EFFECTS OF DIETARY OILS LOW IN n-6:n-3 FATTY ACID RATIO ON CARDIOVASCULAR RISK FACTORS IN MICE: THE IMPACT OF THE SOURCE OF n-3 FATTY ACIDS

By

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ABSTRACT

The impact of the source of n-3 fatty acids on cardiovascular disease has not been fully investigated. This study was carried out to investigate cardiovascular benefits of diets with a low ratio (2:1) of n-6:n-3 fatty acids from different sources, either fish or flaxseed oil, in C57BL/6 mice. Twenty-one mice were divided into 3 groups (n=7) and fed an atherogenic diet supplemented with either a fish or flaxseed oil-based 'designer oil' with low n-6:n-3 fatty acid ratio (treated groups) or safflower oil-based formulation with a high ratio (control group) for 16 weeks. Plasma cholesterol levels declined significantly in both treated groups, by greater than 30%, compared to those in control. The ratio of n-6:n-3 fatty acids in liver was significantly lower in fish and flax groups as compared to control. Our data suggest that lowering dietary ratio of n-6:n-3 fatty acids may significantly reduce cardiovascular risks regardless of the source of n-3 fatty acids.

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5-HPETE	5-hydroperoxyeicosatetranoic acid
5-HPEPE	5-hydroxyeicosapentaenoic acid
AA	arachidonic acid
ALA	alpha-linolenic acid
apoE-KO	apolipoprotein E knock-out
CE	cholesteryl ester
CHD	coronary heart disease
COX	cyclooxygenase
$cPLA_2$	cytoplasmic phospholipase A_2
CRP	C- reactive protein
CVD	cardiovascular disease
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DRI	Dietary Recommended Intakes
EPA	eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	fatty acid(s)
FFA	free fatty acid
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GRAS	generally recognized as safe
HDL-C	high-density lipoprotein cholesterol
IFN-γ	interferon gamma
IL	interleukin
$iPLA_2$	Ca2+ independent phospholipase A_2
LA	linoleic acid
LDL-C	low-density lipoprotein cholesterol
$Lp\text{-}PLA_2$	lipoprotein associated-phospholipase A_2
LPS	lipopolysacharide
LOX	lipoxygenase
LT	leukotriene
LTB_4	leukotriene B_4
MCP	monocyte chemoattractant protein
MUFA	monounsaturated fatty acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin
PGE_2	prostaglandin E_2
PI	phosphatidylinositol
PLA_2	phospholipase A_2
PL	phospholipid
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid

RANTES	regulated on activation normally T-cell expressed and secreted
SCF	stem cell factor
SFA	saturated fatty acid
SM	sphingomyelin
SMC	smooth muscle cell
$sPLA_2$	secratory phospholipase A_2
sTNF RI	soluble tumor necrosis factor receptor I
TBHQ	tert-butylhydroquinone
TC	total cholesterol
TG	triglyceride
TLC	thin layer chromatography
TNF-α	tumor necrosis factor alpha
TX	thromboxane
VEGF	vascular endothelial growth factor

1. Introduction

Cardiovascular disease is the leading cause of disability and mortality in the western world, with diet and lifestyle playing an important role. Epidemiological and clinical studies have reported a beneficial effect of both α-linolenic acid (ALA; 18:3 n-3) and eicosapentaenoic acid (EPA; 20:5 n-3)/docosahexaenoic acid (DHA; 22:6 n-3) on cardiovascular outcomes (Hu et al., 1999; Hu et al., 2002; Djousse et al., 2001) and risk factors, such as improvements in blood lipid profile (Maki et al., 2003; Calebresi et al., 2004), blood pressure (Galeijnse et al., 2002), and inflammatory proteins (Endres et al., 1989; Fritsche et al., 1999; Babu et al., 2003). The most significant dietary source of ALA and EPA/DHA are flaxseed and fish oils, respectively. However, research to date has focused mainly on EPA/DHA, with limited studies on ALA, which has shown thus far that the health benefits of EPA/DHA are greater than those of ALA. ALA is converted to EPA/DHA endogenously; this conversion is limited because the necessary enzymes may also compete for the conversion of the n-6 fatty acid (FA), linoleic acid to arachidonic acid (AA). The competition of n-6 and n-3 FA for the desaturase and elongase enzymes is controversial, particularly for the conversion to DHA, as tissue and species may play a role (Brenner, 1974; Abedin et al., 1999; Barcelo-Coblin et al., 2005). Nonetheless, n-6 and n-3 FA also compete for the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes necessary for the conversion to eicosanoids (Simopoulos, 2002). Anti-inflammatory and anti-aggretory series 3 eicosanoids, namely prostaglandin, thromboxane and leukotrienes, are produced from EPA, whereas the pro-

inflammatory, pro-aggretory series 2 eicosanoids are derived from AA (Simopoulos, 1999; Gallai et al., 1995). The ratio of n-6:n-3 FA is therefore hypothesized to be important. Therefore, a low 2-4:1 n-6:n-3 FA ratio has recently been recommended (Simopoulos, 2002).

Positive effects of fish oil or EPA/DHA have been observed on plasma lipid profile, specifically on reducing triglycerides (TG) (Maki et al., 2003) and potential HDL cholesterol elevating effects (Calebresi et al., 2004). Flaxseed oil or ALA has shown conflicting evidence on plasma lipid profile (Cunnane et al., 1993; Lee & Prasad, 2003). However there is limited evidence of the effects of ALA as part of a low dietary n-6:n-3 ratio under well-controlled conditions. We have shown in a mouse model, namely apolipoprotein E-knockout (apo E-KO) mouse, that fish oil did not reduce plasma TG and was unable to reduce atherosclerotic lesion size in aortic roots (Xu et al., 2007). The apoE-KO mouse model differs from wild-type models in that apoE-KO mice spontaneously develop atherosclerosis. Other studies (Hu et al., 2002) have proposed that the mechanism of action of the reduced cardiovascular risk by fish oil may be due to the incorporation of n-3 FAs, particularly DHA, into tissue cell membranes creating an anti-arrhythmic effect (Schwalfenberg, 2006; Rosenberg, 2002; Simopoulos, 1999; Ander et al., 2004).

Thus, the purpose of the present study was to investigate cardiovascular benefits of diets supplemented with 'designer oils' containing low ratios of n-6:n-3 FA from different sources, either fish oil (containing high amounts of DHA and EPA) or flaxseed oil (containing high amounts of ALA), in C57BL/6 mice. We

have investigated the effects of these oils on food intake, body weight, tissue composition, plasma lipid profile, inflammatory protein profile, plasma bile acids, and cardiac myocyte phospholipase gene expression.

2. Review of Literature

2.1 Atherosclerosis

The leading cause of death in Canada and the world is cardiovascular disease (CVD) (Iuliano, 2001). CVD encompasses many types of conditions, including hypertension, coronary heart disease (CHD), stroke, and congestive heart failure. The most common type of CVD is CHD, which results from a lack of blood flow to the blood vessels nourishing the heart. The major underlying cause of CHD is atherosclerosis (Krummel, 2004). Atherosclerosis is a multifaceted vascular disease that develops from 1) the proliferation of smooth muscle cells (SMC) in the lumen; 2) the development of SMC into a matrix, and 3) the accumulation of blood lipids in that matrix, known as plaque (Krummel, 2004; Skalen et al., 2002). This accumulation of blood lipids at its initial stage is known as fatty streaks and begins at an early age (Iuliano, 2001); diet, lifestyle and genetic factors all play an important role in the progression of the fatty streak. Atherosclerotic lesions are formed in the vessel wall and ultimately this can result in restricted blood flow in the lumen, often causing hypertension if not already present. The lesion may become unstable and rupture causing the formation of a blood clot, known as a thrombus, and consequently could block the blood flow in small-size arteries. Depending on the location of the thrombus, this obstruction

could cause a myocardial infarction or cerebrovascular accident and subsequently death (Krummel, 2004).

Elevated plasma levels of low-density lipoprotein-cholesterol (LDL-C) and decreased levels of high-density lipoprotein-cholesterol (HDL-C) share a significant role in the development of atherosclerosis (Lusis, 2000). Oxidized LDL is known to initiate as well as contribute to the growth of atherosclerotic lesions (Iuliano, 2001). HDL-C has a role in reverse cholesterol transport, via the uptake of excess cellular cholesterol and consequent transport to the liver where it is excreted through bile acid (Brewer 2004). HDL-C may also protect against atherosclerosis by decreasing LDL oxidation via the paraoxonase enzyme bound to HDL, which has been observed in vitro by Mackness et al. (1991). Reducing plasma LDL-C and/or increasing HDL-C concentrations through various methods are associated with significant reductions in the risk of developing CHD (Chapman et al., 2004). Additionally, elevated plasma TG levels are another independent risk factor for CHD (Austin, et al., 1998; Byrne 1999; Malloy & Kane, 2001). Furthermore, inflammation is now understood to be another major contributing factor for CHD development (Willerson & Ridker, 2004; Ross, 1999, Lusis, 2000).

