, Doerrier, & Krauss, 1992)

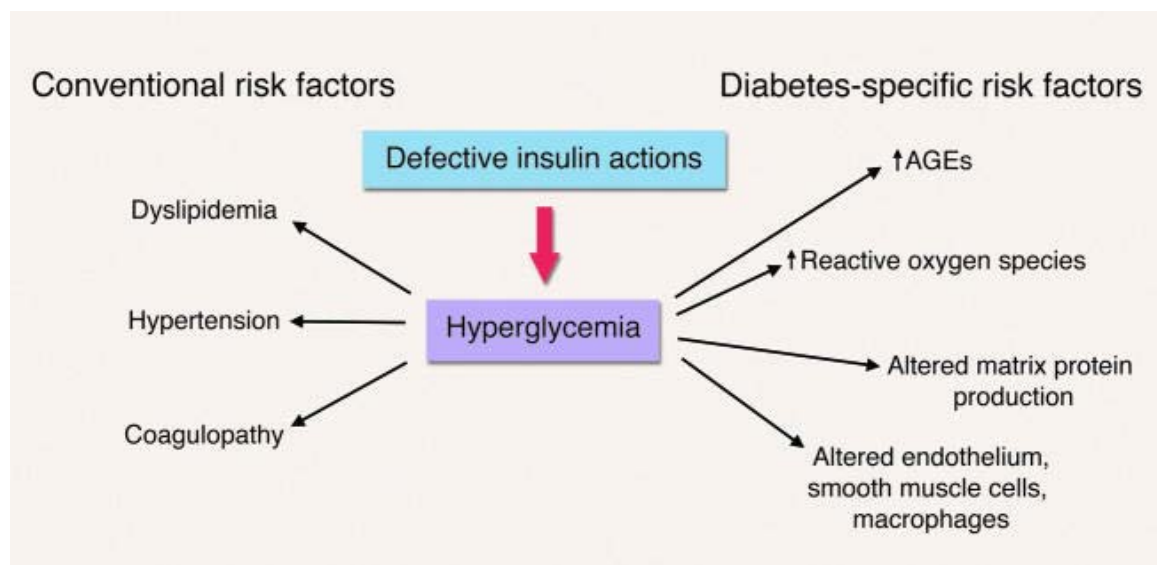


Fig 2: Potential causes of diabetes-induced macrovascular disease. Adapted from “Why does diabetes increase atherosclerosis? I don’t know!”(Goldberg, 2004)

Type 2 Diabetes Mellitus and Coagulopathy

Decreased fibrinolytic system capacity has been observed constantly in association with T2DM (Schneider & Sobel, 2002) and even in association with insulin resistance alone. This is attributable to altered activities of plasminogen activator (PA) (Hamsten, Wiman, de Faire & Blomback, 1985; Schneider, et.al, 1997) and increased concentrations of circulating plasminogen activator inhibitor type-1 (PAI-1), a marker of increased risk of acute myocardial infarction.(Sobel, Frye, & Detre, 2003) Proteolysis mediated by PA s contributes to vascular smooth muscle migration, neointimalization, and activation of matrix metalloproteases that precipitate plaque rupture (Libby, 1995; He, et al., 1989). As a result, the extent and persistence of thrombi are influenced by PA s (Schneider, et.al, 1997) and this is physiologically inhibited by PAI-1 (Pannekoek, et al., 1986). Consequently, fibrinolysis in response to thrombosis depends on a dynamic equilibrium between local concentrations of PA and PAI-1. The inhibition of fibrinolytic system activity in syndromes of insulin resistance and hyperinsulinemia, including T2DM, predisposes to thrombosis and its persistence sets the stage for coronary artery occlusion (Sobel, Woodcock-Mitchell, Schneider, Holt, Marutsuka & Gold, 1998). It has been suggested that increased PAI-1 is strongly associated with insulin resistance and it has been shown to accelerate the development of coronary plaques that precipitate acute coronary syndromes (Sobel, 1999).

Type 2 Diabetes Mellitus and AGEs

Diabetes Mellitus is characterized by hyperglycemia which results in short term metabolic changes and long term irreversible vascular and connective tissue changes including macro-vasculature complications such as atherosclerosis; potentially resulting

in heart disease, stroke and peripheral vascular disease (Forbes, Cooper, Oldfield & Thomas, 2003). Glycation, a chemical modification of proteins with reducing sugars, indicates a possible explanation for the association between chronic hyperglycemia and long term diabetic-specific complications (Dyer, 1993; McCance, et al., 1993). Research suggests that reducing sugars react with amino groups of long lived proteins to form end stage products called advanced glycation end products (AGEs) (Wautier & Guillausseau, 2001; Dyer, 1993; Sensi, et al., 1995). The occurrence of these AGEs favors vasoconstriction, inflammation, and thrombosis leading to CVD.

Smoking

Cigarette smoking is a major health hazard and contributes to cardiovascular morbidity and mortality. It predisposes the individual to different clinical atherosclerotic syndromes which include stable angina, acute coronary syndromes, sudden death, and stroke. Aortic and peripheral atherosclerosis are also increased and this may lead to intermittent claudication and abdominal aortic aneurysms.(Black, 1995) Polycyclic aromatic hydrocarbons found in the tar fraction of cigarette smoke have been shown to accelerate atherosclerosis in experimental models (Penn & Snyder, 1988) Currently, free radical-mediated oxidative stress is a rising crucial step for the development of atherosclerosis.(Gibbons & Dzau, 1994; Kojda & Harrison, 1999; Nedeljkovic, Gokce & Loscalzo, 2003) A reaction between free radicals such as superoxide and nitric oxide (NO) not only decreases NO availability but also generates peroxynitrite and this further augments the cellular oxidative stress(Kojda & Harrison, 1999). Increased oxidative stress with the loss of the protective effect of NO tips the cellular balance towards a

proatherogenic and prothrombotic environment (Nedeljkovic, et al., 2003; Ruberg & Loscalzo, 2002) in smokers.

Diet and Cardiovascular Disease

It was shown that a fat free diet reduces blood cholesterol levels (Mellinkoff, Machella, & Reinhold, 1950) and in the early 50s, Keys identified the relation of blood cholesterol with coronary heart disease. The next year, he indicated the possibility of prevention of coronary heart disease through reduction of dietary fat (Keys, 1953). Studies following this advance suggested that saturated fats had plasma lipid elevating effects while polyunsaturated fats had a lowering effect (Bronte-Stewart, Antonis, Eales, & Brock, 1956). Keys (Keys, 1965) and Hegsted et al (Hegsted, McGandy, Myers, & Stare, 1965) came forth with equations providing a basis of connecting the blood cholesterol levels to the proportions of saturated and polyunsaturated fat in the diet.

Saturated and polyunsaturated fats:

There have been studies describing that an average American diet has 34.3% and 15% of calories coming from total fat and saturated fat, respectively (Berglund, et al., 1999). Epidemiologic studies indicate that saturated fats are for the most part along with genetic makeup and other factors responsible for increasing the blood LDL cholesterol concentration and these elevated levels are accountable for increasing the risk of heart disease (Hu, Manson, & Willett, 2001). It has also been established that elevated cholesterol levels can increase the incidence of atherosclerosis which then increases the risk of coronary artery disease (Katan, et al., 1995; Grundy, 2001)

However, it is also known that from a practical food-choice perspective, it is unfeasible to achieve a nutritionally sufficient diet that has absolutely no saturated fat. The present recommendations to reduce cholesterol levels are focused on lifestyle changes to decrease the intake of saturated fats (<7% of total calories) and instead increase the intake of unsaturated fats (Expert Panel on Detection, 2001). Monounsaturated and polyunsaturated fats are the two unsaturated fats and are mainly found in many fish, nuts, seeds and oils from plants. Both have found to help lower blood cholesterol levels when used in place of saturated fats. Studies show that the Mediterranean style diet which uses olive oil containing high levels of monounsaturated fatty acids has beneficial effects and help reduce cholesterol levels. Another type of diet would be a diet enriched with polyunsaturated fatty acids which are of two types- omega-3 and omega-6 polyunsaturates. But there are certain convictions, certain concerns and a few unknowns surrounding this issue.

Convictions, concerns and unknowns:

The impact of dietary fat on coronary heart disease has been widely studied and the relation between serum cholesterol and coronary heart disease is now decisively established: it is curvilinear and continuous (Martin, Hulley, Browner, Kuller, & Wentworth, 1986). The protective role of polyunsaturated fats has yet to be investigated thoroughly as there are many uncertainties and unknowns since all the long term dietary intervention trials and some of the drug trials have unsettling results with a relative increase in non-cardiovascular mortality or no change in total mortality or both. Studies over the past decade have described that people with coronary heart disease have lower

than expected concentrations of linoleic acid, an essential fatty acid, in platelets and adipose tissue (Riemersma, et al., 1986). A critical question, not yet answered is whether reducing serum cholesterol levels by reducing dietary saturated fats or increasing polyunsaturated fats can actually lead to regression of occlusive atheromas.

Atherosclerosis

Inflammation plays a major role in all phases of atherosclerosis. The response-to-injury hypothesis of atherosclerosis based on numerous pathophysiological observations in humans and animals initially proposed that the first step in atherosclerosis was endothelial denudation (Ross & Glomset, 1973). But most recently, it has been suggested that it is endothelial dysfunction rather than denudation at work. Whichever process is at work, each lesion corresponds to a different stage in a chronic inflammatory process in the artery and if persistent and excessive, this process will result in a complex and advanced lesion. The endothelial dysfunction that results from injury can lead to compensatory responses and these modify the normal homeostatic properties of the endothelium. If the inflammatory response does not counteract or remove the offending agents, this process can continue indefinitely to stimulate migration and proliferation of smooth muscle cells which form an intermediate lesion becoming intermixed with the area of inflammation. If these responses are persistent, they can thicken the artery wall, so that up to a point, the lumen remains unchanged (Glagov, Weisenberg, Zarins, Stankunavicius, & Kolettis, 1987) which is compensated by gradual dilation, an event termed “remodeling.” These responses are mediated by monocyte-derived macrophages at every point of the disease (Jonasson, Holm, Skalli, Bondjers, & Hansson, 1986; van

der Wal, Das, Bentz van de Berg, van der Loos, & Becker, 1989) There is an increased emigration of macrophages and lymphocytes from the blood with continued inflammation and this leads to multiplication and activation of the cells releasing hydrolytic enzymes, cytokines, chemokines and growth factors. They can induce further damage and eventually lead to focal necrosis. Cycles such as those can lead to restructuring of the lesion leading to the formation of an advanced, complicated lesion which at some point may intrude into the lumen and alter the flow of blood when the artery is no longer compensated by dilation.

Oxidation of LDL

It has been established that hypercholesterolemia causes focal activation of endothelium in large and medium-sized arteries. The sign of an early-stage atherosclerotic lesion is the accumulation of lipid-loaded cells which underlie the endothelium of large arteries, namely the fatty streak. Numerous studies have shown that these lipid loaded cells mainly originate from blood-born monocytes and migrate into subendothelial space where they are differentiated into macrophages (Watanabe, Tokunaga, Fan, & Shimokama, 1989) These macrophages engulf a large amount of lipids deposited in the subintimal spaces and take on the appearance of foamy structures, called as foam cells. The infiltration and retention of certain atherogenic lipoproteins like low density lipoproteins (LDL) in the arterial intima start an inflammatory response in the arterial wall (Cybulsky & Gimbrone, 1991; Watanabe, et al., 1985) after being subjected to chemical modification such as oxidation, thereby leading to a series of biological reactions.

All atherogenic lipoproteins, once deposited in the intima, may exert direct or indirect proinflammatory effects (Fan & Watanabe, 2003). It has also been suggested that modification of LDL leads to the release of phospholipids which can activate endothelial cells, particularly at sites of hemodynamic strain. (Kume, Cybulsky, & Gimbrone, 1992) Consequently, hemodynamic strain and the accumulation of lipids may initiate an inflammatory process in the artery leading to inflammatory cytokines, chemokines, oxygen and nitrogen radicals and other inflammatory molecules, and eventually, to inflammation and tissue damage.

Balance between Inflammation and Metabolism

There are two processes that play a role in the progression of atherosclerosis- the balance between inflammatory and anti-inflammatory activity. Metabolic factors affect this in several ways, one contributing to the deposition of lipids in the artery. The altered lifestyles in the Western world have led to a sudden increase in the proportion of the population with obesity and are driving an increase in the prevalence of insulin resistance and T2DM, and it is expected to be followed by an increasing burden of premature cardiovascular disease (McNeill, et al., 2004). In a study by Holvoet et al, they found that metabolic syndrome; a clustering of high blood pressure, high blood glucose levels, high triglycerides, large waist circumference and low HDL cholesterol level, was associated with accelerated atherosclerosis. This was due to an increased accumulation of macrophages in association with endothelial dysfunction (Holvoet, De Keyzer & Jacobs, 2008).

Relative insulin deficiency is the result of insulin resistance with a slow decline in the regulation of blood glucose. It is characterized by hyperinsulinemia and decreased free fatty acid (FFA) uptake and/or increased FFA levels in adipocytes and in the circulation (Nigro, Osman, Dart, & Little, 2006). These FFA are generally associated with obesity and can modulate insulin signaling and this has led to a suggestion that there are molecular processes in the insulin signaling cascade that are susceptible to inhibition or activation thus giving way to a spectrum of insulin-resistant states.

In human and animal studies, it has been demonstrated that with increasing obesity there is an increasing oxidative stress in the fat compartment and in circulating markers of oxidative stress (Furukawa, et al., 2004). There are two potential mechanisms: the decreased production of anti-inflammatory cytokines and the increased levels of systemic oxidative stress mediated via increased oxidative enzymes. (Nigro, et al., 2006) Therefore, not only do the pro-inflammatory cytokines and reactive oxygen species cause peripheral insulin resistance but there is also a direct impact on the endothelium causing endothelial dysfunction and this initiates the atherosclerotic cascade (Ross, 1999). However, the exact mechanisms for the increased susceptibility and progression of atherosclerosis in patients with T2DM are still debatable but there has been substantial work done on the direct actions of insulin on blood vessels and cells of the vasculature (Nigro, et al., 2006). Considerable evidence shows that insulin physiologically targets vascular endothelium and, consequently, a potential link between insulin resistance and atherosclerosis exists. But there is no clarity about the direct actions of insulin in the vasculature. It is still being questioned if insulin actions that are antiatherogenic are

attenuated and those that are proatherogenic are exaggerated or both when insulin resistance is associated with accelerated processes of atherosclerosis.

Insulin resistance which co-exists with compensatory hyperinsulinemia is associated with increased levels of pro-coagulant factors and these add to the enhanced platelet aggregation seen in insulin resistant states. While it is known that insulin levels are typically higher in the fed state, it is postulated that the degree of insulin sensitivity maybe influenced by the composition of diet. Chronic overload of energy consumption stimulates insulin secretion, triglyceride synthesis and fat accumulation in particular visceral obesity with down-regulation of insulin receptors and post receptor signaling, thus promoting hyperinsulinemia and insulin resistance.

Saturated fats and trans-fatty acids are particularly associated with insulin resistance.

Long chain polyunsaturated fatty acids play a physiological role in maintaining cell membrane fluidity and cell signaling. An association between the ratio of omega 6 to omega 3 fatty acids and the prevalence of T2DM is also under research.

Lp-PLA₂

Lp-PLA₂ is a novel inflammatory marker that is being studied broadly for a possible association with cardiovascular events. Lp-PLA₂ has been determined to independently predict future cardiovascular events in several epidemiology studies (Ballantyne, et al., 2004; Blake, Dada, Fox, Manson, & Ridker, 2001; Koenig, Khuseyinova, Lowel, Trischler, & Meisinger, 2004; Oei, et al., 2005; Packard, et al., 2000). Most of these studies have shown that elevated levels of Lp-PLA₂ (mass or activity) are an independent predictor of future events. There is growing evidence that elevated Lp-PLA₂ may mean

increased risk for recurrent events in patients with clinically manifested CVD.

(Blankenberg, et al., 2003; Brilakis, et al., 2005) There has been controversy about the role of this enzyme in relation to atherosclerosis with initial studies suggesting an atheroprotective effect through its actions in hydrolyzing polar phosphatidylcholines (Brilakis, et al., 2005; Stafforini, McIntyre, Zimmerman, & Prescott, 1997; Watson, et al., 1995). However, recent studies suggest that the actions of Lp-PLA₂ are actually pro-atherogenic (MacPhee, et al., 1999)

Lp-PLA₂ in circulation is principally bound to LDL in humans (Stafforini, et al., 1999) and the substrate for the enzyme is provided by the oxidized phospholipid component of LDL (Mertens & Holvoet, 2001; Zalewski & Macphee, 2005). Following oxidation of LDL, Lp-PLA₂ acts very rapidly to hydrolyze one of the fatty acid moieties of the phospholipid and produces oxidized non-esterified fatty acid (NEFA) and lysophosphatidylcholine (lysoPC), both of which are inflammatory molecules (MacPhee, et al., 1999).

Most of the total body oxidized LDL exists within the intima (when compared to circulation) and therefore, the products of Lp-PLA₂ activity are produced within the vessel wall where the inflammatory processes of atherosclerosis occur. Both the inflammatory by-products of Lp-PLA₂ are highly soluble in circulation and by diffusion throughout atheroma, affect the various cell types in atherosclerosis (Zalewski & Macphee, 2005). It is not yet clear if the oxidative stress which is associated with T2DM is attributable to Lp-PLA₂ activity. A recent animal study demonstrated that the expression of Lp-PLA₂ by bone- marrow derived leukocytes was considerably up-regulated in the presence of AGEs (Shi, et al. 2005) However, in few other studies,

participants with diabetes did not markedly differ in their Lp-PLA₂ levels from participants without diabetes (Persson, Nilsson, Nelson, Hedblad, & Berglund, 2007). Hence, it is an area that needs to be explored in association with metabolic disorders.

Omega 3 PUFA and inflammation

Some reports have shown Omega-6 fats to be as high as 20-25 fold more common in the typical western diet than omega-3 fats (Simopoulos, 1991). The consumption of a diet rich in linoleic acid (LA: 18:2omega-6) present in vegetable oils like soy, corn, safflower and sunflower oils is mainly the reason for the preponderance of omega-6 fats. The omega-3 homologue of alpha linolenic acid (ALA: 18:3 ω -3), on the other hand, seen in leafy green vegetables, flaxseed and canola oils is consumed less. It has been proven that even moderate dietary supplementation with omega 3 PUFA significantly increases their level in circulating monocyte cells within two weeks (Gibney & Hunter, 1993). Once ingested, these 18 carbon fatty acids can undergo desaturation and elongation to form 20 carbon fatty acids.

LA is converted to AA (Arachidonic acid, 20:4 ω-6) and ALA is converted to EPA (Eicosapentaenoic acid, 20:5 ω -3). These 20 carbons, Omega-6 and omega 3 fatty acids compete in the formation of prostaglandins. EPA is in competition with AA for the synthesis of prostaglandins and leukotrienes at the cyclooxygenase and 5-lipoxygenase level. The EPA and docosahexaenoic acid (DHA) from fish or fish oil leads to mechanisms that decrease inflammation including: 1) decrease in PGE₂ (Prostaglandin E₂) metabolite production 2) decrease in LTB₄ (Leukotriene B₄) formation, an inducer of inflammation and a strong inducer of leukocyte chemotaxis and adherence 3) decrease

in TXA2 (thromboxane A2), a vasoconstrictor and a potent platelet aggregator with an 4) increase in prostacyclin PGI3 (vasodilator, inhibitor of platelet aggregation) (Simopoulos, 1986). EPA is also a 5-lipoxygenase substrate and can lead to the formation of LTB5, but this leukotriene has little inflammatory activity compared to LTB4, formed by AA. (Goldman, Pickett, & Goetzl, 1983; James, Cleland, Gibson, & Hawkes, 1991)

Many factors are responsible and contribute the complex process of inflammatory reactions. Microbiological, immunological and toxic agents can start the inflammatory response by stimulating various cellular and humoral mediators and through that process, excessive amounts of interleukins are released and play a very important role in the early phase of inflammation. Animal and human studies support the hypothesis that omega-3 PUFA suppress cell mediated immune responses by possibly increasing the membrane fluidity and altering the expression of membrane proteins. Pro-inflammatory eicosanoids of AA metabolism are released from the membrane phospholipids during this course and when EPA competes with AA for enzymatic metabolism, it results in the production of less inflammatory and chemotactic derivatives.

Therefore, an increase in dietary omega-3 fats can alter the balance of eicosanoids produced to a less inflammatory mixture. The cytokines IL-1 β and TNF- α have been known to have pro-inflammatory cellular actions which include increasing expression of adhesion molecules which are required for leukocyte extravasation and helping in the production of collagenases. Inclusion of omega-3 fats in the diet has also been shown to suppress the monocyte synthesis of both TNF- α and IL-1 β in both healthy (Endres, et al., 1989) and in patients with rheumatoid arthritis (Kremer, et al., 1990). Ingestion of

omega-3 fatty acids leads to suppression of thromboxane A₂ (TXA₂) synthesis by platelets and by gradient preparations of blood mononuclear cells.

James, et al., concluded that the results provided evidence of a role for TXA₂ as an autocrine or paracrine facilitator of cytokine synthesis. They suggested that TNF- α and IL-1 β synthesis were inhibited by TXA₂ and this inhibition was overcome by the addition of an active TXA₂ analogue. Also, cytokine synthesis was inhibited by TXA-receptor antagonists. Omega-3 fatty acids also inhibits PGE₂ synthesis leading to the inhibition of TXA₂ synthesis and this might provide a very important yet only one element of a complex mechanism responsible for inhibition of cytokine synthesis by omega-3 fatty acids (James, et al., 2000).

An alternate approach to increasing the cellular EPA concentrations is to enhance ALA intake, a progenitor of EPA. It was demonstrated by Caughey, et al., that when flaxseed oil, which contains \approx 56% ALA, was used by healthy male volunteers in their domestic food preparation, leukocyte EPA concentrations were increased and both IL-1 β and TNF- α production were suppressed by \approx 30% after 4 wk. Dietary ALA has also been shown to elevate membrane EPA levels in vivo, but the conversion is dependant on omega 6 PUFA intake and it has been recommended that studies should modify the omega 6: omega 3 to limit the influence of omega 6 (Arterburn, Hall, & Oken, 2006).

Omega 3, Lp-PLA₂ and ox-LDL

Results from several studies evaluating the effect of omega 3 PUFA on Lp-PLA₂ and ox-LDL show a potential mechanism between them and hence the need for further research. As previously mentioned, accumulation of ox-LDL is key in the formation of

foam cells in early atheroma. Plasma levels of ox-LDL have been associated with both inflammation and subclinical atherosclerosis development (Hulthe & Fagerberg, 2002) and are also correlated with metabolic syndrome risk factors (Sigurdardottir, Fagerberg & Hulthe, 2002). The most abundant oxidized phospholipid in ox-LDL is oxidized phosphatidylcholine (oxPC) which is a specific substrate for Lp-PLA₂, which releases a highly atherogenic lipid, lysophosphatidylcholine (LPC) (Tokumura, Toujima, Yoshioka & Fukuzawa, 1996; Stremmler, Stafforini, Prescott, Zimmerman & McIntyre, 1989). Thus in plasma, Lp-PLA₂ is thought to circulate bound to ox-LDL, but the relations between Lp-PLA₂ and ox-LDL in atherosclerotic lesions is not well understood. (Vickers et. al., 2009)

Several studies have shown the effect of omega 3 PUFAs on CVD, a few in regard to the mechanism of omega 3s. In fact, Browning (Browning, 2003) proposed an anti-inflammatory impact of EPA and DHA suggesting a reduction in pro-inflammatory blood plasma concentrations of eicosanoids, leukotrienes and cytokines mediated by omega 3 fatty acids. The basic mechanism by which omega 3 fatty acids appear to alleviate risk for CHD begins with enrichment of membrane phospholipids with EPA and DHA (Harris, 2007). Once these long chain omega 3 fatty acids are resident in the cell membranes, they are believed to have at least four separate effects. First, they may alter membrane properties because of their highly unsaturated nature (Stillwell & Wassall, 2003) which can in turn alter the manner in which the membranes interact with their ligands (Ma et al, 2004). Secondly, altering membrane fatty acid composition can affect the ability of membrane-associated proteins to associate with the membrane or interact with other protein complexes involved with cell signaling systems. Recent studies have

demonstrated that several signaling proteins are enriched in lipid rafts and can be displaced from membrane rafts by EPA. The raft lipid composition is also altered after EPA supplementation (Li et al, 2005). Also, a variety of cell stressors initiate intracellular G-protein linked responses, one of which is the activation of phospholipase A₂ (PLA₂) (Calder, 2006). PLA₂-liberated omega-3 fatty acids may directly modify the activity of ion channel themselves, resulting in altered resting membrane potentials which abort the arrhythmic role of partially depolarized myocytes (Leaf, Kang, Xiao & Billman, 2006). Finally, intracellular omega-3 fatty acids are also able to serve as ligands for a variety of nuclear receptors which impact inflammatory responses and lipid metabolism. Omega-3 fatty acids elicit hypotriglyceridemic effects by coordinately suppressing hepatic lipogenesis, upregulating fatty oxidation in the liver and skeletal muscle through PPAR activation, and enhancing flux of glucose to glycogen through downregulation of HNF-4 α . Omega-3 fatty acids may also act by enhancing postprandial chylomicron clearance through reduced VLDL secretion and by directly stimulating lipoprotein lipase activity. These combined effects support the use of omega-3 fatty acids as a valuable clinical tool for the treatment of hypertriglyceridemia. (Davidson, 2006; Deckelbaum, Worgall & Seo, 2006). In all probability, as a consequence of these membrane effects, omega-3 FA reduces the activity of inflammatory cells and levels of certain inflammatory mediators (Calder,2006), which may ultimately result in reduced arterial plaque fragility (Thies et al, 2003).