2.1.1 Inflammation, cytokines and atherosclerosis

Cytokines are small peptides that are produced by an immune response to an inflammatory stimulus and released by macrophages, endothelial cells, and adipocytes; they can behave in an autocrine, paracrine, or endocrine manner

(Babcock et al., 2004; Bousserouel et al., 2003; Fisman et al., 2003; Grundy, 2006). There are a number of proinflammatory cytokines known to be associated with CVD and atherosclerosis. Some cytokines such as IL-12 and TNF-α directly influence the development and growth of atherosclerotic lesions, while others like IL-17 stimulate the production of other cytokines or proteins that are key markers of inflammation, such as C-reactive protein (CRP) (Mori & Beilin, 2004). Conversely, there are also anti-inflammatory cytokines as well as those with no known influence or a controversial influence on atherosclerosis. A summary of the effects of cytokines on atherosclerosis is found in **Table 1**.

2.2 n-3 polyunsaturated fatty acids

FA are classified as either saturated or unsaturated; unsaturated FA can be further classified as monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA). Saturated FA are fully saturated with hydrogen atoms and no double bonds present; examples of saturated FA include stearic acid and palmitic acid. Dietary intake of saturated fat is known to be detrimental to heart health by elevating both TC and LDL-C. Dietary Recommended Intakes (DRI) released by the National Academy of Sciences (2005) state to limit our dietary intake of saturated fat to less than 10% of an individual's total energy intake. MUFA contain one double bond in their structure; an example of a MUFA is oleic acid. High MUFA intake, typical of the Mediterranean diet, has been shown to have positive health benefits with respect to blood lipid profile (Ginsberg et al., 1999). Therefore, it is recommended to include MUFA in our daily diets, while

Table 1. **Classification of cytokines by their effect on or relationship with atherosclerosis**

Classification of Effect	Cytokines	Effect on Atherosclerosis	Model	Reference
Beneficial	IL-5	Major factor in immunoglobulin secretion of B-1 cells	IL-5 -/- mice	Kopf et al., 1996
			NA	Takatsu, 1998
	IL-10	Major anti-inflammatory cytokine; overexpression reduces atherosclerosis	LDL receptor KO mice	Pinderski et al., 2002
	IL-11	May influence cytoprotection in myocardium	NA	Fisman et al, 2003
	IL-13	Promotes cell survival by preventing apoptosis	Cell culture	Evans & Kilshaw, 2000
Harmful	IL-1β	Contributes to development of lesions by activating SMCs	Cell culture from Wistar rats	Bousserouel et al., 2003
	IL-2	Involved in T-cell activation; growth promoting factor of T lymphocytes	NA	Fayad et al., 2006
	IL-6	Major procoagulant cytokine; regulates acute phase proteins; increases plasma fibrinogen	NA	Koh et al., 2005
			NA	Heinrich et al., 1990
	IL-12	Accelerates atherosclerotic lesion development	ApoE KO mice	Lee et al., 1999
			ApoE KO mice	Davenport & Tipping, 2003
	IL-17	Strong positive association with CRP and increases production of IL-6; increased IL-17 during acute myocardial infarction and unstable angina	Coronary artery disease patients	Hashmi & Zeng, 2006
	MCP-1	Involved in SMC migration; found in atherosclerotic plaques;	NA	Charo & Peters, 2003
		Precursor to foam cells	Pigs	Gerrity & Naito, 1980
	MCP-5	Expressed in plaque macrophages and concentrations increased with plaque progression	ApoE KO mice	Lutgens et al., 2005
	RANTES	Associated with development of internal carotid artery	Patients with	Ghilardi et al., 2008

		occlusive disease;	internal carotid artery occlusive disease	
		RANTES antagonist limits plaque formation	LDL receptor KO Mice	Braunersreuther et al., 2008
	TNF-α	Major inflammatory cytokine; stimulates production of MCP-1;	NA	Libby et al. 2005
	sTNF RI	Expressed in atherosclerotic lesions	Human cell culture	Barath et al., 1990
		Associated with carotid atherosclerosis	Stroke-free patients	Elkind et al., 2002
Controversial	IL-3	Beneficial in terms of glucose metabolism; harmful in terms of CVD	NA	Fisman et al., 2003
	IL-4	Have been expressed in human plaques and also supports cell survival against TNF-alpha.	Pig aorta endothelial cells	Grehan et al., 2005 Fisman et al., 2003
		May regulate 15-lipoxygenase leading to LDL oxidation	Cultured human monocytes	Conrad et al., 1992
	IFN-γ	Has both beneficial (increases superoxide distmutase – plaque stabilization) and harmful effects (induces vascular smooth muscle cell apoptosis – plaque destablilization); overall effects on atherosclerosis are harmful	Mouse peritoneal macrophages Human vascular smooth muscle cells	Alfaro Leon & Zuckerman, 2005 Rosner et al., 2006
	G-CSF	Effect on atherosclerosis is unclear	Human aortic endothelial cells	Rajavashisth et al., 1990
		May have protective and regenerative properties in post-infarction myocytes	NA	Takano et al., 2006
		Improves signs and symptoms of peripheral artery disease to same extent as bone marrow transplant	Patients with atherosclerotic peripheral artery disease	Arai et al., 2006
	GM-CSF	Deficiency increased atherosclerosis in apoE KO mice given hypercholesterolemic diet	ApoE KO mice	Ditiatkovski et al., 2006

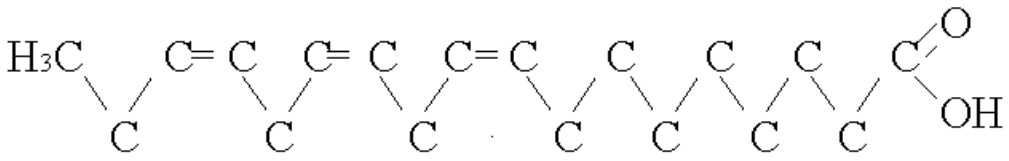
		Increases atherogenesis by affecting monocyte and SMC gene expression; upregulates histamine	Human and C57BL/6J mouse atherosclerotic lesions	Murata et al., 2005
	Thrombopoietin	Levels may be influenced by atherosclerosis;	Patients with coronary atherosclerosis	Cotton et al., 2003
		May be useful in the treatment of acute ischemia and cardiotoxicity	H9C2 cell line	Li et al., 2006
	VEGF	Closely related to atherosclerosis accelerating factor;	Adult outpatients	Kimura et al., 2006
		Reduce atherosclerotic developmemt	ApoE KO mice	Fabrizio Rodella et al., 2006
Unknown effects	IL-9	Their effects on atherosclerosis have not yet been established	NA	Fisman et al., 2003
	SCF	Expressed in human endothelial and smooth muscle cells; role not established, further research is needed	Human arterial endothelial and smooth muscle cells	Miyamoto et al., 1997
		Suppresses apoptosis	Mouse mast cells	Iemura et al., 1994

IL, interleukin; MCP, monocyte chemoattractant protein; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon gamma; RANTES, regulated on activation normally T-cell expressed and secreted; SCF, stem cell factor; TNF-α, tumor necrosis factor alpha; sTNF RI, soluble tumor necrosis factor receptor I; VEGF, vascular endothelial growth factor; ApoE KO, apolipoprotein E knock-out.
NA, not applicable (information attained via review article)

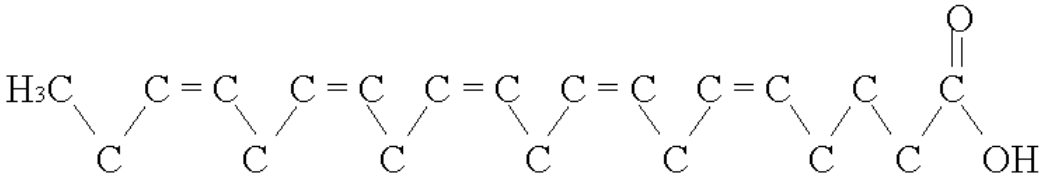
still maintaining a diet with between 20-35% of total energy from fat (National Academy of Sciences, 2005).

PUFA include more than one carbon-carbon double bond in their structure. The first carbon double bond can occur on either carbon 6 or carbon 3 from the methyl end, which is referred to as either n-6 or n-3 PUFA, respectively. There are many n-3 FA, the most important being α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whose structures are shown in **Figure 1**. n-3 FA are necessary for tissue membranes, most importantly in the nervous system and retina; a deficiency in n-3 FA can lead to scaly dermatitis, learning deficiencies, sensory neuropathy, and visual problems (National Academy of Sciences, 2005; Yamamoto et al., 1987; Holman et al., 1982; Holman & Johnson, 1982). Dietary ALA is provided mainly from plant sources (**Table 1**) while EPA and DHA are provided from marine sources (**Table 2**). Humans lack the enzymes necessary to synthesize these FA, therefore they are known as essential FA. ALA can be converted to DHA and EPA endogenously, although this conversion is limited; thus the essentiality of these FA seems controversial (Cunnane, 2003).