Other studies proposed the relation between the intake of omega 3s and CHD suggesting that CHD is generally high in countries where the estimated intake of EPA and DHA is extremely low (long chain omega-3 fatty acid intake \leq 0.07% energy) (

Hibbein, Nieminen, Blasbalg, Riggs & Lands, 2006). Also, the greater intake of EPA and DHA reduce all-cause mortality, cardiac and sudden death, and stroke (Wang, et al., 2006). Similarly, low intakes of ALA can also be found in developing countries where cardiovascular disease is on the rise (Reddy, 2004). A few cohort studies show an association between low ALA intake and the risk of fatal coronary heart disease (CHD) (Hu, et al., 1999; Pietinen, et al., 1997). Some epidemiological studies (Baylin, Kabagame, Ascherio, Spiegelman & Campos, 2003; Rastogi, et al., 2004; Dolecek, 1992), but not all (Hu, et al., 1999; Albert, et al., 2005) have established a strong inverse association between dietary ALA and risk of myocardial infarction. In one study, both ALA and LA substituted diets (17% of total energy) were shown to lower serum lipids as well as biomarkers of inflammation (Zhao, et al., 2004). The association between ALA and MI could be due to potential anti-inflammatory properties of ALA that were found in some studies (Ferrucci, et al., 2006; Lopez-Garcia, et al., 2004). These studies also showed association between increased ALA intake and decreased plasma concentration of markers of inflammation like C-reactive protein. The potential anti-inflammatory effects of ALA could be mediated, in part, through its conversion to EPA and DHA through enzymes like desaturase and elongase (Nakamura & Nara, 2004). However, other studies (Burdge, 2004; Goyens, Spilker, Zock, Katan & Mensink, 2006) suggested that this conversion is generally low (<8%) suggesting that ALA may exert direct protective anti-inflammatory effects. In contrast, ALA has been shown to inhibit the production of nitric oxide, which through its vasodilator actions, serves as an important regulatory molecule in the maintenance of vascular homeostasis (Sladek, et al., 2007). Some studies have suggested Omega 3 PUFAs have also been shown to suppress oxidized LDL and

reduce cardiovascular disease risk. Finnegan et al., 2003 suggested that dietary EPA and DHA at an intake of 1.7 g/day increased the potential susceptibility of LDL oxidation in vitro, whereas increasing dietary ALA had no effect on LDL susceptibility to oxidation. One study by Egert et.al, 2007 has shown that the ex vivo susceptibility of LDL to oxidation is mainly dependent on dietary factors, especially antioxidants and the fatty acid composition of the diet. A diet rich in monounsaturated fatty acids (MUFAs) leads to an enrichment of MUFAs in the LDL particle and thereby to a higher resistance to the oxidative processes as compared to diets rich in omega-6 polyunsaturated fatty acids (PUFAs), especially linoleic acid (C18:2). This study demonstrated that the enrichment of the diets with ALA, EPA or DHA changed the omega-3 fatty acid profiles in the LDL particles confirming that the ingested fatty acids were effectively incorporated. An increased dietary intake of ALA, EPA or DHA led to a considerable enrichment of the respective fatty acid in the LDL particles to make them more prone to oxidation. It was shown that fish oil supplementation containing omega-3 PUFA compared to supplementation with omega-6 PUFA or MUFA oils reported a reduction in lag time, an indicator of enhanced oxidizability, and a reduction in oxidation rate, an indicator of decreased oxidizability (Pederson, et al., 2003; Sorenson, et al., 1998; Suzukawa, Ishikawa, Yoshida and Nakamura, 1995). This contradictory result suggests that lipids present on LDL surface are initially more readily oxidized in LDL particles from individuals consuming fish oil but that the extent of oxidation is lower. In addition, dietary ALA also caused EPA enrichment. Their study also suggested that dietary EPA seemed to be preferentially incorporated in the LDL particles, and, simultaneously increased their DHA content. Their conclusions were that perhaps ALA enrichment did

not enhance LDL oxidizability, but the effects of EPA and DHA on the *ex vivo* LDL oxidation were somewhat contradictory, possibly in part due to their complex metabolic pathways with a variety of possible conversion and retro-conversion reactions. Whatever the mechanism, all of the above studies suggest there is some sort of association between omega 3 PUFAs and inflammation and myocardial infarction. They show the need for further clarification and this study hopes to show the omega 3 PUFAs help in reducing the concentration and activity of ox-LDL as well as Lp-PLA₂. This in turn helps in reducing the atherogenic activity of the plaques and decreases the risk of a myocardial infarction in glucose sensitive and intolerant subjects.

CHAPTER III

METHODS AND PROCEDURES

Methods and Procedures

Before beginning this study, approval from the Colorado State University Committee on Human Research was obtained. The subjects were required to sign an Informed Consent that explained the dietary obligations and risks that could be associated with being involved in this study and also testing procedures.

Subjects

Fifty-nine subjects (44 females, 15 males) were recruited by mailed letters, a local senior club newsletter and fliers placed at doctors' offices and a local. Participants were 50-80 years of age and weight stable for a minimum of twelve months prior to the study. They ranged from sedentary to moderately active (exercise no more than 3 days/week, 1 hour/day). Activity level was assessed by both an initial phone screening and the Stanford Physical Activity Questionnaire administered before study initiation. Because of our interest in recruiting pre-diabetic and normal glucose tolerant subjects as well, we screened them using a FreeStyle Flash blood glucose meter following a twelve hour fast. Subjects were classified as diabetic by self report of a clinical diagnosis as well as with a fasting blood glucose level of > 125 mg/dl. Pre-diabetic individuals were those with a fasting blood glucose level between 100 and 125 mg/dl. Of the fifty nine subjects, twenty four had glucose levels in the pre-diabetic range and thirty five within a healthy fasting

blood glucose range. A telephone screening interview helped us eliminate individuals with known cardiovascular disease (CVD) or any other inflammatory condition.

Exclusionary criteria also consisted of women who were pregnant, smoking, on hormone therapy and consumption of prescription drugs or over the counter drugs that were known to alter inflammatory markers which included oral contraceptives. Individuals were also excluded if they had either been chronically consuming an omega-3 supplement for a year or more. However, due to the significant number of interviewees consuming baby aspirin on a regular basis, these individuals were allowed into the study on the basis that the frequency and dosage of the drug usage would not change during the course of the study.

Experimental Design:

Participants were initially screened on the telephone to determine their eligibility. Following initial acceptance into the study, the subjects were asked to set a preliminary appointment at the Human Performance Clinical/Research Laboratory at Colorado State University to complete the Informed Consent, Psychosocial Questionnaire, Health History Questionnaire, and Stanford Physical Activity Questionnaire. During this session, a research assistant measured fasting blood glucose and instructed the participant in proper completion of the three day diet record. This diet record was to include 2 week days and 1 weekend day that best represented the participant's typical diet and was used to determine the individual's average daily caloric intake. Based on the calculation of total energy from the baseline three-day food records, subjects were assigned a supplement dosage that represented 5.3% of average caloric intake, resulting in range from 6 g/day to 16 g/day of the provided oils. Because we wanted to compare a similar

amount of omega-3 fatty acids using fish oil and flaxseed oil (e.g. EPA/DHA vs ALA) we used the studies of Emken et al., showing that in a typical U.S diet ~300 mg of omega-3 (EPA/DHA) would be synthesized from ~2 g of ALA; therefore we estimated that ~7 grams of ALA= ~1 gram of EPA/DHA (Emken, Adlof & Gulley, 1994). To ensure similar intakes of fat between the groups we supplemented the fish oil group with olive oil. Specifically, the ALA group was supplemented with flaxseed oil (Spectrum Naturals, Inc. Boulder, CO, 0.57 g alpha-linolenic acid/capsule) where 3% of their calories came from ALA and the other 2.3% from other fats contained in the flax oil capsule. The EPA/DHA group was supplemented with fish oil (Spectrum Naturals, Inc. Boulder, CO, 180 mg eicosapentaenoic acid/capsule and 120 mg docosahexaenoic/capsule) where 0.45% of their calories came from EPA/DHA, 0.65% from other fats in the fish oil, and the other 4.2% of their calories came from olive oil (Seagate, Inc San Diego, CA 800 mg MUFAs/capsule). The control group used olive oil, (Seagate, Inc San Diego, CA 800 mg MUFAs/capsule) where 5.3% of the calories came from olive oil. A week after their initial visit, participants were scheduled for baseline testing which included anthropometric measurements (height, weight and waist circumference), body composition which was determined by dual energy x-ray absorptiometry (DEXA, Lunar DPX-IQ v.4.5c), fasting blood sample, blood pressure recordings, and initiation of supplementation protocol. Subjects were asked to consume the assigned supplement daily for eight weeks, while keeping their physical activity, weight and medications the same. Each participant received individual counseling on dietary modifications to reduce the consumption of saturated fat, decrease/eliminate omega-3 fatty acid intake and decrease/eliminate antioxidant intake. Diets were to remain

isocaloric by substituting the same number of calories reduced in high fat foods with the fats provided by the supplements. All subjects participating in the study were blinded to the specific dietary supplementation that was provided. For the duration of the study (8 weeks), subjects met with a research assistant once a week in order to take anthropometric measurements, provide supplementation and monitor any changes in their activity level, medications or illness. Subjects were asked to return any remaining capsules from the previous week and were supplied with a new set of capsules. The remaining capsules that were returned were counted and compared against the known number of capsules previously supplied to the subject to determine his/her compliance. It was considered non compliant if the daily intake deviated more than one gram from the prescribed amount and the weekly capsule counts were used to determine this average daily consumption. Compliance to the dietary intake recommendations was assessed by a 3-day diet record that was completed by each participant every other week. At the end of week four and week eight of the study, testing periods were held similar to baseline testing (fasting blood draw, measurement of anthropometrics and DEXA analysis at the end of week 8) Subjects were instructed to maintain their normal diet and physical activities to avoid any fluctuations in their weight and to minimize further changes.

Anthropometrics

A physician's balance scale was used to measure body weight (nearest 0.1 kg) and height (nearest 0.5 cm). Waist circumference was measured at the level of the umbilicus using a Gulick spring-loaded measuring tape. Medium length DEXA scans were used to assess percent body fat.

Dietary data

Food Intake and Analysis System (FIAS v 3.99 University of Texas School of Public Health, 1998), which utilizes the United States Department of Agriculture (USDA) database of food composition information (including individual fatty acids) for over 7300 foods was used to analyze the food records.

Blood Collection

Venous blood samples were obtained from the antecubital region after an overnight fast and these blood samples were centrifuged for 15 minutes (2800 rpm, 0° C, S4180 rotor; Beckman GS-15R Centrifuge) to separate plasma. Three wash cycles with EDTA and 10 minutes centrifugation were used to isolate red blood cells. Samples of red blood cells (with equal part K₃ – EDTA) and plasma were stored at -80° C until analysis.

Assays

Total cholesterol, triglycerides and HDL-C were measured using commercially available kits (Waco Chemicals USA Inc, Virginia) and automated microplate reader (Biotek Instruments, Inc, Vermont) Ox-LDL concentration was determined by using the Mercodia Oxidized LDL Competitive ELISA. Lp-PLA₂ concentration and enzyme activity were determined by enzyme linked immunosorbant assay (ELISA) and calorimetric activity method respectively. Both assays were performed by diaDexus, Inc. Fatty acid composition of red blood cell membranes and flaxseed oil and fish oil capsules were evaluated by gas liquid chromatography (6890A Series Gas Chromatographer, Model G1530A, Agilent Technologies)

Statistical Analysis

Descriptive statistics (means and standard deviations) were performed as well as a one-way ANOVA for comparison of baseline values for age, anthropometrics, percent body fat, Lp-PLA₂ levels, LDL-C, HDL-C, triglycerides, cholesterol, cholesterol/HDL ratio, oxidized LDL, oxidized LDL/LDL ratio and medication use between controls and the ALA and EPA/DHA intervention groups. BMI, Lp-PLA₂ activity, triglycerides, and oxidized LDL values were log transformed due to their skewed distributions. A Kruskal-Wallis test was used to compare gender and medication use between the groups. Because the dietary data and erythrocyte membrane fatty acid data were not normally distributed even after log transformations, a Wilcoxon signed rank test was used to compare the mean grams for the dietary variables and the mean membrane fatty acid change from baseline to post-testing between the control, flaxseed oil and fish oil groups. A comparison across groups was calculated for changes scores using the Kruskal-Wallis test. The omega-6/omega-3 ratio was calculated using the sum of arachidonic acid and linoleic acid divided by the sum of ALA, EPA and DHA.

Univariate spearman correlation coefficients were also determined for the relationship of baseline Lp-PLA₂ mass and activity with the variables listed above. Change scores were also calculated for each of these variables comparing pre to post values and spearman correlation coefficients were determined for changes in Lp-PLA₂ mass and activity and the above listed variables.

A repeated measures general linear model (GLM) was used to determine time x group interactions for BMI, WC, percent fat, Lp-PLA₂ levels, LDL-C, HDL-C, triglycerides, cholesterol/HDL-C ratio, oxidized LDL and oxidized LDL/LDL ratio

between the baseline and post-test control, ALA and EPA/DHA groups. Age, gender, and medication use were controlled for in these analyses. Statistical significance was accepted at $p < .05$. All analyses were run using SPSS 13.0 (Statistical Package for Social Science, SPSS Inc., Chicago, IL) for Windows.

CHAPTER IV

RESULTS

Results

Fifty nine subjects (44 females, 15 males), mean age 61 years, completed the eight weeks protocol and were included for the analysis. Insulin data was not included for two subjects whose results were not within a normal physiological range. Descriptive statistics at baseline for the control, ALA and EPA/DHA groups are shown in Table 1.

Descriptive statistics at baseline for the control, ALA and EPA/DHA groups

	Control group N=19	ALA group N=20	EPA/DHA group N=20	p- value
	Mean \pm S.D.	Mean \pm S.D.	Mean \pm S.D.	
Age	58.6 + 6.3	63.4 + 8.2	62.1 + 7.7	0.13
Gender (% female)	74%	65%	85%	0.35
BMI (kg/m ²)	25.5 + 3.6	25.2 + 3.1	26.3 + 4.9	0.76
WC (cm)	86.6 + 9.8	85.6 + 10.6	85.0 + 13.9	0.9
Percent Fat (%)	32.3 + 8.3	31.3 + 7.9	35.9 + 6.1	0.2
Lp-PLA ₂ mass (ng/mL)	204.1 + 58.8	219.9 + 64.0	222.9 + 56.8	0.58
Lp-PLA ₂ activity (nmol/min/mL)	142.4 + 28.9	145.8 + 29.1	140.1 + 36.6	0.78
Glucose mg/dL	95.6 + 19.8	97.2 + 18.4	94.4 + 21.6	0.9
LDL-C (mg/dL)	136.2 + 42.6	117.2 + 27.3	128.9 + 27.2	0.2
HDL-C (mg/dL)	42.8 + 13.2	44.2 + 13.4	39.5 + 12.6	0.5
Triglycerides (mg/dL)	94.1 + 58.6	80.6 + 40.6	90.1 + 37.7	0.76
Total Cholesterol (mg/Dl)	197.8 + 43.3	177.5 + 30.2	185.5 + 29.2	0.19
Oxidized LDL (U/L)	70.6 + 30.9	64.2 + 23.6	66.7 + 22.9	0.81
Ox. LDL/LDL ratio	0.53 + 0.22	0.56 + 0.21	0.53 + 0.21	0.79
Medications				
Statins	21%	15%	15%	0.85
Anti-inflammatory	32%	60%	25%	0.06
Blood sugar	0%	0%	5%	0.38

BMI, body mass index; WC, waist circumference, Lp-PLA₂, lipoprotein associated phospholipase A₂, LDL-C, low density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol

On average, the subjects in all the three groups (Control, ALA and EPA/DHA groups) were in their early 60s, overweight according to BMI and pre-diabetic. The subjects were randomized into these groups and there were no significant differences between the groups for age, gender, body mass index (BMI), waist circumference (WC), percent body fat or fasting glucose level. However, the ALA group included more than half of them on anti-inflammatory medications (60% in the ALA compared to 32% in control and 25% in EPA/DHA group).

Dietary analysis

Analysis of food records showed that the habitual diets of all subjects were similar to a typical American diet and there were no differences in nutrient intake between the Control, ALA, and EPA/DHA in daily caloric intake at baseline compared to the average intake for the duration of the study as shown in table 2. As expected, monounsaturated fat intake significantly increased in the control group and the EPA+DHA group due to olive oil supplementation, while polyunsaturated fat intake significantly increased in the ALA group. Although polyunsaturated fats did not significantly increase in the EPA+DHA group, the omega-6: omega-3 ratio did significantly decrease, which was also demonstrated in the ALA group.

	Control Group N=19			ALA Group N=20			EPA/DHA Group N=20		
	Baseline	Average intake weeks 1-8		Baseline	Average Intake weeks 1-8		Baseline	Average Intake weeks 1-8	
	Mean (%) \pm S.D.	Mean (%) \pm S.D.	p-value	Mean (%) \pm S.D.	Mean (%) \pm S.D.	p-value	Mean (%) \pm S.D.	Mean (%) \pm S.D.	p-value
Total Calories	1888.1 \pm 453.0	1985.4 \pm 394.4	0.38	1782.0 \pm 432.0	1858.5 \pm 441.9	0.09	1753.9 \pm 380.2	1792.2 \pm 318.9	0.45
Total Fat (g%)	75.7 (36%) \pm 25.3	85.3 (39%) \pm 17.9	0.01	72.8 (36%) \pm 25.2	81.5 (38%) \pm 22.7	0.01	70.4 (36%) \pm 24.2	83.0 (41%) \pm 19.5	0.01
PUFA (g%)	14.9 (7%) \pm 5.4	15.4 (7%) \pm 3.6	0.69	15.3 (7%) \pm 7.1	22.5 (10%) \pm 7.4	0	16.1 (8%) \pm 8.1	17.0 (8%) \pm 5.0	0.23
MUFA (g%)	29.8 (14%) \pm 11.0	37.6 (17%) \pm 8.7	0	27.9 (14%) \pm 11.1	29.7 (14%) \pm 8.5	0.29	28.1 (14%) \pm 11.0	35.4 (17%) \pm 9.3	0.01
SFA (g%)	25.4 (12%) \pm 10.5	24.5 (11%) \pm 6.8	0.57	24.3 (12%) \pm 7.9	23.9(10.2%) \pm 7.0	0.74	20.9 (11%) \pm 7.0	23.9 (12%) \pm 6.3	0.12
ALA (g%)	1.1 (0.6%) \pm 0.4	1.3 (0.6%) \pm 0.3	0.13	1.4 (0.7%) \pm 0.5	7.2 (3%) \pm 1.7	0	1.7 (0.8%) \pm 2.0	1.6 (0.8%) \pm 0.7	0.22
EPA (g%)	0.01 (0.01%) \pm 0.02	0.03 (0.01%) \pm 0.04	0.19	0.05 (0.03%) \pm .09	0.03 (0.02%) \pm 0.04	0.53	0.05 (0.03%) \pm 0.83	0.41 (0.22%) \pm 0.07	0
DHA (g%)	0.03 (0.01%) \pm 0.04	0.05 (0.02%) \pm 0.06	0.03	0.09 (0.05%) \pm 0.10	0.06 (0.03%) \pm 0.04	0.23	0.06 (0.04%) \pm 0.11	0.31 (0.16%) \pm 0.05	0
n6/n3 ratio	12.0 \pm 4.1	10.5 \pm 2.1	0.13	8.9 \pm 3.4	2.0 \pm 0.5	0	10.1 \pm 4.4	6.3 \pm 1.4	0

Table 2: Dietary intake presented as means and percentage of total calories at baseline and average intake for weeks 1-8 based on self-reported daily diet records

	Control Group N=18			ALA Group N=19			EPA/DHA Group N=19		
	Baseline	8-weeks	p-value	Baseline	8-weeks	p-value	Baseline	8-weeks	p-value
	Mean % ± S.D.	Mean % ± S.D.		Mean % ± S.D.	Mean % ± S.D.		Mean % ± S.D.	Mean % ± S.D.	
SFA									
16:0 palmitic acid	21.0 ± 3.8	20.2 ± 1.2	0.53	19.8 ± 1.2	19.7 ± 0.8	0.84	20.4 ± 1.0	20.7 ± 1.0	0.3
18:0 stearic acid	18.5 ± 1.3	18.4 ± 0.8	0.96	18.5 ± 1.4	18.5 ± 1.1	0.57	18.6 ± 1.2	18.5 ± 1.0	0.21
MUFA									
18:1 oleic acid	13.8 ± 1.0	14.0 ± 1.2	0.49	12.9 ± 1.1	13.0 ± 1.2	0.73	13.1 ± 0.8	13.6 ± 1.0	0
PUFA									
18:2 linoleic acid	10.8 ± 1.7	11.0 ± 1.1	0.69	10.3 ± 1.8	10.4 ± 1.5	0.68	11.0 ± 1.7	11.0 ± 1.8	0.47
18:3 ALA	0.1 ± 0.3	0.0 ± 0.0	0.31	0.1 ± 0.5	0.1 ± 0.4	0.65	0.0 ± 0.0	0.0 ± 0.0	1
20:4 Arachidonic acid	15.4 ± 2.9	16.0 ± 1.1	1	15.4 ± 1.6	15.1 ± 1.8	0.33	16.2 ± 1.6	15.6 ± 1.2	0.01
20:5 EPA	0.0 ± 0.0	0.0 ± 0.0	1	0.1 ± 0.3	0.4 ± 0.6	0.03	0.0 ± 0.0	0.6 ± 0.7	0.01
22:6 DHA	3.8 ± 1.3	3.8 ± 1.0	0.01	4.4 ± 1.0	4.2 ± 1.1	0.06	4.4 ± 1.1	5.3 ± 1.0	0
n6/n3 ratio	7.2 ± 1.6	7.5 ± 1.8	0.01	5.9 ± 1.7	6.0 ± 2.0	0.9	6.7 ± 2.3	4.9 ± 1.5	0

Table 3: Percentage of fatty acids present in erythrocyte cell membranes before and after the intervention

Table 3 shows the percentage of fatty acids present in erythrocyte membranes before and after intervention as expected EPA and DHA significantly increased in the fish oil group; however ALA did not increase in the ALA group nor did MUFA's increase in the control group.

No other macronutrient changes were found in any groups. To explain the correlation of changes in Lp-PLA₂ concentration and activity with other descriptive variables such as total cholesterol, triglycerides, HDL, LDL and ox-LDL to LDL ratio in glucose sensitive and glucose intolerant subjects, Table 4 and 5 are respectively used. In glucose sensitive subjects, where blood glucose level is less than 100mg/dl, there is a significant correlation between Lp-PLA₂ activity and ox-LDL at the 0.01 level and a significant correlation between Lp-PLA₂ activity and the ratio of Ox-LDL to LDL at the 0.05 level. There was no significant correlation between Lp-PLA₂ mass and the other descriptive variables including ox-LDL and HDL.

Table 4: Correlation coefficients between Lp-PLA₂ mass and activity and the descriptive variables in glucose sensitive subjects (N=35)

	Lp-PLA ₂ mass r	Lp-PLA ₂ activity r
Glucose	-0.106	- 0.064
Oxidized LDL	-0.126	0.550**
Total Cholesterol	-0.302	0.110
Triglycerides	-0.188	0.022
HDL	0.216	-0.100
LDL	-0.254	0.192
Ox LDL/LDL	0.162	0.383*

*p<0.05

**p<0.01

In glucose intolerant subjects, there was no significant correlation between Lp-PLA₂ mass and activity and the variables as shown in Table 5.

Table 5: Correlation coefficients between Lp-PLA₂ mass and activity and the descriptive variables in glucose intolerant subjects (N=24)

	Lp-PLA ₂ mass r	Lp-PLA ₂ activity r
Glucose	0.212	0.365
Oxidized LDL	0.058	0.262
Total Cholesterol	0.186	0.100
Triglycerides	0.337	0.210
HDL	-0.093	-0.095
LDL	0.245	0.136
Ox LDL/LDL	-0.295	0.088

*p<0.05

**p<0.01

In all subjects, both glucose sensitive and intolerant, there is a significant correlation between Lp-PLA₂ activity and ox-LDL at 0.01 level and between Lp-PLA₂ activity and the ratio of ox-LDL to LDL at p< 0.05 level as shown in Table 6. No significant correlation is seen between Lp-PLA₂ mass and the descriptive variables. However, Lp-PLA₂ activity was significantly associated with ox-LDL at 15% of variance and was also significantly associated with ox-LDL/LDL ratio.

Table 6: Correlation coefficients between Lp-PLA₂ mass and activity and the descriptive variables in all subjects

	Lp-PLA ₂ mass r	Lp-PLA ₂ activity r
Glucose	-0.066	0.165
Oxidized LDL	-0.062	0.394**
Total Cholesterol	-0.066	0.091
Triglycerides	0.006	0.161
HDL	0.092	-0.156
LDL	-0.048	0.154
Ox LDL/LDL	-0.007	0.259*

*p<0.05

**p<0.01

Changes in fasting plasma Lp-PLA₂ concentration and activity

To examine the potential effects of the eight week omega-3 fatty acid supplementation on Lp-PLA₂ activity and concentration in high and low ox-LDL groups, multivariate analysis was performed while controlling for age, gender, anti-inflammatory drugs, blood sugar medications, represented in Figures 1 and 2. Calculating the median value of ox-LDL at 62.3, any value falling below that number was considered low ox-LDL group and any value equal to or greater than that number was considered high ox-LDL group. To explain this within-subjects and between-subjects factors are used.

Changes in Lp-PLA₂ concentration in low ox-LDL group:

The two within-subject factors used are pre concentrations of Lp-PLA₂ measure at baseline and post concentrations of Lp-PLA₂ after 8 weeks of ALA or EPA/DHA supplementation and the between-subject factors are the three groups.

Within-Subjects Factors

Measure: MEASURE_1

factor1	Dependent Variable
1	LPPLA2_Conc_Pre
2	LPPLA2_Conc_Post

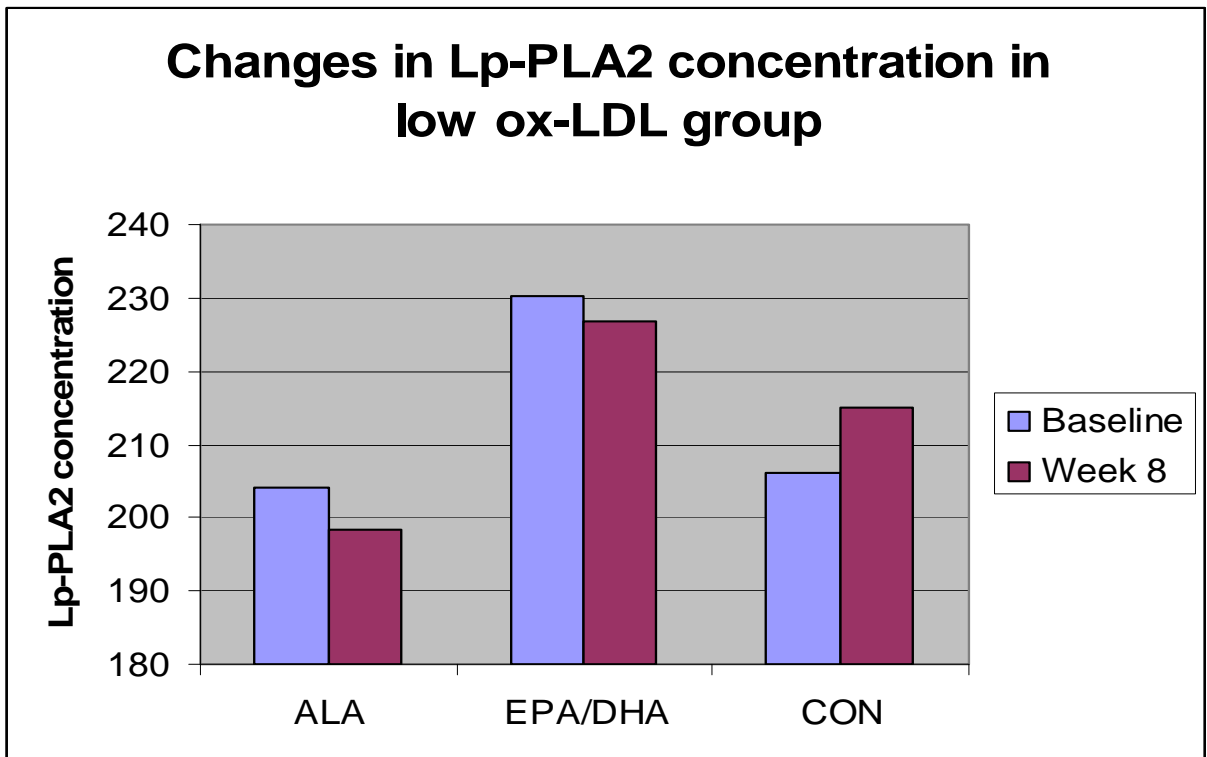
Between-Subjects Factors

		Value Label	N
Group	1.00	CON	8
	2.00	ALA	12
	3.00	FISH	9

The pre (baseline) and post (week 8) concentration levels of Lp-PLA₂ did not show any significant change from baseline to 8-weeks in any groups in the low ox-LDL group

during the intervention as shown in Figure 1 below, although a minor non-significant increase in Lp-PLA₂ concentration in the control group (baseline= 206 ng/mL+62.29; week 8= 215.01 ng/mL+73.26) was observed. The overall time group interaction was not significant for the association (p value= 0.825).