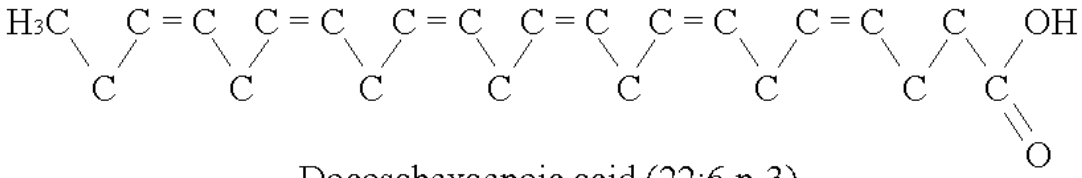
Figure 1. **Chemical structures of α-linolenic acid, docosahexaenoic acid, and eicosapentaenoic acid**



Alpha-linolenic acid (18:3 n-3)



Eicosapentaenoic acid (20:5 n-3)



Docosahexaenoic acid (22:6 n-3)

Table 2. **Dietary sources of α-linolenic acid**

Dietary Source	(g oil/100 g sample)	% 18:3 n-3 of total lipid
Flaxseed	29.3	43.3
Poppy seed	42.3	1.3
Sesame	40.5	0.7
Kidney bean	1.2	45.7
Hazelnut	49.2	0.5
Peanut	37.9	0.6
Almond	40.8	0.2
Walnut	50.8	11.6

(Adapted from Maguire et al., 2004; Ryan et al., 2007)

Table 3. **Dietary sources of docosahexaenoic and eicosapentaenoic acid**

Dietary Source (100 g edible portion)	DHA and EPA (g)[1]	Reference
Anchovy	1.4	Mahan & Escott-Stump, 2004[2]
Bass, farmed	1.0	Cahu et al., 2004
Bass, wild	0.2	Cahu et al., 2004
Bluefish	1.2	Mahan & Escott-Stump, 2004[2]
Cod, wild	0.1	Cahu et al., 2004
Herring, Atlantic	1.6	Mahan & Escott-Stump, 2004[2]
Lobster	0.4	Mahaffey et al., 2007
Mackerel	1.8	Mahaffey et al., 2007
Mahimahi, wild, Hawaii	0.2	Ako et al., 1994
Nori	0.1	Ako et al., 1994
Perch, wild	0.5	Cahu et al., 2004
Pollock	0.3	Mahaffey et al., 2007
Salmon, pink	1.0	Mahan & Escott-Stump, 2004[2]
Sardines	1.0	Mahaffey et al., 2007
Shrimp	0.4	Mahaffey et al., 2007
Trout	0.6	Mahaffey et al., 2007
Tuna, albacore	1.3	Mahan & Escott-Stump, 2004[2]

DHA, decosahexaenoic acid; EPA, eicosapentaenoic acid

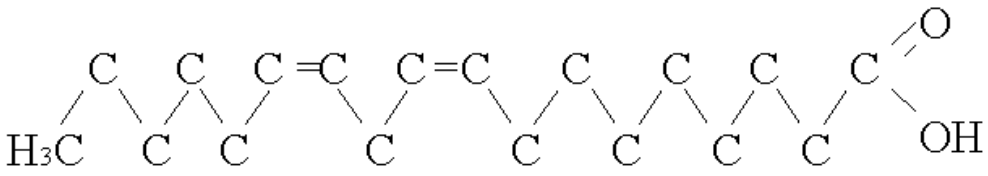
[1]Important note: Values vary throughout season and dietary changes (Cahu et al., 2004)

[2]Adapted from Mahan & Escott-Stump, 2004, which was modified from Simopoulos AP, Kifer RR, Martin RE. The health effects of polyunsaturated fatty acids in seafoods. Washington D.C., 1986, Academic Press and Human Nutrition Information Service, USDA: Provisional table on the content of omega-3 fatty acids and other fat components in selected foods, HNIS/PT-103, 1988.

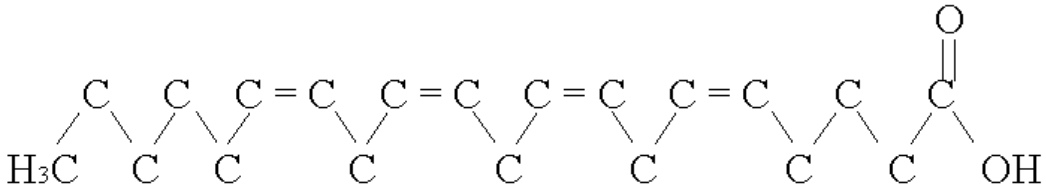
The main n-6 FA is linoleic acid (18:2, LA), which can then be converted endogenously to arachidonic acid (20:4, AA); these structures are illustrated in **Figure 2**. Linoleic acid is an essential FA; a deficiency of dietary n-6 FA can lead to rough, dry, and scaly dermatitis (National Academy of Sciences, 2005). It is recommended to consume less than 10% of total energy from PUFA. In 1990, Health Canada recommended a daily dietary intake of 1500 mg n-3 FA and 9000 mg n-6 FA for men and 1100 mg n-3 and 7000 mg n-6, respectively, for women;

or 0.5% of energy for n-3 and 3% for n-6, which provides a n-6:n-3 FA ratio of 6/1 (Ferrier et al., 1995; Scientific Review Committee, 1990). Canada was the first country to make separate recommendations for each PUFA, both n-3 FA and n-6 FA (Scientific Review Committee, 1990). These remain as the current n-3 and n-6 FA recommendations in Canada, with no differentiation between ALA and EPA/DHA. However, the recently released Canada's Food Guide now recommends consuming at least two meals containing fish per week and 2-3 Tbsp oils such as canola, soybean, or olive per day.

Figure 2. Chemical structures of arachidonic acid and linoleic acid



Linoleic acid (18:2 n-6)



Arachidonic acid (20:4 n-6)

2.2.1 n-3 polyunsaturated fatty acids and atherosclerosis

Numerous studies have reported beneficial effects and/or associations of dietary n-3 FA, from both ALA, and DHA and EPA on atherosclerosis and its

risk factors (Rosenberg, 2002; Mori et al., 1997; Zaloga et al., 2006; Baylin et al, 2003; Takahashi et al., 2005). Furthermore, epidemiological studies have revealed that both dietary ALA and EPA/DHA intake are positively associated with reduced CVD risk (Djousse et al., 2001; Hu et al., 1999, Hu et al., 2002; Albert et al., 1998; Daviglus et al., 1997, Thies et al., 2003). Much of the work done on n-3 FA has been regarding DHA and EPA found in fish oil; recently there has been much interest on the health benefits of ALA and flaxseed. The research to date on flaxseed and ALA has been promising.

Positive effects on plasma lipids have been shown by both types of n-3 FA. A reduction in plasma TG has been observed as a result of long-chain n-3 FA intake (Harris & Bulchandani, 2006; Harris, 1989; Kudo & Kawashima, 1997; Maki, 2003). There are several possible mechanisms: 1) n-3 FA are susceptible to β-oxidation (Guo et al., 2005) and decrease the uptake of non-esterified FA by the liver, which reduces the availability of the FA necessary for TG synthesis; 2) n-3 FA may reduce the activity of enzymes essential for TG synthesis (diacylglycerol acyltransferase and phosphatidic acid phosphohydrolase); or 3) phospholipid synthesis is increased, which reduces the diacylglycerol required for TG synthesis (Harris & Bulchandani, 2006; Schwalfenberg, 2006). Increases, although small, in HDL-C as a result of long-chain n-3 FA intake have also been observed (Calabresi et al., 2004; Ferrier et al., 1995; Breslow, 2006). Effects of fish oil on plasma total, LDL cholesterol, and apoB has been inconsistent; elevated LDL-C and ApoB levels have been observed with DHA supplementation (Goyens & Mensink, 2006; Maki et al., 2003). Conversely, long-chain n-3 FA

have been shown to reduce LDL particle size (Mori et al., 2000; Contacos et al., 1993). Flaxseed has been shown to reduce total and LDL cholesterol (Prasad et al., 1998) by 9% and 18% respectively (Cunnane et al., 1993). However, the cholesterol lowering properties of flaxseed are likely due to the presence of lignan (dietary fibre) not ALA (Babu et al., 2003; Lee & Prasad, 2003). Flaxseed oil had no significant effects on serum cholesterol concentrations in hypercholesterolemic rabbits (Lee & Prasad, 2003). Lignans are not found in significant amounts in flaxseed oil (Flax Council of Canada).

Many of the beneficial effects on cardiovascular outcomes are related to the incorporation of n-3 FA, especially DHA and EPA, into cell membranes and their consequent antiarrhythmic effect (Kang & Leaf, 1994; Kang & Leaf, 1995). Arrhythmia refers to the abnormal rhythm of the heart beats, either too fast, too slow, skipped or extra beats. This condition occurs as a result of poor electrical signaling in the heart muscle to other chambers (atria or ventricles) for a fully coordinated beat. Cunnane et al. (1993) also showed that ALA was readily incorporated into plasma and erythrocyte membranes. Nevertheless, DHA and EPA are incorporated into plasma and tissue membranes at a faster rate than ALA (Simopoulos, 1999). Furthermore, Wang et al. (2005) reported that DHA, not EPA or ALA, is likely responsible for the antiarrhythmic effect. The antiarrhythmic effect is most likely due to the improvement in membrane fluidity (less rigid) with the FA incorporation into cardiac cell membrane, thereby preventing atrial fibrillation (rapid twitching of cardiac muscle fibre) by altering the sodium and calcium ion channels (Schwalfenberg, 2006; Rosenberg, 2002). In

addition, Mozaffarian et al. (2006) have observed slower heart rates, reduced atrioventricular conduction, and lower risk of prolonged ventricular repolarization in participants with higher fish consumption compared to those with the lowest intake. This finding indicates an overall improvement in heart rhythm. Improved membrane fluidity may also influence the binding of cytokines to the membrane (Ergas et al., 2002).

The drawback of the incorporation of n-3 FA into cell membranes is an increased risk for lipid peroxidation as oxidative stress is known to be a factor in atherogenesis (Mori & Beilin, 2004; Iuliano, 2001). ALA is less susceptible to oxidation compared to DHA and EPA because of less double bonds compared to EPA and DHA (Finnegan et al., 2003; Egert et al., 2007). However, it was shown that the production of reactive oxygen species was reduced with the incorporation of DHA into cell membranes. Therefore, n-3 FA may actually reduce oxidative stress (Mori & Beilin, 2004; Ergas et al., 2002). The effect of all n-3 FA on lipid peroxidation requires further investigation.

Perhaps the greatest effect of n-3 FA is their anti-inflammatory activities, possibly via the shift in eicosanoid production, which will be explained shortly. Also, as mentioned previously, inflammation is now known to play a significant role in atherosclerosis. Another possible mechanism by which n-3 FA may reduce inflammation is via an interaction with peroxisome proliferators activated receptor-alpha, a transcription factor that breaks down leukotrienes (Ergas et al., 2002). Positive effects of dietary n-3 in either humans or animals on specific cytokines including IL-1β, IL-6, TNF-α, IL-12, and interferon-δ (Endres et al.,

1989; James et al., 2000; Fritsche et al. 1999) have been reported. Dietary flaxseed, containing ALA, has also been shown to reduce expression of the inflammatory markers IL-6, mac-3, and VCAM-1 and subsequently reduce atherosclerosis in LDL-R-KO mice (Dupasquier et al., 2007). Other studies (Babu et al., 2003) have also demonstrated anti-inflammatory effects of flaxseed, at least in part being attributed to ALA (Zhao et al., 2004). The effect of n-3 FA on many other cytokines has yet to be investigated. Other cardiovascular effects of n-3 FA include a reduction in platelet aggregation, platelet count (Mori et al., 1997; Yamada et al., 1998), and blood pressure (Geleijnse et al., 2002; Breslow, 2006).

The effect of n-3 FA on atherosclerosis is ultimately a major cardiovascular outcome as well. An epidemiological study by Hino et al. (2004) showed an inverse association between long-chain n-3 FA intake and carotid intima-media thickness, a marker of atherosclerosis, using ultrasonography. In addition, Thies et al. (2003) have shown that long-chain n-3 FA enhance plaque stability. Despite these positive results, 3 major clinical trials failed to show that n-3 FA prevent restenosis (reoccurrence of a clogged artery) after angioplasty (Leaf et al., 1994; Cairns et al., 1996; Johansen et al., 1999). Similarly, we have recently shown in apo E-KO mouse, that 1% dietary fish oil for 14 weeks was unable to reduce atherosclerotic lesion size in aortic roots (Xu et al., 2007). A possible explanation is that this outcome may be exclusive to this animal model.

Overall, the effects of both ALA and EPA/DHA appear to be beneficial with respect to cardiovascular risk. However EPA/DHA has been shown to be more potent in their risk reduction in clinical trials, as summarized in **Table 4**.

Some discrepancies may also be accounted for by differences in study populations, such as genetics and metabolic conditions. Nevertheless, low n-3 FA intake is now being recognized as a relevant risk factor for CVD, which has led to the investigation of a marker to assess n-3 FA status. A current area of interest is the 'omega-3 index', which is the percent n-3 FA of total lipid in plasma membranes. The clinical applicability is being investigated.

2.3 Dietary n-6:n-3 ratio

In Paleolithic times, people consumed a diet with an n-6:n-3 ratio of approximately 1-2/1 (Eaton & Konner, 1985). Currently, the ratio of the typical western diet is 20-30/1. According to the Multiple Risk Factor Intervention Trial, a longitudinal trial with over 12000 male participants, the average intake of long-chain n-3 FAs was approximately 175 mg/day, with 20% reporting no intake (Dolecek & Granditis, 1991). This intake is well-below the desirable range. The increase in the n-6:n-3 ratio is due to both a decrease in fish intake and the emphasis on mass agricultural production, which has reduced the n-3 content in many foods (Simopoulos, 1999; Simopoulos, 1995). Furthermore, sources of n-6 are much more varied than sources of n-3 and there are many limitations to fish intake, a major source of n-3. Even populations with typically high n-3 FA intake, for example Japan, are decreasing their n-3 intake while also increasing

Table 4. Summary of studies comparing the effects of DHA/EPA intake to ALA intake on cardiovascular risk factors and tissue fatty acid composition

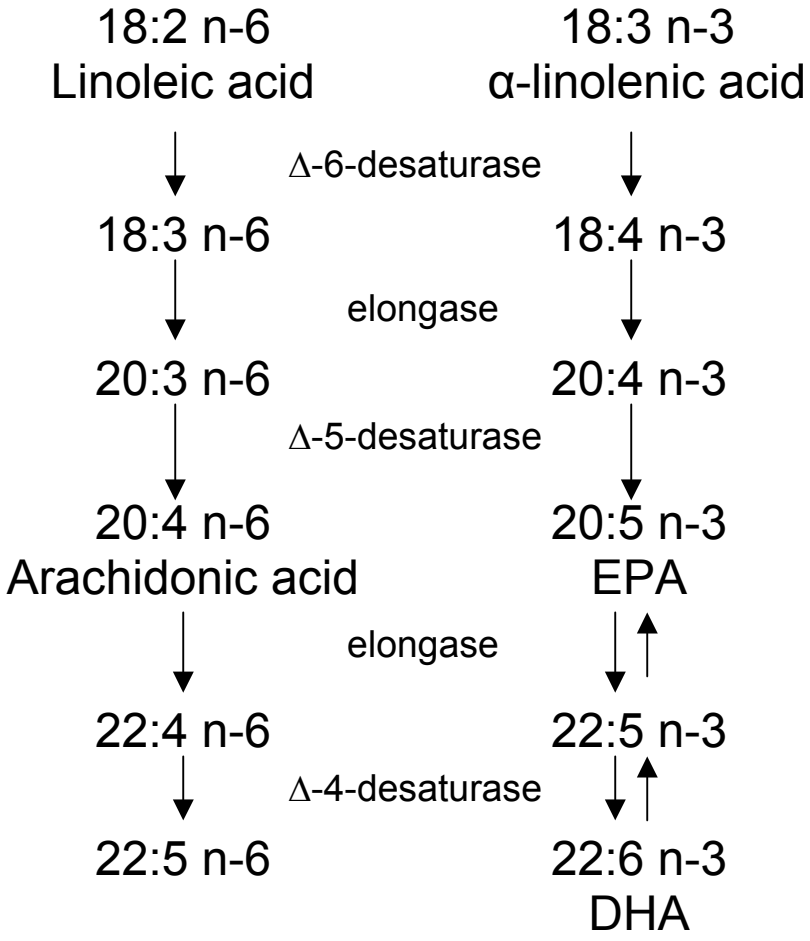
Reference	Subjects (n per treatment group)	Source/ratio	Duration	Outcome
Goyens & Mensink, 2006	Mildly hypercholesterolemic subjects (37)	6.8 g ALA/d or 1.05 g EPA + 0.55 g DHA/d	6 weeks	-EPA/DHA increased LDL-C and apoB compared to ALA -EPA/DHA increased tissue factor pathway inhibitor, which helps regulate blood coagulation -other plasma lipids or markers of endothelial function did not differ between groups
Wilkenson et al., 2005	Mildly hypertriglyceridemic male subjects (57)	15 g ALA/d (0.5/1 ratio) or 3 g EPA/DHA/d (5.2/1 ratio)	12 weeks	- EPA increased in plasma erythrocytes in ALA group; DHA was increased in the EPA/DHA group - ALA reduced plasma total cholesterol and TG - EPA/DHA also reduced plasma TG and small, dense LDL - no difference between groups in plasma clotting factors
Finnegan et al., 2003	Moderately hyperlipidemic men and women (30)	0.7 or1.5 g EPA/DHA/d or 5 or 10 g ALA/d	6 months	- No difference between groups in any factors relating to coagulation or fibrinolytic factors
Abedin et al., 1999	Female 3-week old guinea pigs (14)	7% ALA or 1% AA + 0.7% DHA or 3% AA + 2.1% DHA (ad libitum)	12 weeks	- ALA increased both liver and heart phospholipid ALA, EPA, and DHA however, liver and heart EPA and DHA were not increased to the same extent as AA+DHA groups
Finnegan et al., 2003	Moderately hyperlipidemic men	0.7 or1.5 g EPA/DHA/d or 5 or	6 months	- All treatment groups showed significant improvements in plasma EPA, however ALA did

	and women (3)	10 g ALA/d		not improve plasma DHA - plasma TG was reduced in EPA/DHA groups at 2 months but not sustained at 6 months - no treatment effects on other plasma lipids - EPA/DHA increased susceptibility to LDL-oxidation in vitro
Egert et al., 2007	Healthy young adults (15-17)	2.5 g ALA/d or 1.1 g EPA/d or 1.1 g DHA/d	3 weeks	- ALA significantly increased plasma TG - Experimental diets had no significant effect on other plasma lipids - Both EPA and DHA increased susceptibility to LDL-oxidation ex vivo - ALA increased plasma ALA and EPA but not DHA
Yamada et al., 1998	5-week old Sprague-Dawley rats (8)	5% ALA or 4.6% EPA or 4.8% DHA (all 5/1 n-6:n-3 ratio)	2 weeks	- DHA reduced platelet aggregation compared to control, ALA and EPA did not; all 3 groups did not differ significantly - EPA and DHA significantly reduced plasma TC and TG compared to ALA; plasma HDL did not differ between groups -ALA, EPA, and DHA reduced prostacyclin production compared to control in aorta

their n-6 intake, as they are influenced by western culture (Sugano & Hirahara, 2000).