Figure 1: Lp-PLA₂ concentration in ALA, EPA+DHA and control groups in low ox-LDL group at baseline and 8 weeks

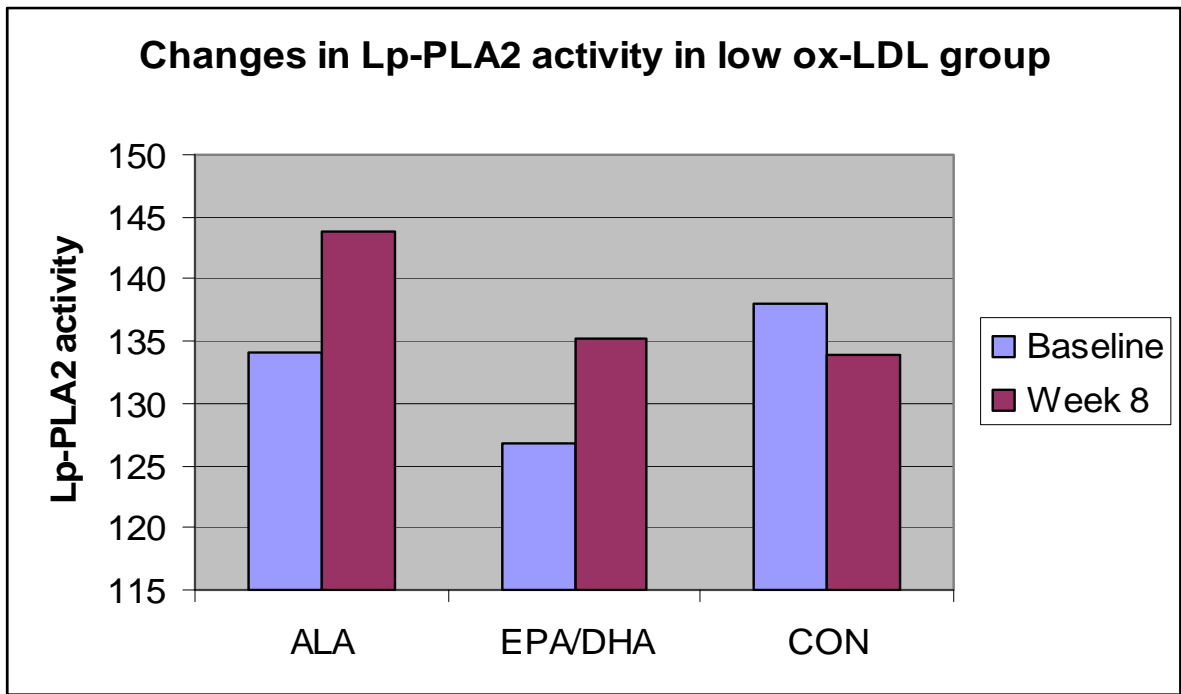


Changes in Lp-PLA₂ activity in low ox-LDL group:

The pre and post activity levels of Lp-PLA₂ did not show any significant change from baseline to 8-weeks in the low ox-LDL group as shown in Figure 2 below. A slight decrease in Lp-PLA₂ activity in control group between the baseline and 8 week was observed (CON: baseline =137.97 mmol/min/mL+27.50; week 8= 133.92

mmol/min/mL+28.56) while other groups showed a slight increase in the values. The overall time group interaction was not significant for the association (p value= 0.515).

Figure 2: Lp-PLA₂ activity (mmol/min/mL) in ALA, EPA+DHA and control groups in low ox-LDL groups at baseline and 8 weeks



Changes in Lp-PLA₂ concentration in the high ox-LDL group:

Within-Subjects Factors

Measure: MEASURE_1

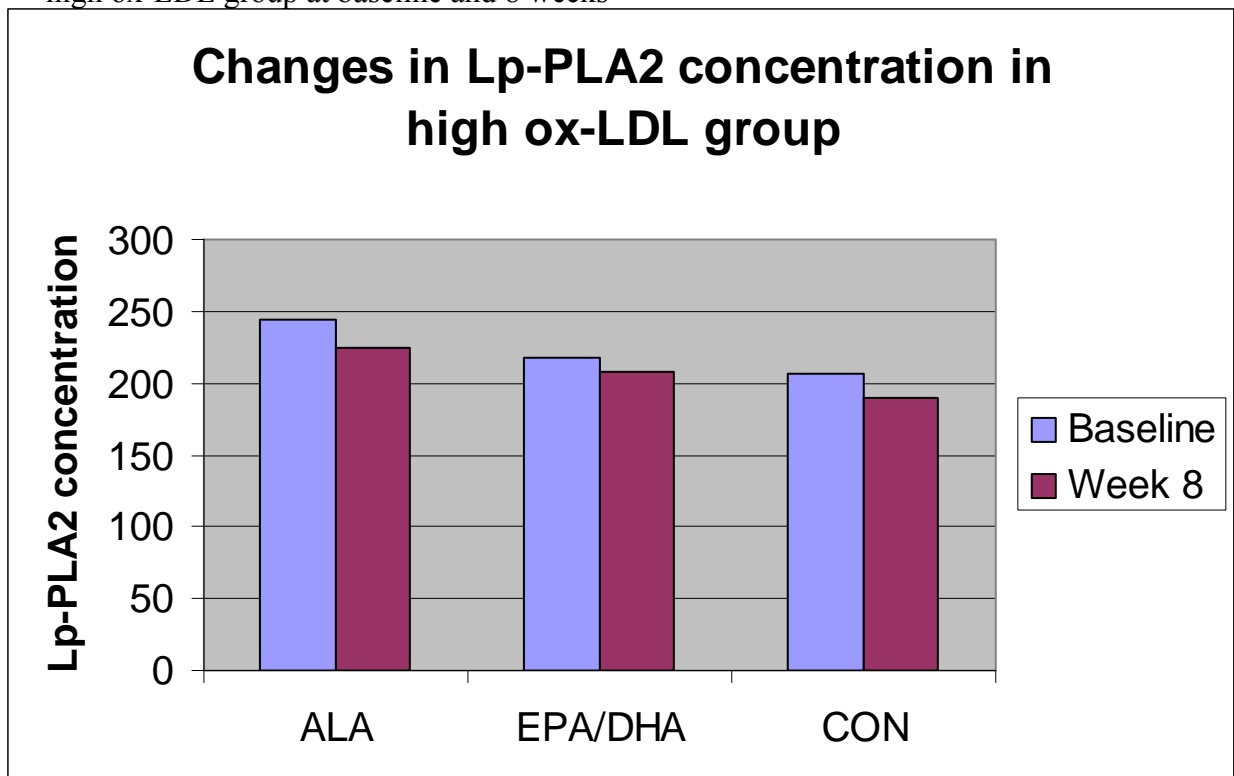
factor1	Dependent Variable
1	LPPLA2_Conc_Pre
2	LPPLA2_Conc_Post

Between-Subjects Factors

		Value Label	N
Group	1.00	CON	10
	2.00	ALA	8
	3.00	FISH	11

The pre and post concentration levels of Lp-PLA₂ did not show any significant change from baseline to 8-weeks in the high ox-LDL group as shown in Figure 3 below. (All groups showed a slight decrease in the values at baseline and 8 weeks). The overall time group interaction was not significant for the association (p value= 0.682).

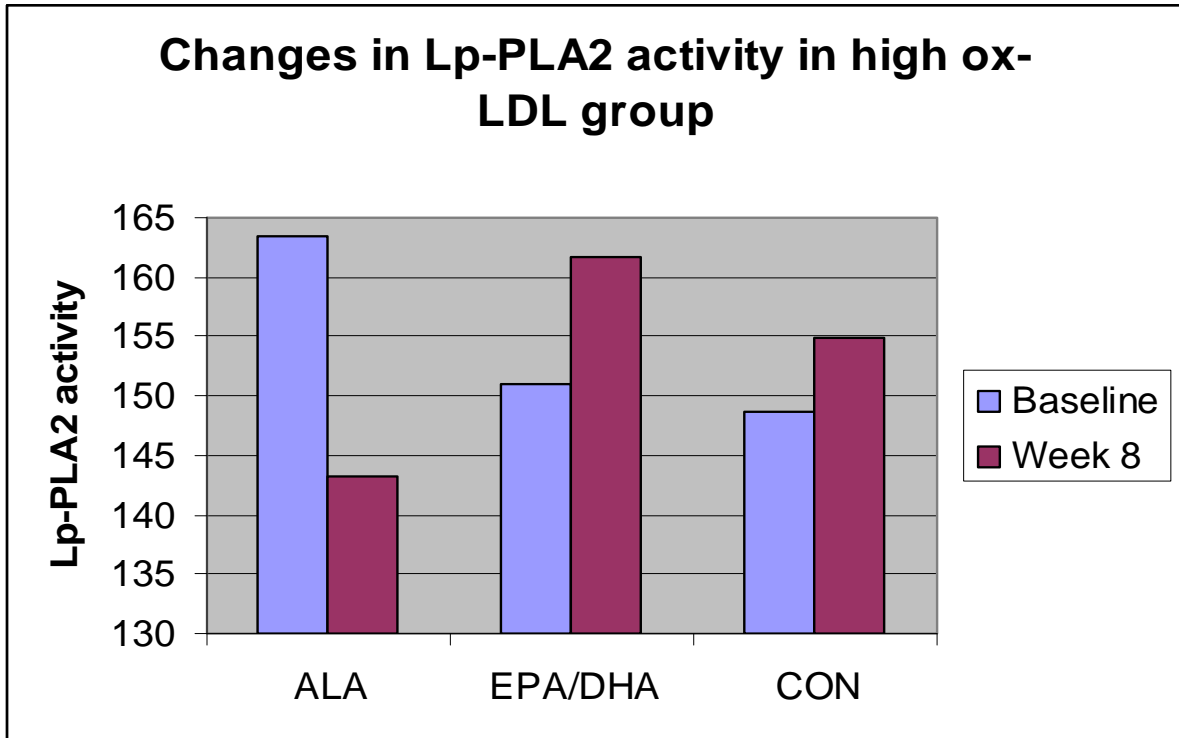
Figure 3: Lp-PLA₂ concentration (ng/mL) in ALA, EPA+DHA and control groups in high ox-LDL group at baseline and 8 weeks



Changes in Lp-PLA₂ activity in the high ox-LDL group:

The pre and post activity levels of Lp-PLA₂ did not show any significant change from baseline to 8-weeks in the high ox-LDL group as shown in Figure 4 below (A slight decrease was observed in the ALA group- baseline= 163.42 mmol/min/mL+27.29; week 8= 143.25 mmol/min/mL+14.52, while the other groups showed a slight increase in their values). The overall time group interaction was not significant for the association (p value= 0.168).

Figure 4: Lp-PLA₂ activity (mmol/min/mL) in ALA, EPA+DHA and control group in high ox-LDL group at baseline and 8 weeks



Changes in fasting plasma ox-LDL

To explain the effect of ALA and EPA/DHA supplementation on ox-LDL, the table below shows that there was no significant change in plasma ox-LDL levels at baseline and after the eight week diet supplementation in all the three groups. No significant changes are seen in Lp-PLA₂ mass or activity or LDL-C over the 8 week period, but we see a decreasing trend for LDL-C in all the three groups. Increasing trends in HDL-C values were also observed. There was a slight decrease in the triglyceride value in the ALA and EPA/DHA groups.

Table 7: Statistics at baseline and Week 8 for the control, ALA and EPA/DHA groups

	Control Group N=19		ALA Group N=20		EPA/DHA Group N=20		p- value
	Baseline Mean \pm S.D.	8-weeks Mean \pm S.D.	Baseline Mean \pm S.D.	8-weeks Mean \pm S.D.	Baseline Mean \pm S.D.	8-weeks Mean \pm S.D.	
Lp-PLA ₂ mass (ng/mL)	204.1 \pm 58.8	200.4 \pm 65.0	219.9 \pm 64.0	209.1 \pm 63.7	222.9 \pm 56.8	216.4 \pm 57.1	0.91
Lp-PLA ₂ activity (nmol/min/mL)	142.4 \pm 28.9	148.4 \pm 43.8	145.8 \pm 29.0	143.5 \pm 23.6	140.0 \pm 36.5	149.8 \pm 39.9	0.62
HDL-C (mg/dL)	42.8 \pm 13.1	48.7 \pm 18.1	44.2 \pm 13.4	44.9 \pm 13.4	39.5 \pm 12.6	45.0 \pm 15.9	0.23
Triglycerides (mg/dL)	94.1 \pm 58.6	96.5 \pm 58.0	80.6 \pm 40.6	77.0 \pm 43.4	90.1 \pm 37.7	83.1 \pm 28.9	0.19
LDL-C (mg/dL)	136.2 \pm 42.5	118.9 \pm 40.7	117.1 \pm 27.3	105.7 \pm 28.5	128.9 \pm 27.2	125.5 \pm 35.3	0.24
Oxidized LDL (U/L)	70.6 \pm 30.9	70.2 \pm 31.5	64.2 \pm 23.6	65.5 \pm 25.1	66.7 \pm 22.9	69.8 \pm 21.6	0.29

Correlation of changes in ox-LDL with changes in Lp-PLA₂ concentration and activity

Subjects were grouped as glucose sensitive and glucose intolerant based on the level of their fasting blood glucose. In glucose intolerant subjects, the level of blood glucose is greater or equal to 100 mg/dl, whereas in glucose sensitive subjects, the fasting blood glucose levels are lower than 100 mg/dl. In glucose intolerant subjects, changes in ox-LDL were positively correlated with changes in Lp-PLA₂ concentration but no such correlation was seen with changes in Lp-PLA₂ activity at p<0.05. The values are shown Table 8 below.