Both n-3 and n-6 FA require the same enzymes for converting linoleic acid (LA) and ALA in the endoplasmic reticulum (ER) to AA and DHA/EPA respectively, as seen in **Figure 3.** There may be competition between these two essential FA (LA and ALA), specifically for the rate-limiting desaturases (Brenner, 1974). Hence, the desaturation step is slower than the elongation one (Personal communication, S. Cunnane, June 21, 2007). Therefore an increase of dietary LA or ALA results in limited metabolism of the other (Schwalfenberg, 2006). It is well-accepted that approximately 2-3% of ALA is converted to EPA and 0.6% to DHA (Personal communication, S Cunnane, June 21, 2007; S Innis, June 21, 2007). The conversion has been shown to be tissue dependent by several studies (Abedin et al., 1999; Barcelo-Coblin et al., 2005), and also thought to be species dependent. Additionally, the conversion may be altered throughout the lifespan. It is questionable whether some tissues, or even humans in general, possess sufficient Δ-4 desaturase, if any, for the conversion of docosapentaenoic acid (DPA) to DHA (Sprecher, 1999).

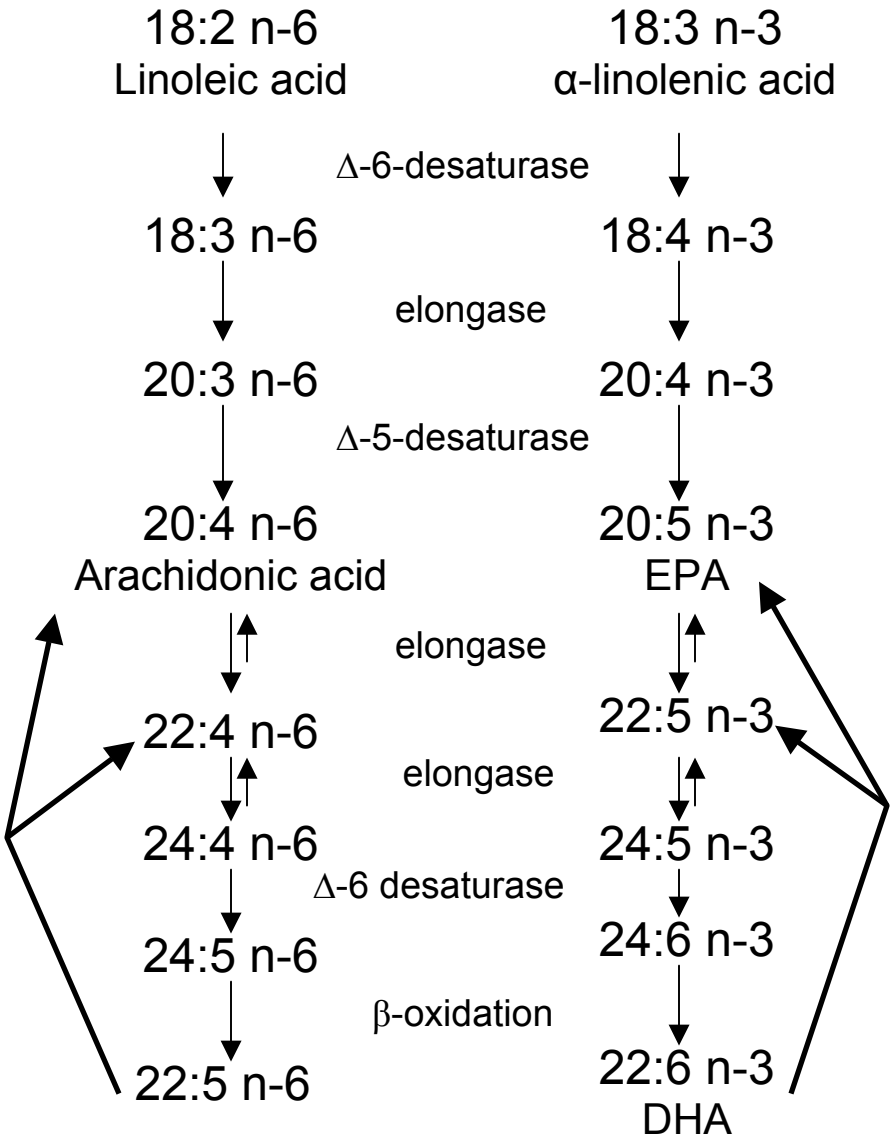
Figure 3: Conversion pathways of linoleic acid and α-linolenic acid
(Adapted from Abayasekara & Wathes, 1999)



Sprecher and colleagues (1995; 1999; 2000) have reviewed the research refuting the existence of the delta-4 desaturase enzyme, seen in Figure 3. It is their conclusion that 22:5(n-3) is converted to 24:5(n-3) then 24:6(n-3) in the ER. The peroxisome receives 24:6(n-3) from the ER, where it can then be converted via β-oxidation to 22:6(n-3) (DHA). The revised pathway is illustrated in **Figure 4**. It is Sprecher and colleagues' conclusion that n-6 FA follow a similar metabolic pathway. Thus it seems n-6 and n-3 FA may also compete for the

enzymes in the degradation-resynthesis cycle of long-chain FA. This revised pathway is now generally more accepted amongst the scientific community. Nevertheless, the n-6:n-3 ratio appears to remain critical.

Figure 4. Revised conversion pathways of linoleic acid and α-linolenic acid (Sprecher et al. 1995; 1999; 2000)



Other dietary components have been shown to influence the conversion of ALA to EPA/DHA. Trans FA interfere with the desaturase and elongase enzymes necessary for essential FA (Simopoulos, 1999). Even saturated and monounsaturated (n-9) FA may suppress some of the health benefits of n-3 FA by altering its incorporation into cell membranes, as shown in plasma and liver of Wistar rats (MacDonald-Wicks & Garg, 2004). Therefore, not only is the ratio of n-6:n-3 FA important for the body to receive the benefits of n-3 FA, but also total dietary fat composition (Simopoulos, 1999). In addition, ALA is a shorter chain length than EPA/DHA, and therefore it is also oxidized directly for energy, whereas EPA is preferentially incorporated into membranes (de Deckere et al., 1998). The n-6:n-3 ratio may be especially important for vegetarians and vegans, as they have limited, if any, intake of EPA or DHA; their sole dietary source of n-3 FA may be ALA (Davis & Kris-Etherton, 2003).

It is well known that the ratio of n-6:n-3 FA must be reduced; however the extent of reduction is relatively unknown. Controversy exists over the most appropriate dietary n-6:n-3 FA ratio; many countries provide their own separate recommendations. Many also believe that the ratio is not useful because ALA and EPA/DHA are inherently different and therefore should have separate recommendations (de Deckere et al., 1998). Current recommendations by Simopoulos (2002) are to consume a 1-2:1 dietary n-6:n-3 FA ratio, which is closer to human's traditional intake. Most countries and health organizations (including the World Health Organization) only make separate recommendations as to the total amount of n-6 and n-3 FA, not regarding the ratio.

2.3.1 Dietary n-6:n-3 fatty acid ratio and atherosclerosis

Decreasing the dietary ratio of n-6:n-3 FA has implications not only with respect to potentially amplifying the beneficial effects of n-3 FA, but also by reducing the atherogenic effects of chronic production of series 4 leukotrienes and series 2 prostaglandins. The reason for this effect is that EPA competes with AA for the same enzymes, namely lipoxygenase (LOX) and cyclooxygenase (COX), in eicosanoid production (**Figure 5**). Eicosanoids are paracrine hormones that affect blood vessel contraction, platelet aggregation, blood clotting, and regulation of cytokine production (Babcock et al., 2004; Ettinger, 2004). Phospholipase A_2 releases AA and EPA, the substrate used to produce eicosanoids, from the cell membrane (Berne et al., 2004; Murphy & Ward, 2005). The functions of both AA- and EPA-derived eicosanoids are listed in **Table 5**. Overall, eicosanoids produced from AA produce a strong inflammatory response, whereas those produced from EPA produce less inflammatory response, which may prevent blood clots from forming in the arteries. Dietary supplementation of n-3 FA confirms that PGE_2 and LTB_4 production is decreased in multiple sclerosis patients (Gallai et al., 1995).

Figure 5. Metabolism of arachidonic acid and eicosapentaenoic acid via the cyclooxygenase and lipoxygenase pathway
AA, arachidonic acid; EPA, eicosapentaeinoic acid; 5-LOX, 5-lipoxygenase; COX, cyclooxygenase; 5-HPETE, 5-hydroperoxyeicosatetranoic acid; 5-HPEPE, 5-hydroxyeicosapentaenoic acid.
(concept adapted from Simopoulos, 2002; Berne et al., 2004)

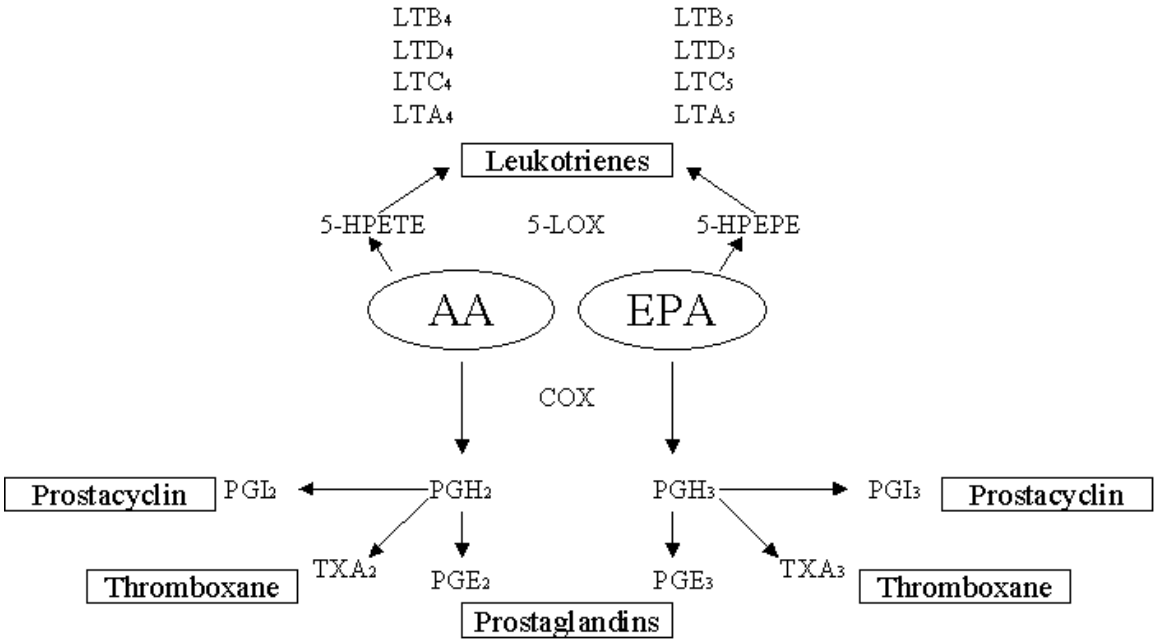


Table 5. Eicosanoid functions

Eicosanoid	Function
PGE_2	Produces general inflammatory responses and weak vasodilator
PGI_2	Produces inflammatory response and vasodilator
PGI_3	Active vasodilator and inhibitor of platelet aggregation
TXA_2	Potent platelet aggregator and vasoconstrictor
TXA_3	Weak platelet aggregator and vasoconstrictor
LTC_4	Induces smooth muscle contraction and increases microvascular permeability
LTB_4	Induces inflammation and leukocyte chemotaxis and adherence
LTB_5	Weak inducer of inflammation and chemotactic agent

(Adapted from Simopoulos, 1999; Gallai et al., 1995, Flax Council of Canada; Murphy & Ward, 2005)

Many studies have confirmed that a low dietary n-6:n-3 FA ratio may reduce cardiovascular risk; however comparisons between ALA and EPA/DHA as part of the ratio have been scarce. The theory of the dietary n-6:n-3 ratio assumes that effects of all n-3 FA, namely ALA and EPA/DHA, have comparable effects. However, literature does not support equivalent effects of both classes of n-3 FAs. Another argument against assessing the n-6:n-3 FA ratios, is also that it suggests that lowering one's n-6 FA intake will also provide a physiological benefit, ie. lower tissue AA; however, this effect has yet to be consistently shown. Also, the desirable ratio may differ throughout the lifespan. Finally, because the competition for desaturase and elongase enzymes is controversial and the competition for COX and LOX is believed to be more important, the AA/EPA ratio may be of more clinical importance (Harris, 2006). Nevertheless, studies directly comparing similar and low dietary n-6:n-3 ratios from both plant and marine sources have been scarce.

Of those studies focusing on the ratio of n-6:n-3, results have been inconsistent and have not always compared ALA to EPA/DHA. A low dietary LA/ALA ratio (2.8/1) has been observed to decrease platelet aggregation in comparison to a higher ratio with identical amounts of dietary monounsaturated fat (Freese et al., 1994). Yamada et al. (1996) have also shown that reducing dietary n-6:n-3 ratio reduces platelet count. Other key studies investigating n-6:n-3 ratio on cardiovascular risk are summarized in **Table 6**.

Table 6. Summary of studies investigating low n-6:n-3 ratio on cardiovascular risk

Reference	Subjects (n per treatment group)	Design	Source/dietary ratio	Duration	Outcome
Kiecolt-Glaser et al., 2007	Elderly men and women (43)	Observational	Mean n-6:n-3 ratio of plasma PL is 14.13	Not applicable	- Both serum IL-6 and TNFα were significantly associated with plasma PL n-6:n-3 ratio
Sanders et al., 2006	Men and post-menopausal women (43-59)	Randomized parallel design	n-6:n-3 ratio of 11.4 (control); 2.9 (EPA/DHA); 4.4 (ALA) 2.4 (EPA/DHA +ALA) 6.6 (moderate ALA)	6 months	- plasma TG decreased only in those subjects that received additional EPA/DHA - plasma fibrinogen was not affected by dietary intervention
Griffen et al., 2006	Men and post-menopausal women (43-59)	Randomized parallel design	n-6:n-3 ratio of 11.4 (control); 2.9 (EPA/DHA); 4.4 (ALA) 2.4 (EPA/DHA +ALA) 6.6 (moderate ALA)	6 months	- no difference in insulin sensitivity or plasma glucose between the groups - the EPA/DHA + ALA group had increased HDL compared to control - fasting TG was decreased only in the groups receiving EPA/DHA - quartiles of n-6:n-3 ratio (regardless of group) and LDL, HDL, LDL density, fasting and post-prandial TG were not significantly associated
Liou et al., 2007	Healthy men 20-45 y of age (22)	Randomized cross-over	LA:ALA ratio of 4:1 or 10:1 (equal ALA/diet)	4 weeks	- plasma LA was increased and EPA decreased during the 10:1 diet -plasma AA:EPA was lowered after the 4:1 diet -The ratio did not alter plasma LDL, HDL, CRP or platelet aggregation

Table 6 continued

Minihane et al., 2005	Healthy adult Indian Asian men (14-15)	Randomized double blind parallel study	Moderate n-6:n-3 ratio (9/1) or high n-6:n-3 ratio (16/1) with identical amounts of total n-3	6 weeks	- no effect of diet on total n-6 or AA in platelet PL fatty acid composition - total n-3 in platelet PL fatty acids was significantly higher (5%) in those on moderate n-6:n-3 ratio diet compared to high ratio - no difference between groups in plasma lipids, insulin, glucose, or CRP
Yamashita et al., 2005	Double homozygous apoE -/- deficient and C57BL/6 mice	Animal study (4 groups)	n-6:n-3 ratios of 0.29;1.43, 5.00 and 8.00 (from different proportions of safflower and flaxseed oil)	16 weeks	- plasma TG and LDL was significantly lower in the lowest n-6:n-3 ratio group compared to the other groups and HDL was higher - atherosclerosis was statistically less in the lowest ratio group compared to the group with the highest ratio and showed a dose dependent decrease with decreasing n-6:n-3 ratio
Ezaki et al., 1999	Healthy elderly 67-91 y of age (20)	Cross-over	n-6:n-3 ratio of 4:1 or 1:1 (lowered by replacing soybean oil with Perilla oil)	10 months	-serum ALA increased at 3 months in 1:1 group - serum EPA and DHA were increased at 10 months -serum cholesterol, TG, glucose, insulin, platelet count, and platelet aggregation were not altered by the ratio

2.4 Phospholipids and phospholipases

As previously mentioned, many of the beneficial effects of n-3 FA are associated with their incorporation into membrane phospholipids. Phospholipids are similar in structure to TG, but differ in that they contain a phosphate group at position three. A saturated FA is usually found on carbon 1, and a highly PUFA on carbon 2 of the glycerol backbone. The types of phospholipids and the FA within those lipids are continually changing in response to their environment and dietary lipid intake. The main phospholipids are sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), lysophosphatidylcholine (PC), phosphatidylinositol, and phosphatidylethanolamine (Ettinger, 2004). Phosphatidylcholine is the major phospholipid in the cell membrane. Sphingomyelin differs from the other phospholipids in that it has a sphingosine base rather than a glycerol base; this phospholipid is located in all cell membranes but found in greater amounts in the nervous system, specifically the myelin sheath (Ettinger, 2004). Lysophospholipids differ from other phospholipids in that they are missing a FA at the sn-2 position, as a reflection of phospholipase activity.