Table 8: Correlation of changes in ox-LDL with changes in Lp-PLA₂ concentration and activity in glucose intolerant subjects

		Change_oxLDL
change_LpPLA2mass	Correlation Coefficient	0.451 *
	p-value	0.031
	N	23
change_LpPLA2activity	Correlation Coefficient	-0.003
	p-value	0.989
	N	23

*. Correlation is significant at the 0.05 level (2-tailed).

In glucose sensitive subjects, where blood glucose is less than 100 mg/dl, changes in ox-LDL were positively correlated with changes both in the concentration and activity of Lp-PLA₂ as shown in Table 9.

Table 9: Correlation of changes in ox-LDL with changes in Lp-PLA₂ concentration and activity in glucose sensitive subjects

		change_oxLDL
change_LpPLA2mass	Correlation Coefficient	0.396 *
	p-value	0.019
	N	35
change_LpPLA2activity	Correlation Coefficient	0.349 *
	p-value	0.04
	N	35

*. Correlation is significant at the 0.05 level (2-tailed).

CHAPTER V

DISCUSSION

Discussion:

The purpose of this study was to determine if there was a correlation between Lp-PLA₂ and ox-LDL as well as the effect of ALA and EPA/DHA diet supplementation on the same in glucose sensitive and glucose intolerant subjects. The diets were fairly isocaloric as evident by their total calories from baseline to 8-weeks; further the ALA, EPA and DHA and control groups all significantly increased their intake of ALA, EPA/DHA and MUFA's respectively. Assessment of dietary intake through food records is commonly subject to bias and to avoid such a bias, percentage of erythrocyte membrane fatty acids was measured. Changes in erythrocyte membrane fatty acids during a period of controlled fat feeding are assessed as a potential short-term marker of compliance (Poppitt, Kilmartin, Butler & Keogh, 2005). The results of this study indicate the EPA/DHA group was compliant but we did not see significant changes in MUFA or ALA in these groups respectively. The strength of correlation between dietary intake and biomarker appears to vary considerably between individual fatty acids (Zock et al., 1997). It would be imagined that biomarkers of the omega 3 and omega 6 PUFAs, such as ALA, or linoleic acid (LA), would have the strongest association with intake since the inability to generate double-bonds more than 9 carbons from the carboxyl or delta end of

the fatty acid ensure these PUFA may be derived from diet alone (Leaf et al., 1995 & Kobayashi et al., 2001), which is in contrast to what is seen here. The MUFAs on the other hand with an even number of carbon atoms may be less well correlated with intake (Beynen, 1980 & Hunter, 1992) since their derivation is not dependent on intake from diet alone. Whether the subjects did not comply or the fatty acids were not incorporated is difficult to determine.

Our first hypothesis was to show that baseline Lp-PLA₂ concentration and activity would be positively correlated with baseline oxidized LDL (ox-LDL) in fasting plasma samples of both glucose sensitive and glucose intolerant subjects with the associations being stronger among glucose intolerant subjects. But the results do not entirely support this hypothesis.

Correlation of Lp-PLA₂ concentration and activity with ox-LDL at baseline

In glucose sensitive subjects, a positive correlation between Lp-PLA₂ activity and ox-LDL is seen at $p < 0.01$ level but no such correlation is seen between Lp-PLA₂ concentration and ox-LDL. The results of this study also do not represent a baseline significant relation between Lp-PLA₂ concentration and activity and ox-LDL in glucose intolerant subjects. Lp-PLA₂ mass and activity represent potentially different physiologically relevant species of Lp-PLA₂. The mass assay represents Lp-PLA₂ in which the epitopes are exposed to the antibody in the presence of intact lipoproteins, while the activity assay represents Lp-PLA₂ after disruption of lipoproteins by detergent. Thus Lp-PLA₂ mass represents “exposed” Lp-PLA₂ and Lp-PLA₂ activity represents total Lp-PLA₂ in plasma. Both Lp-PLA₂ activity and LDL-cholesterol (LDL-C) concentration

are associated with early onset atherosclerosis, endothelial dysfunction, and future cardiac events (Lavi et al, 2007; Koenig, Twardella, Brenner & Rothenbacher, 2006). Previous studies have shown that in T2DM, people tend to have higher low-density lipoprotein (LDL) cholesterol and triglycerides. In T2DM, LDL particles tend to be smaller and denser, and this likely increases their risk for atherosclerosis (Feingold, Grunfeld, Pang, Doerrier, & Krauss, 1992). Since LDL is the substrate for oxidation, concentrations of ox-LDL depend on the sensitivity of LDL particles to oxidation, small dense LDLs contain smaller amounts of antioxidants and are, therefore more prone to oxidation (Lamarche, 1998). As Stafforni et al, 1999 and Gazi et al, 2005 have shown that the activity of Lp-PLA₂ is mainly associated with smaller LDL particles, it is expected that glucose intolerant subjects would have higher levels of ox-LDL and these levels would be positively correlated with Lp-PLA₂ concentration and activity.

Furthermore, studies have shown that Lp-PLA₂ activity and LDL-C concentration are positively correlated (Persson, Nilsson, Nelson, Hedblad & Berglund, 2007; Guerra, Zhao, Mooser, Stafforini, Johnston & Cohen, 1997), an observation consistent with the knowledge that the major portion of Lp-PLA₂ in the circulation is bound to LDL. The positive relationship between Lp-PLA₂ activity and LDL-C concentration and their association with cardiovascular disease probably reflects the enzymatic action of Lp-PLA₂ on LDL particles, which produces end products with pro-inflammatory activity that may account for many of the atherogenic properties of oxidized LDL (Tew et al, 1996; Macphee et al, 1999). And it is surprising that our results do not represent this theory. Perhaps, there are other variables playing a significant role in glucose intolerant subjects like HDL-C or lipid composition of lipoproteins which was not a part of our study but

have shown to affect the Lp-PLA₂ activity (Stafforini, Carter, Zimmerman, McIntyre & Prescott, 1989).

Effect of an eight week ALA or EPA+DHA supplementation on plasma Lp-PLA₂ concentration and activity in low and high ox-LDL groups

Our results also did not show any significant change in the concentration or activity of Lp-PLA₂ either in low ox-LDL group or high ox-LDL group after ALA or EPA/DHA supplementation in diet. No particular trend was observed, however, a slight decrease in Lp-PLA₂ concentration in all the three groups (CON, ALA, and EPA/DHA) was seen in higher ox-LDL group in agreement with our second hypothesis. But Lp-PLA₂ activity only decreased non-significantly in ALA group and not the control or EPA/DHA group in high ox-LDL group. Both control and EPA/DHA groups use olive oil as diet supplementation which contain high levels of monounsaturated fatty acids which have shown to help reduce cholesterol levels (Hodson, Skeaff, & Chisholm, 2001; Knoop, et al., 2004). The anti-atherogenic properties of olive oil due to minor polar compounds (MPC) found in the same has been attributed to Lp-PLA₂ activity depression (Dell'Agli, M., et al., 2006), which is in contradiction to our results. Few studies have shown the preferential association of Lp-PLA₂ activity and not mass in glucose intolerant subjects with LDL (Packard, et al, 2000; Ballantyne, et al, 2004; Koenig, Khuseyinova, Lowel, Trischler & Meisinger, 2004; Persson, Nilsson, Nelson, Hedblad & Berglund, 2007), in particular the denser, smaller LDL particles versus larger particles (Gazi, Lourida, Filippatos, Tsimihodimos, Elisaf & Tselepis, 2005; Karabina, Liapikos, Grekas,

Goudevenos & Tselepis, 1994), but no such correlation is seen here. Perhaps, the higher oxidized state (elevated circulating levels of Ox-LDL) plays a part since we observe a non-significant decrease in Lp-PLA₂ activity in the control group in lower ox-LDL group. Also, no such observation is made in the EPA/DHA group where there was a slight increase in the activity level. Overall, there was no significant change in the concentration and activity in Lp-PLA₂ over this 8 week period of ALA or EPA/DHA diet supplementation.

Research explains that eicosanoids derived from omega-3 PUFAs (ALA) tend to inhibit platelet aggregation and are anti-inflammatory (Simopoulos, 1991; Simopoulos, 1999) and an increased intake of ALA may actually help in reducing the production of atherosclerotic mediators and lessen the progression of CVD (James, Gibson, & Cleland, 2000). Along with the inflammatory process, an elevated concentration of serum LDL cholesterol is a major risk factor for CHD (Program, 1994) and diets high in PUFA are associated with lower LDL levels (Hodson, Skeaff, & Chisholm, 2001). Oxidative modification of LDL is considered a key factor in promoting atherosclerosis (Holvoet, 2004). Lp-PLA₂ in circulation is principally bound to LDL in humans (Stafforini, et al., 1999) and the substrate for the enzyme is provided by the oxidized phospholipid component of LDL (Mertens & Holvoet, 2001; Zalewski & Macphee, 2005). There is evidence suggesting that elevated Lp-PLA₂ may mean increased risk for recurrent events in patients with clinically manifested CVD (Blankenberg, et al., 2003; Brilakis, et al., 2005). Therefore, reducing the concentration and activity of Lp-PLA₂ may attenuate the atherosclerotic process. And therefore, diet supplementation of ALA or EPA/DHA should have decreased the concentration and activity of Lp-PLA₂ by lowering the ox-

LDL levels but we see no such change. This could be possibly because of a smaller study population or lower dosages of ALA or EPA/DHA consumption, but Pedersen et al., (2009) used moderate doses (2 g) of omega-3 PUFA and higher doses (6.6 g) of omega-3 PUFA to study the effect of marine omega-3 PUFA on plasma Lp-PLA₂ levels in healthy subjects but found no such effect on plasma levels of Lp-PLA₂.

Effect of an eight week ALA or EPA+DHA supplementation on ox-LDL

There were no significant changes in fasting plasma ox-LDL after the diet supplementation in any of the groups, but a decreasing trend in LDL-C was observed. As previously mentioned, diabetics tend to have higher levels of small dense LDL which are more prone to oxidation and hence higher levels of ox-LDL. Also, the activity of Lp-PLA₂ is mainly associated with smaller LDL particles and these levels would positively correlate with Lp-PLA₂ concentration and activity. Recent studies have also shown a certain link between ox-LDL and Lp-PLA₂ by observing that ox-LDL, and more specifically its unhydrolyzed oxidized phospholipids, can up-regulate Lp-PLA₂ expression in monocytes through the PI3K and p38 MAPK pathway. In turn, Lp-PLA₂ promotes lipoprotein uptake in macrophages (Wang, Li, Yang, Xu, Zha & Wang, 2009). After 8 week ALA or EP/DHA diet supplementation, our results also did not show any significant change in the concentration or activity of Lp-PLA₂ and hence we would not expect to find any changes in fasting plasma ox-LDL levels either. Mata et al., showed that dietary fatty acids produce significant changes in LDL fatty acid composition and plasma lipoprotein levels in healthy men and women consuming a regular diet. Moreover, their results demonstrated that dietary changes modified the susceptibility of LDL to

undergo in vitro oxidative modification and monocyte adhesion to human endothelial cells in vitro. Oxidative modification of LDL involves peroxidation of PUFAs. Dietary omega 3 fatty acids are incorporated into lipoproteins, thereby potentially affecting the susceptibility of LDL to oxidative modification. There are conflicting results, however, between studies on the effects of omega 3 fatty acids supplementation on LDL oxidizability (Harats et al., 1991; Harris et al., 1993; Oostenbrug et al., 1994 & Suzukawa et al., 1995), whereas other studies (Nenseter et al., 1992; Bittolo-Bon et al., 1993; Frankel et al., 1994 & Bonanome et al., 1996) showed no effect of dietary omega fatty acids on LDL oxidation. Monocytes, endothelial cells, and smooth muscle cells are able to oxidize LDL by cell-derived oxidants like H₂O₂ and superoxide radicals (Garner & Jessup, 1996). Since omega 3 fatty acids supplementation may reduce free-radical production in stimulated human monocytes and polymorphonuclear cells (Fisher et al., 1986 & Fisher et al., 1990), changes in cellular free-radical production due to omega 3 fatty acids may in turn influence cell-mediated oxidative modification of LDL in vivo. Whether supplementation with omega 3 fatty acids leaves the LDL particles more prone to oxidative modification and influences the cells' ability to oxidize LDL has not yet been conclusively resolved. Furthermore, there may be other key elements playing a role like high concentrations of antioxidants which have been shown to counteract the susceptibility of omega 3 fatty acids-enriched LDL to oxidative modification (Harats et al., 1991 & Oostenbrug et al., 1994). There was no significant change in LDL-C values as well but a decreasing trend was observed which supports that polyunsaturated fatty acids have a modest LDL-cholesterol- lowering effect (Mensink & Katan, 1992)

Triglyceride values show a decreasing trend in ALA and EPA/DHA groups. Even the 2007 National Lipid Associations (NLA) safety task force concluded that omega 3 fatty acids are a safe therapeutic option for lowering triglycerides. One study suggested the fact that the most significant reduction in triglyceride clearance was achieved with the PUFA (omega-3) diet suggests that this phenomenon is related to the degree of unsaturation but the mechanism of action of omega-3 fatty acids in the reduction of triglycerides remains unclear. There is evidence that omega-3 fatty acids increase triglyceride clearance from circulating VLDL particles by increasing lipoprotein lipase (LPL) activity (Bays, 2007). Perhaps, this mechanism played a part in the decreasing values of triglycerides. There is an increasing trend of HDL values in all groups which is in agreement with some studies that have shown an increase of HDL-C with high doses of omega-3 fatty acids. Studies have shown that only when the diet contains more than 14% of energy as omega-6 polyunsaturated fatty acids are LDL and HDL cholesterol concentrations reduced (Shepherd, Packard, Patsch, Gotto & Taunton, 1978; Matson & Grundy, 1985) These results accord with those of Mensink and Katan, 1989 who observed no significant change in HDL cholesterol concentration on exchange of 6.5% of total dietary energy as saturated fat for unsaturated fat. The doses of omega-3 fatty acids in our study is not as high as 14% of energy, and no significant change is seen in HDL values, only increasing trends, in agreement with other studies. There may be other disadvantages with high intakes of omega-3 fatty acids as well such as an increased susceptibility of LDL to peroxidation (Nenseter & Drevon, 1996).

Effect of an eight week ALA or EPA+DHA supplementation on correlation of Lp-PLA₂ concentration and activity with ox-LDL

The results of this study represent a significant correlation between changes in Lp-PLA₂ concentration and activity with changes in ox-LDL in glucose sensitive subjects and between changes in Lp-PLA₂ concentration with changes in ox-LDL in glucose intolerant subjects but no such correlation was seen with changes in Lp-PLA₂ activity at $p < 0.05$. Overall these results are in agreement with our hypothesis which states that changes in fasting plasma Lp-PLA₂ concentration and activity after isocaloric diet supplementation with ALA, EPA/DHA will be positively correlated with change in ox-LDL after the dietary supplementation. But we also hypothesize that these changes will be greater in glucose intolerant subjects, which is only partially true. No correlation is observed between the changes in Lp-PLA₂ activity and changes in ox-LDL in glucose intolerant subjects.

Few studies have shown that the Lp-PLA₂ enzyme mass is preferentially distributed on small dense LDL particles, however, the enzyme activity in these particles is less than expected from the concentration (Gazi, Lourida, Filippatos, Tsimihodimos, Elisaf & Tselepis, 2005). Van Berkel, De Rijke & Kruijt, (1991) have shown that fully oxidized LDL does not exist in the circulation. In vitro oxidative damage to LDL could be one possibility while the other possibility is even if such highly oxidized particles would enter the circulation, they would be rapidly cleared in the liver via scavenger receptors. In contrast, circulating minimally oxidized LDL, in which oxidative modification has not been adequate to cause changes by scavenger receptors, was demonstrated (Avogaro,

Bon & Cazzolato, 1988). And therefore is suggested by them that all assays for ox-LDL most likely detect minimally oxidized LDL. Since LDL is the substrate for oxidation, concentrations of ox-LDL depend on the sensitivity of LDL particles to oxidation, small dense LDLs contain smaller amounts of antioxidants and are, therefore more prone to oxidation (Lamarche, 1998). Stafforni et al, 1999 and Gazi et al, 2005 have shown that the activity of Lp-PLA₂ is mainly associated with smaller LDL particles. So, it is possible that the activity of Lp-PLA₂ being measured is not completely accurate since we are, in actuality measuring the minimally oxidized LDL. Again, this phenomenon could be attributed to the increased susceptibility of small dense LDL to oxidation as previously discussed because, according to data published previously by Gazi et al, 2005 and others, the enzyme activity is significantly reduced during lipoprotein oxidation (Liapikos et al, 1994; Dentan, Lesnik, Chapman & Ninnio, 1994). Another possibility of the reduced Lp-PLA₂ specific activity could also be attributed to the well-known differences in lipid composition of small dense LDL particles compared with buoyant LDL particles (Tselepis, Dentan, Karabina, Chapman & Ninnio, 1995). An alternative suggestion could be the existence of an inactive form of Lp-PLA₂ in plasma that could be preferentially associated with lipoprotein subfractions migrating at the dense portion of the gradient, i.e., LDL-5 and HDL. According to Gazi et al, 2005, HDL-Lp-PLA₂ activity does not significantly contribute to the total plasma enzyme activity, whereas the HDL-Lp-PLA₂ mass significantly influences the total plasma enzyme mass. Whatever the mechanisms which affect the concentration or activity of Lp-PLA₂, more research needs to be conducted to evaluate if there are other unknown variables playing a part in the correlation of Lp-PLA₂ and ox-LDL.

Over the past twenty years significant evidence has emerged supporting the cardioprotective effect of omega-3 fatty acids (Dolecek, 1992; Browning, 2003; Harris, 2007; Hibbein, Nieminen, Blasbalg, Riggs & Lands, 2006 & Wang, et al., 2006). A number of mechanisms may be responsible for this effect, including the prevention of arrhythmias, lowering heart rate and blood pressure, lowering triglycerides and, of specific interest in this study, decreasing inflammation (Harris, 2007). Moreover, Lp-PLA₂ has been repeatedly shown to have a pro-inflammatory, pro-thrombotic effect that has not yet been observed in relation to omega-3 fats (Sudhir, 2005; Ballantyne et al., 2004; Koenig et al., 2004 & Oei et al., 2005). Lp-PLA₂ in circulation is principally bound to LDL in humans (Stafforini, et al., 1999) and the substrate for the enzyme is provided by the oxidized phospholipid component of LDL (Mertens & Holvoet, 2001; Zalewski & Macphee, 2005). Some studies have suggested omega 3 fats have also been shown to suppress oxidized LDL and reduce cardiovascular disease risk (Finnegan et al., 2003). The present study evaluated the effects of an eight week ALA and EPA+DHA supplementation on concentration and activity of Lp-PLA₂ and plasma levels of ox-LDL. Upon evaluating changes within the ALA and EPA+DHA and control groups over the eight week period, no significant changes in Lp-PLA₂ concentration or activity were observed either in low ox-LDL group or high ox-LDL group. No significant changes in plasma levels of ox-LDL were observed from baseline to week-8, but a decreasing trend in LDL-C was observed. Triglyceride values also show a decreasing trend in ALA and EPA/DHA groups while there was an increasing trend of HDL values in all groups. The results of this study do not represent a baseline significant relation between Lp-PLA₂ concentration and activity and ox-LDL in glucose intolerant subjects. At week 8, a

significant correlation between changes in Lp-PLA₂ concentration and activity with changes in ox-LDL in glucose sensitive subjects was observed. Finally, changes in Lp-PLA₂ concentration with changes in ox-LDL in glucose intolerant subjects was observed at week 8.

CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The aim of this study was to determine the effects of ALA and EPA/DHA diet supplementation on Lp-PLA₂ concentration and activity, ox-LDL and the correlation between them in glucose sensitive and glucose intolerant male and female weight stable adults over the age of 50 years.

Fifty nine adults, (44 females, 15 males), were recruited from the Fort Collins community and completed the eight week protocol. All participants completed an informed consent and health history questionnaire and were free of any known cardiovascular disease or inflammatory conditions. Fasting blood glucose was measured prior to study entry in order to obtain a range of glucose concentrations for the purpose of assessing changes between hyperglycemic and normoglycemic individuals.

Anthropometric values (height, weight and waist circumference) were measured on a weekly basis, 3-day diet records assessed on a bi-weekly basis (a total of four throughout the study), and DEXA and venous blood draw were performed at baseline, week four and week eight of the study. Participants were instructed to make no alterations in activity level or medications for the duration of the study. The ALA, EPA + DHA and control groups were assigned supplementation based on 5.3% of total caloric intake and were given specific instructions to reduce fat from other sources in order to maintain an isocaloric diet.

Results show that there was no significant change in the concentration or activity of Lp-PLA₂ either in low ox-LDL group or high ox-LDL group after ALA or EPA/DHA supplementation in diet. No particular trend was observed, however, a slight decrease in Lp-PLA₂ concentration in all the three groups (CON, ALA, EPA/DHA) was seen in higher ox-LDL group. But Lp-PLA₂ activity only decreased in ALA group (baseline 163.425 mmol/min/mL; week 8 143.25 mmol/min/mL,) and not the control or EPA/DHA group in high ox-LDL group. A minor increase in Lp-PLA₂ concentration in the control group (baseline: 206 ng/mL; 8 weeks: 215.01 ng/mL) in low ox-LDL group and a slight decrease in Lp-PLA₂ activity in control group was observed (baseline 137.97 mmol/min/mL; 8 week 133.925 mmol/min/mL). There were no significant changes in fasting plasma ox-LDL after the diet supplementation in any of the groups, but a decreasing trend in LDL-C was observed. Triglyceride values also show a decreasing trend in ALA and EPA/DHA groups while there was an increasing trend of HDL values in all groups. In glucose sensitive subjects, a positive correlation between Lp-PLA₂ activity and ox-LDL is seen at $p < 0.01$ level but no such correlation is seen between Lp-PLA₂ concentration and ox-LDL at baseline. The results of this study also do not represent a baseline significant relation between Lp-PLA₂ concentration and activity and ox-LDL in glucose intolerant subjects. A significant correlation between changes in Lp-PLA₂ concentration and activity with changes in ox-LDL in glucose sensitive subjects was observed at 8 weeks. Also, changes in Lp-PLA₂ concentration with changes in ox-LDL in glucose intolerant subjects was observed but no such correlation was seen with changes in Lp-PLA₂ activity at $p < 0.05$ or $p < 0.01$.

While this study targeted a population fitting an age demographic in which advanced stages of inflammation may be assumed, considering the role of hyperglycemia in the inflammatory process, more significant changes could have been observed with involving more participants with impaired fasting glucose or clinical type 2 diabetes. Future research should focus on a population with increased prevalence of type 2 diabetes, metabolic syndrome or dyslipidemia, as well as consider extending the omega-3 fatty acid supplementation beyond eight weeks. Both omega-3 and omega-6 polyunsaturated fatty acids are essential nutrients in human diets but the optimal levels of these fatty acids, both absolute and relative to each other, remain uncertain. The potential for chronic, prolonged consumption of omega-3 fats to significantly alter Lp-PLA₂, ox-LDL and other inflammatory markers also needs to be studied. Further investigation is also necessary in order to better understand the mechanisms by which omega-3 fatty acids reduce cardiovascular disease risk, particularly in relation to the complex processes of inflammation in promoting atherosclerosis

Conclusion

An eight week supplementation of ALA or EPA + DHA resulted in no significant changes in Lp-PLA₂ concentration or activity or changes in ox-LDL, but a significant correlation between changes in Lp-PLA₂ concentration and activity with changes in ox-LDL in glucose sensitive subjects and changes in Lp-PLA₂ concentration with changes in ox-LDL in glucose intolerant subjects was observed at the end of the study.

Recommendations

1. A larger study population with an increased prevalence of type 2 diabetes, metabolic syndrome or dyslipidemia would perhaps reflect more advanced stages of oxidative stress and inflammation and may result in more impressive changes due to the intervention.
2. Higher dosages or more chronic, prolonged consumption of ALA or EPA + DHA may allow for a better understanding of the regulation of inflammatory processes than what was observed in this study.
3. The “upstream” promoters of Lp-PLA₂ such as macrophages, T cell lymphocytes and mast cells, or “downstream” products of Lp-PLA₂ such as oxidized fatty acids and lysoPC need to be further investigated to provide a better picture of its activity in response to omega-3 supplementation.
4. Research needs to be focused on other dietary interventions like anti-oxidants that may affect plasma levels of ox-LDL

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APPENDICES

APPENDIX A
HEALTH HISTORY QUESTIONNAIRE

HUMAN PERFORMANCE CLINICAL/RESEARCH LABORATORY
COLORADO STATE UNIVERSITY
CONFIDENTIAL HEALTH HISTORY QUESTIONNAIRE

STUDY _____ DATE _____ SUBJECT ID # _____

Reviewed by (must be PI): _____

PLEASE PRINT

GENERAL MEDICAL HISTORY

Do you have any current medical conditions? YES NO
If Yes, please explain:
(including pregnancy or inflammatory disease)

Have you had any major illnesses in the past? YES NO
If Yes, please explain:

Have you ever been hospitalized or had surgery? YES NO
If Yes, please explain: (include date and type of surgery, if possible)

Have you ever had an EKG? YES NO
If Yes, please explain:

Have you been diagnosed with diabetes? YES NO
 If Yes, please explain:

Age at diagnosis _____

Are you currently taking any medications, including aspirin, hormone replacement therapy, or other over-the-counter medications?

YES NO

If Yes, please explain:

Medication Reason Times taken per Day Taken for how long?

SPECIFIC MEDICATIONS

		YES	NO
Aspirin	(chronically)	<input type="checkbox"/>	<input type="checkbox"/>
Ibuprofen		<input type="checkbox"/>	<input type="checkbox"/>
Acetaminophen		<input type="checkbox"/>	<input type="checkbox"/>
Steroids (cortisone, etc.)		<input type="checkbox"/>	<input type="checkbox"/>
Other anti-inflammatories		<input type="checkbox"/>	<input type="checkbox"/>
Oral contraceptives		<input type="checkbox"/>	<input type="checkbox"/>
Weight loss medications		<input type="checkbox"/>	<input type="checkbox"/>
Insulin sensitizers		<input type="checkbox"/>	<input type="checkbox"/>

FAMILY HISTORY

	Age (if alive)	Age of Death	Cause of Death
Father	_____	_____	_____
Mother	_____	_____	_____
Brothers/Sisters	_____	_____	_____

_____	_____	_____
_____	_____	_____
_____	_____	_____

Do you have a family history of any of the following: (Blood relatives only, please give age at diagnosis if possible)

Diagnosis	YES	NO	Relation	Age at
a. High blood pressure	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
b. Heart Attack	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
c. Coronary bypass surgery	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
d. Stroke	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
e. Diabetes	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____
f. Obesity	<input type="checkbox"/>	<input type="checkbox"/>	_____	_____

MENSTRUAL HISTORY

When was your last menstrual period?