Phospholipases function by cleaving off FAs or the base (attached to the phosphate group) from the PL; there are four main types of phospholipases, A, B, C, and D. For the purposes of this study we will only be investigating the expression of phospholipase A_2 (PLA_2). Currently, the most important phospholipase with respect to atherosclerosis is lipoprotein-associated PLA_2 (Lp-PLA_2), which is strongly and independently associated with atherosclerosis. Lp-PLA_2 has been identified as a potential area for developing therapeutic agents to

inhibit (Jenny, 2006). Other major phospholipases include cytosolic PLA_2 ($cPLA_2$), which may be particularly important for AA metabolism; Ca2+ independent PLA_2 ($iPLA_2$), which may be essential in phospholipid remodeling (Kudo & Murakami, 2002); and secretory PLA_2 ($sPLA_2$), which is a controversial player in atherosclerosis and inflammation (Menschikowski et al., 2006). Recently, Bostrom et al. (2007) were the first to show that $sPLA_2$ contributes to atherogenesis. Results reported by Kugiyama et al. (2000) also support the role of $sPLA_2$ in adverse cardiovascular outcomes; their results showed an increased independent risk of coronary events in patients with unstable angina with elevated levels of $sPLA_2$. AA requires PLA_2 for its release from membranes and subsequent production of pro-inflammatory and pro-aggretory eicosanoids via COX and LOX. Paradoxically, n-3 FA, particularly EPA, also require PLA_2 for its release from cell membranes. Therefore, PLA_2 presents as yet another enzyme for which n-6 and n-3 FA compete for. However, likely due to the greater presence of n-6 FA in cell membrane, PLA_2 is often associated with a pro-atherogenic state, particularly $cPLA_2$ (Kronke et al., 2002; Burgermeister et al., 2000). EPA and DHA have also been shown to modulate PLA_2 activity in peritoneal macrophages (Tappia et al., 1995).

2.5 Source and cardiovascular benefits of n-3 fatty acids

2.5.1 Fish oil

Fish high in DHA and EPA are mostly cold-water or ocean fish as opposed to fresh-water fish (Bistrian, 2004). This is due to the tendency of shorter, more

saturated FA to solidify in colder temperatures, whereas longer highly unsaturated FA such as DHA and EPA remain in liquid form (Ettinger, 2004). Beneficial effects of fish oil and DHA and EPA on CVD have been previously discussed.

Some limitations of fish oil supplements are poor patient acceptability possibly because of the fishy taste, belching, nausea and diarrhea in those consuming supplements regularly. Fish are also known to be high in methyl-mercury due to bioaccumulation; hence mercury poisoning is a reasonable danger with regular fish consumption, especially because of its high absorption (95-100%) (Mozaffarian & Rimm, 2006; Innis et al., 2006). Fish particularly high in mercury include shark, swordfish, king mackerel, and tilefish (Food and Drug Administration, 2004). Less mercury is found in fish oil alone compared to fish (Foran et al., 2003). Therefore, eating fish species known to contain high quantities of mercury should be cautioned particularly in young children and pregnant/breastfeeding women (Helland et al., 2001). Allergy to fish and seafood is also common. Furthermore, dioxin concentration and other sources of contamination can be a safety issue in fish oil supplements; an issue further reviewed by Bays (2007). Fish oil may also worsen hyperglycemia at higher doses (Breslow, 2006). Finally, the Acceptable Macronutrient Distribution Range according to the DRI's for n-3 FA is 0.6-1.2 g/day (National Academy of Sciences, 2005). There is however no defined upper limit, although the United States Food and Drug Administration do specify that 3g of n-3 FA from fish oil per day is Generally Recognized as Safe (GRAS). There is a lack of sufficient evidence for adverse effects of high n-3 intake, however some studies have

indicated that there may be an increased risk for lipid peroxidation. There may also be increased risk of bleeding/hemorrhage. Therefore caution should be taken with high n-3 FA intake, especially for those taking blood thinners or who suffer from bleeding disorders (Lichtenstein, 2005).

2.5.2 Flaxseed oil

Flaxseed is grown in the mid-western United States and Canada where the climate is cool (Morris, 2001). Just over 40% of flaxseed is composed of lipid, 28% is fibre, 20% protein, 7% moisture, and 4% ash. The FA composition of flaxseed oil is summarized in **Table 7**. The ratio of n-6:n-3 FA in flaxseed oil is 0.3/1, which is unique in comparison to other fat and oil sources (Flax Council of Canada). To increase the shelf-life of flaxseed oil though, it should be stored in the refrigerator and in a closed opaque container (Morris, 2001).

Table 7. Fatty acid composition of flaxseed oil

Type of Fatty Acid	Proportion (%)
n-3 polyunsaturated fatty acids	57
n-6 polyunsaturated fatty acids	16
Monounsaturated fatty acids	18
Saturated fatty acids	9

(Source: Flax Council of Canada publications)

Beneficial effects of ALA on cardiovascular risk factors have been previously discussed. Other components of flaxseed, besides ALA, have been shown to possess health benefits. For instance, flaxseed can be used to treat constipation because of its potent laxative effect with high lignan content (Morris,

2001). The cholesterol-lowering properties of flaxseed are also attributed to lignan. Lignan is also a form of phytoestrogen, of which beneficial effects have been observed with respect to breast and prostate cancer, osteoporosis, cognitive function, inflammation, reproduction, as well as CVD (Dixon, 2004).

2.5.3 n-3 polyunsaturated fatty acid-enriched foods

With emphasis being placed on consuming more dietary n-3 FA, health professionals have been encouraging greater fish intakes. Another strategy is to increase the n-3 FA content of more widely consumed foods to obtain their health benefits (Abayasekava & Wathes, 1999). One of the first enriched foods was eggs produced from chickens fed a fish oil or enriched n-3 diet (Surai & Sparks, 2001). A DHA-enriched egg has an n-6:n-3 ratio of 3.5:1 compared to 24.7:1 in a normal egg (Gerbi et al., 2004). Eggs that are enriched with n-3 FA have also been shown to be equally effective in their cardiovascular benefits as compared to supplements or foods with naturally occurring n-3 FA (Maki et al., 2003; Ferrier et al., 1995).

The incorporation of n-3 FA via direct EPA/DHA or ALA into animal feeds to improve meat FA composition is also being explored as an option to increase n-3 FA intake (Rymer & Givens, 2005). However, adverse effects in these animals, such as effects on reproduction and fertility, need to be investigated further (Abayasekara & Wathes, 1999). Production of hormones is influenced by prostaglandin synthesis, which in turn may affect and potentially inhibit ovulation and thus effecting fertility. The Fat-1 gene has also been identified as being key in the conversion of n-6 FA to n-3 FA (Lai et al., 2006).

Expression of this gene in pigs has resulted in high n-3 FA meat from this animal. This product has yet to enter the food market, but presents as a major breakthrough in improving the n-3 FA intake in the population. The sensory and safety issues must be evaluated.

Enrichment of dairy products by feeding dairy cattle high n-3 FA diet is also a possibility. Finally, n-3 FA can be added after production to dairy products with algae oil; this has been shown in yogurts (Chee et al., 2005). Unfortunately, organoleptic properties may be affected; however microencapsulation of n-3 FA may lessen the detrimental effects. Daily intake of milk enriched with n-3 FA, folic acid, and vitamin E were shown to significantly reduce serum TG, total cholesterol, LDL cholesterol, Apo B, glucose and homocysteine in patients with metabolic syndrome (Benito et al., 2006; Carrero et al., 2004). Clearly, there is evidence for efficacy of these products and an existing market. Additional food products will continue to be developed to join the 'functional' eggs, milk, cheese, yogurt, and meats already manufactured. (Kolanowski et al., 2001).

3. Study rationale

Controversy exists over the appropriate ratio of n-6:n-3 FA in the diet. Beneficial effects of fish oil and DHA/EPA have been shown; health benefits of flaxseed oil and ALA have also been shown, but to a lesser extent. A major limitation of flaxseed oil and ALA may be the limited conversion to DHA and EPA. Our search on PubMed revealed that there is limited information on the cardiovascular effects of diets containing low n-6:n-3 FA ratio and otherwise

comparable amounts of other FA including saturated, MUFA and PUFA when used in "free living" conditions. More importantly, the impact of the source of n-3 FA (fish or plant) in such diets has not been investigated. Therefore, to the best of our knowledge, this study would be an original study to address these important questions in a well-defined animal model of human disease. We chose a wild-type mouse model because it best reflects the human conditions we wished to explore; a lack of any type of familial hypercholesterolemia and a major effect of diets on plasma lipid levels. C57BL6 wild-type mice were chosen because they have shown greater response to high cholesterol/high fat diets compared to other types of wild-type mice (Paigen et al., 1985).