1. Currently bleeding
2. Last period less than three months ago
3. Last period less than 6 months ago
4. Last period less than 12 months ago
5. More than 12 months without a period

TOBACCO HISTORY (check one)

TOBACCO USE

None

Quit (when) _____

Cigarette

Cigar

Pipe

Chew Tobacco

Snuff

Total years of tobacco use _____

CURRENT

(if applicable)

per day

Cigarette _____

Cigar _____

Pipe _____

Chew Tobacco _____

Snuff _____

CARDIORESPIRATORY HISTORY

Are you presently diagnosed with heart disease?

YES	NO
<input type="checkbox"/>	<input type="checkbox"/>

Do you have any history of heart disease?

<input type="checkbox"/>	<input type="checkbox"/>
--------------------------	--------------------------

Do you have a heart murmur?	<input type="checkbox"/>	<input type="checkbox"/>
Occasional chest pain or pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Chest pain or pressure on exertion?	<input type="checkbox"/>	<input type="checkbox"/>
Episodes of fainting?	<input type="checkbox"/>	<input type="checkbox"/>
Daily coughing?	<input type="checkbox"/>	<input type="checkbox"/>
High blood pressure?	<input type="checkbox"/>	<input type="checkbox"/>
Shortness of breath?		
At rest?	<input type="checkbox"/>	<input type="checkbox"/>
lying down?	<input type="checkbox"/>	<input type="checkbox"/>
After 2 flights of stairs?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have asthma?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a history of bleeding disorders?	<input type="checkbox"/>	<input type="checkbox"/>
Do you have a history of problems with blood clotting?	<input type="checkbox"/>	<input type="checkbox"/>

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

<u>MUSCULOSKELETAL HISTORY</u>	YES	NO
Any current muscle injury or illness?	<input type="checkbox"/>	<input type="checkbox"/>
Any muscle injuries in the past?	<input type="checkbox"/>	<input type="checkbox"/>
Do you experience muscle pain at rest?	<input type="checkbox"/>	<input type="checkbox"/>
Do you experience muscle pain on exertion?	<input type="checkbox"/>	<input type="checkbox"/>
Any current bone or joint (including spinal) injuries?	<input type="checkbox"/>	<input type="checkbox"/>
Any previous bone or joint (including spinal) injuries?	<input type="checkbox"/>	<input type="checkbox"/>
Do you ever experience painful joints?	<input type="checkbox"/>	<input type="checkbox"/>
Do you ever experience swollen joints?	<input type="checkbox"/>	<input type="checkbox"/>

Do you ever experience edema (fluid build up)?

Do you have pain in your legs when you walk?

If you checked YES to any of the above, you will be asked to clarify your response by an investigator so we can be sure to safely determine your ability to participate.

NUTRITIONAL SURVEY

How many times do you usually eat per day? _____

What time of day do you eat your largest meal? _____

How many times per week do you eat out ? _____

Are you taking any diet supplements? YES NO .

IF YES, PLEASE PROVIDE DETAILS:

How many times per week do you normally eat the following:

Ground beef__ Sausage_____ Bacon_____ Beef_____

Pork_____ Cheese_____ Fish_____ Poultry_____

Shellfish_____ Fried Foods__ Breads_____ Cereals_____

Fruits_____ Vegetables__ Eggs_____ Desserts_____

Other_____ (describe)

How many servings per week of the following do normally consume:

Whole milk__ 2% Milk_____ Skim milk__ Buttermilk_____

Coffee_____ Tea_____ Soft-Drinks__ Beer_____

Wine_____ Liquor_____ Water_____

Have you ever dieted? YES NO

If YES, have you dieted within the past 12 months or are you currently on a diet?

YES NO

If YES, please describe the diet:

a). Name (if applicable): _____

b). Prescribed by a Physician/nutritionist? YES NO

c). Have you lost weight? YES NO

d). Duration of diet _____

What was your weight 12 months ago? _____

What is your current weight? _____

Have you dieted other than in the past 12 months? YES NO

If YES, please answer the following:

a). How many times have you dieted?

b). How old were you?

c). Weight loss (amount)?

You may be asked to complete a more detailed diet survey if you are volunteering for a research study.

PHYSICAL ACTIVITY SURVEY

Compared to a year ago, how much regular physical activity do you get? (Check one)

- Much less
- Somewhat less
- about the same
- somewhat more
- much more

Have you been exercising regularly for the past three months?

YES NO

If YES, what type of exercise do you regularly participate in? (check those that apply)

	<input type="checkbox"/>	Days per week	Minutes per session	Intensity <small>(1=easy,10=very hard)</small>
Walking	<input type="checkbox"/>	_____	_____	_____
Running	<input type="checkbox"/>	_____	_____	_____
Cycling	<input type="checkbox"/>	_____	_____	_____
Swimming	<input type="checkbox"/>	_____	_____	_____
Aerobics	<input type="checkbox"/>	_____	_____	_____
Weight Training	<input type="checkbox"/>	_____	_____	_____
Martial Arts	<input type="checkbox"/>	_____	_____	_____
Other (describe)		_____	_____	_____

EDUCATION: Please check the highest degree obtained:

- Grade School
- Junior High
- High School
- College Degree
- Master's Degree
- Doctorate

**COLORADO STATE UNIVERSITY
INFORMED CONSENT TO PARTICIPATE IN A RESEARCH PROJECT**

TITLE OF PROJECT: Effect of dietary fat type on emerging cardiovascular risk factors in individuals with impaired glucose tolerance or type 2 diabetes

NAME OF PRINCIPAL INVESTIGATOR: Tracy L. Nelson, MPH, Ph.D.

NAME OF CO-INVESTIGATORS: Matthew S. Hickey, Ph.D. and John Hokanson, PhD

CONTACT NAME AND PHONE NUMBER FOR QUESTIONS/PROBLEMS: Tracy Nelson, 491-6320

SPONSOR OF THE PROJECT: USDA-Colorado Agricultural Experiment Station

PURPOSE OF THE RESEARCH: To determine the effect of a specific type of dietary fat on cardiovascular risk factors specifically those that may be associated with hardening of the arteries. It is important to identify factors that may be promote or protect individuals from cardiovascular disease so that it may possibly be prevented.

PROCEDURES/METHODS TO BE USED: You will be asked to complete a health history questionnaire prior to participation, which will take about 15-20 minutes. If you agree to participate and the investigators determine you are eligible, you will be scheduled for several visits to the Human Performance Clinical/Research Laboratory (HPCRL). You will not be able to participate in this study if you currently have any type of malignancy, and women who are pregnant will also be excluded. These exclusion factors will be determined from a health history questionnaire you will complete before starting the study. In addition, if your fasting glucose levels are below a certain level or your normal diet contains an unusually high intake of a specific type of fat, you will not be eligible for the study. Should you meet any of these exclusion criteria, we will fully inform you as to the reason for the exclusion.

The preliminary tests include:

1). **Finger prick** (this will take about 2 minutes) – Your finger will be pricked with a small needle while you are sitting down. The blood spot will be used to determine your fasting glucose level. We will ask that you not eat anything 10-12 hours before reporting to the HPCRL. This procedure will be performed during the screening phase to determine your eligibility for the study.

2). **Blood pressure and heart rate** (this will take about 5 minutes)– This will be taken while you are in a sitting position. Your heart rate will be obtained at the same time.

3). **Body composition (fat and lean tissue)** (this will take about 15 minutes)- This will be performed using a machine called a dual energy X-ray absorptiometer (DEXA). This unit uses 2 low energy X-rays to determine the amount of body fat you have. You should be advised that the amount of radiation exposure in this procedure is very low, about

1/10,000 of the normal radiation exposure you receive yearly from what is called “background” radiation from the

Page 1 of 5 Subject’s Initials _____ Date

environment. Put another way, the exposure from a DEXA scan is less than the normal exposure in a flight from Denver to Chicago, and about 1/250th the exposure from a normal stomach X-ray you might receive at a hospital. This test will be performed in room 124 in the HPCRL. You will be asked to lie quietly on a bed in shorts and a T-shirt for about 15 minutes while the scan is performed. **This procedure will be performed twice: once during the preliminary phase and once at the end of the protocol.**

4). Blood Sample: (this will take about 10 minutes) You will be asked to report to the HPCRL for a blood sample-you must not eat or drink (except water) anything for 10-12 hours before this sample is taken. You will lie on a cot, and a blood sample will be taken from a vein in your forearm. We will be taking a 10 cc sample, which is equivalent to about 2 teaspoons. The blood will be analyzed for the presence of the immune system factors thought to be involved in cardiovascular disease as well as genetic variants that may be associated with these factors. Genetic variations are simply areas of DNA that vary among individuals. This is similar to hair color varying among individuals. There are many people with brown hair and many people with blond hair – just as we find there are many people with certain DNA variations and others with other DNA variations. In addition, we will measure glucose, insulin and fat levels in your blood. This procedure will take about 10 minutes. **This procedure will be performed 3 times:** once during the screening period at the beginning of the study, at 4 weeks and after the completion of the protocol.

4). Diet Analysis: You will be asked to complete a 7 day diet diary in which all food and drink you eat will be recorded. We will analyze your diet using a computer program and determine the percentage of fat, carbohydrate and protein as well as vitamin and mineral status. **This procedure will be performed once during the screening phase to determine your normal diet intake and determine your eligibility for the study. The diet analysis will be repeated once per week during the study so 9 analyses will be performed.**

Study Protocol:

You will be asked to participate in this intervention designed to alter your dietary intake of a certain fat. This will be conducted on a supervised outpatient basis. You will be provided with a diet menu that includes a list of recommended items to meet the diet goals. All experimental groups will be provided with a specific type of fat supplement that can be picked up (weekly) at the HPCRL. The study diets will have roughly the same amount of carbohydrate, fat, and protein as your normal diet. All members of the study will be asked to complete weekly body weight logs and will complete 7 day diet diaries throughout the study. You can expect this study to last 8 weeks. The objective of this research project is to study adaptations to a change in

Signature

Date

RISKS INHERENT IN THE PROCEDURES:

1). DEXA: The risks associated with the DEXA are very low. The radiation you will receive in this study is less than 1/3000th of the FDA limit for annual exposure. Put another way, you could receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation

exposure. In this study, you will receive 2 scans over an 8 week period. The more radiation

you receive over the course of your life, the more is the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.

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2). Blood Samples: The risks associated with blood drawing include hematoma/bruising, slight risk of infection, local soreness, and fainting. These are all very minor risks and if present, are generally resolved in less than a day.

3). Diet Supplement: There is no risk associated with the diet supplement used in this study. It is a commercially available liquid form of a specific type of dietary fat that we believe is a "healthy fat". We have used this supplement in previous studies at CSU, and it can be purchased in any health food store.

BENEFITS:

You will receive detailed diet and body composition data, and information on cardiovascular risk factors.

CONFIDENTIALITY:

Your data will be coded and kept in a locked file cabinet on the CSU campus. A copy of the coded data must be sent to the sponsor of this project. However, you will not be identified in relation to your data at any point. We will be investigating genetic variations that may be associated with immune system parameters as well as nutrient uptake by different cells in the

body. There are many factors associated with the development of disease. These types of genetic tests may help us understand how disease develops but they will not provide us with information about whether they were there cause of a disease developing. That is to say if you were to develop cardiovascular disease, these markers don't provide enough information to say they were the cause of the cardiovascular disease. Therefore, we will not be contacting you with your results regarding these tests since they are not diagnostic in any way.

Investigator or Co-investigator Signature

Date

Page 5 of 5 Subject's Initials _____

Date

**Research Integrity & Compliance Review Office
Office of the Vice President for Research
321 General Services Building - Campus Delivery 2011
Fort Collins, CO
TEL: # (970) 491-1553
FAX: # (970) 491-2293**

NOTICE OF APPROVAL FOR HUMAN RESEARCH

DATE: July 15, 2009
TO: Nelson-Ceschin, Tracy, Health & Exercise Science
Israel, Richard, Health & Exercise Science, Swiss, Evelyn,
RICRO, Hickey, Matthew, Health & Exercise Science
FROM: Barker, Janell, CSU IRB 1
PROTOCOL TITLE: Effect of Dietary Fat Type on Emerging Cardiovascular Risk
Factors in Individuals with Impaired Glucose Tolerance or
Type 2 Diabetes
FUNDING SOURCE: US Department of Agriculture
PROTOCOL NUMBER: 09-1001H
APPROVAL PERIOD: Approval Date: June 07, 2009 Expiration Date: June 06, 2010

The CSU Institutional Review Board (IRB) for the protection of human subjects has reviewed the protocol entitled: Effect of Dietary Fat Type on Emerging Cardiovascular Risk Factors in Individuals with Impaired Glucose Tolerance or Type 2 Diabetes. The project has been approved for the procedures and subjects described in the protocol. This protocol must be reviewed for renewal on a yearly basis for as long as the research remains active. Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.

If approval did not accompany a proposal when it was submitted to a sponsor, it is the PI's responsibility to provide the sponsor with the approval notice.

This approval is issued under Colorado State University's Federal Wide Assurance 00000647 with the Office for Human Research Protections (OHRP). If you have any questions regarding your obligations under CSU's Assurance, please do not hesitate to contact us.

Please direct any questions about the IRB's actions on this project to:

Janell Barker, Senior IRB Coordinator - (970) 491-1655

Janell.Barker@Research.Colostate.edu

Evelyn Swiss, IRB Coordinator - (970) 491-1381 Evelyn.Swiss@Research.Colostate.edu

Barker, Janell

Includes: Approval is to continue to analyze data collected per approved protocol

Approval Period:	June 07, 2009 through June 06, 2010
Review Type:	EXPEDITED
IRB Number:	00000202
Funding:	US Department of Agriculture