4. Study hypotheses and objectives

4.1 Hypotheses

The source (fish oil or flaxseed oil) of dietary n-3 FA will not be a major determinant of benefits of diets low in n-6:n-3 FA (2:1) on cardiovascular risk reduction in wild-type mice, when other classes of FA are adequately provided through diet.

4.2 Objectives

The purpose of the study is to investigate cardiovascular benefits of diets supplemented with 'designer oils' containing identical low ratios of n-6:n-3 FA from different sources, either fish oil (containing high amounts of DHA and EPA) or flaxseed oil (containing high amounts of ALA), in C57BL/6 mice. The specific objectives include:

1. To assess the effects of these oils on plasma total cholesterol, TG, HDL, and non-HDL cholesterol;

2. To determine the effects of these oils on plasma inflammatory markers and gene expression of cytokines in the spleen;

3. To determine the effects of these oils on liver and heart FA composition.

5. Materials & Methods

5.1 Experimental Animals

Twenty-one, six week old C57BL/6 wild type mice were obtained from the Central Animal Facility (Winnipeg, MB, Canada). They were given three weeks to acclimate to our facilities, while being given free access to standard mouse chow and water. The mice were maintained in a temperature-controlled room, with a 12-hour light: 12 hour dark cycle. At 9 weeks the experimental diets were introduced to the experimental animals.

5.2 Ethics

Animal care for the study, as well as all experimental protocol has received approval from the Animal Care Committee on the use of animals in Research at the University of Manitoba, Winnipeg, Manitoba, Canada.

5.3 Experimental design

The mice were divided into three groups (n=7) receiving three different diets during the experimental course of 16 weeks; the group names are control, flax, and fish. An n=7 was determined by using the estimated standard deviation of outcome measures and the desired power to calculate the necessary number of mice per group to detect significant differences. Due to the social nature of mice it was recommended by our animal holding facility that they be housed together. Unfortunately, some mice had to be housed separately because of fighting within

the cages. Four weeks has been shown to be sufficient time for the maximal incorporation of n-3 FA into cardiac cell membranes in rats (Owen et al., 2004). However, 16 weeks of feeding allowed for sufficient time to collect blood lipids and cytokines, as well as to assess the long-term effects of these diets on our selected parameters. Following 16 weeks of feeding, animals were sacrificed using CO_2 (Othman et al., 2008) and the tissues and blood were collected.

There were several mice that had to be euthanized throughout the study due to weight loss, dehydration, and jaundice. At 12 weeks of age, one mouse from the control group and one mouse from the fish group were euthanized; at 22 weeks one mouse from the fish group and at 24 weeks old one mouse from the control group were euthanized. At sacrifice, many of these mice presented with an enlarged gallbladder, with suspected cholesterol gallstones due to their pale colour, and the liver appeared pale. This may be due to excess cholic acid in the diet.

A second trial was conducted to collect additional tissues, due to a lack of adequate samples for all biochemical assays available from the first trial. Trial two consisted of 18 C57BL/6 mice, 12 four-week old mice and 6 five and a half-week old mice (n=6 per group), also purchased from Central Animal Facility (Winnipeg, MB, Canada). Mice were given one week to acclimate to the facilities, while being given free access to water and standard mouse chow. The second trial had an experimental course of 6 weeks, with groups receiving identical experimental diets to trial one. Hearts from trial two were used for lipid extraction; see sections 5.5.4 and 5.5.5. Owen et al. (2004) reported that 28 days

is the maximum time required for n-3 FA to reach the highest concentration in cardiac cell membranes in rats; therefore we consider 6 weeks to be a sufficient experimental course. Spleens were used for analysis of gene expression; see section 5.5.8. Spleens were selected for cytokine analysis because the spleen is the site of the second highest white blood cell population in the body and a major area of immune regulation. Bone marrow is the site of the highest white blood cell population; however we were unsuccessful in collecting sufficient bone marrow for analysis. One mouse in the flax group had to be sacrificed prematurely (week 4) due to similar symptoms as described above.

5.4 Experimental diets

The diet mixes were prepared as outlined in **Table 8,** with the 'designer oil' mixture added to mouse chow; water was then added to produce a pasty consistency and pellets were formed. They were dried in an oven at approximately 45°C overnight. They were then stored in closed, opaque containers at -20°C until used. 'Designer oils' were formulated for selected n-6:n-3 FA ratio and added to regular PicoLab mouse chow and 2% cholesterol to produce atherogenic chow pellets. Differing amounts of fish and flaxseed oil were used in each of the experimental groups because the focus of the study was on the ratio of n-6:n:-3 fatty acids not the total amounts of each oil used. Fish oil was a generous gift from Pronova Biocare, Sandefjord, Norway and flaxseed oil was purchased from DYETS Inc; Bethlehem, PA. The diet was prepared fresh bi-weekly and stored in a cold room.

Table 8. Experimental diet composition

Oil/fat	Control	Flax	Fish
Safflower oil	75 g	25 g	35 g
Fish oil	0	0	36 g
Flax oil	0	48 g	0
Beef tallow	25 g	27 g	29 g
Cholesterol	20 g	20 g	20 g
Cholic Acid	2 g	2 g	2 g
TBHQ[a]	9 mg	9 mg	9 mg
Chow	878 g	878 g	878 g
Total	1000 g	1000 g	1000 g

[a] TBHQ, tertiary-butylhydroquinone

Harris (2006) summarized a number of studies investigating n-3 FA and found that one of the most common oils used in the control diet was safflower oil; this is likely because safflower oil has a high proportion of linoleic acid (18:2 n-6) and a low proportion of n-3 FA. Tert-butylhydroquinone (TBHQ) was added to reduce autooxidation (Fritsche et al., 1999). Cholesterol was added at 2% w/w to formulate an atherogenic diet. Cholic acid (0.2% w/w) was added to enhance cholesterol absorption. Mice are resistant to increased plasma lipids and consequently atherosclerosis; therefore they required an atherogenic diet. This aspect of the animal model may be considered a limitation because it does not reflect human metabolism. The nutrient compositions of the diets are in **Table 9**. The major lipid composition and individual FA profile of the diet is listed in **Table 10**.

Table 9. Nutrient composition of the experimental diets (per 100g)

Ingredients	Control	Flax	Fish
Protein (g)	18	18	18
Carbohydrate (g)	46.5	46.5	46.5
Fat (g)	17.9	17.9	17.9
Approximate ratio n-6:n-3	25:1	2:1	2:1
Cholesterol (g)	2	2	2
Cholic acid (g)	0.2	0.2	0.2
Ash and vitamin (g)	4.2	4.2	4.2
Fibre (g)	2.4	2.4	2.4
Moisture (g)	8.8	8.8	8.8
Total (g)	100	100	100

Fat ('designer oils') were added to chow diet

5.5 Data collection

5.5.1 Food intake and body weight

Food intake was measured three times weekly (Monday, Wednesday, Friday); attempts were made to take the measurements at identical times each day. Food given and food discarded was weighed to obtain a food weight for the given cage. Total intake was determined for each group on a per week basis. Body weight was measured at baseline and each week thereafter.

Table 10. Fatty acid composition (%) of experimental diets as a percent of total lipid

Fatty acid	Control diet	Flaxseed diet	Fish diet
14:0	0.55	0.55	0.67
14:1	0.02	0.02	0.03
15:0	0.06	0.06	0.07
16:0	12.43	12.47	12.73
16:1 (7 + 5)	1.37	1.43	1.90
17:0	0.23	0.23	0.20
18:0	5.64	6.44	6.69
18:1 (9 + 7)	20.74	23.68	21.74
18:2 (6)	53.72	35.13	35.72
18:3 (6)	0.04	0.05	0.19
18:3 (3)	1.49	15.88	1.92
20:0	0.42	0.36	0.46
20:1	0.36	0.49	0.90
20:2	0.11	0.12	0.20
20:3 (6)	0.03	0.05	0.12
20:4 (6)	0.08	0.10	0.49
20:3 (3)	0.01	0.02	0.06
20:5 (3)	0.27	0.28	7.04
22:0	0.19	0.19	0.23
22:1	0.08	0.34	0.45
22:4 (6)	0.01	0.01	0.03
22:5 (6)	0.01	0.02	0.08
22:5 (3)	0.07	0.06	0.82
22:6 (3)	0.25	0.26	4.57
24:0	0.12	0.13	0.10
24:1	0.09	0.06	0.18
Type of fat (% w/w)			
Σ saturated fat	19.65	20.42	21.14
Σ MUFA	22.68	26.02	25.21
Σ PUFA	56.09	51.97	51.23
n-6:n-3 ratio	25.91	2.15	2.55

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids
Fat ('designer oils') were added to chow diet

5.5.2 Blood collection

Blood samples were taken at baseline and every subsequent four-week period. A maximum of 0.6% of mouse body weight was collected at each blood collection, approximately 170 ul. At baseline as well as week 4, 8 and 12, blood samples were taken with heparin in the syringe from the jugular vein of lightly anesthetized animals. Heparin was used for preventing blood coagulation. At week 16 (end-point), blood samples were taken at sacrifice via cardiac puncture, and also with heparin. The blood samples were centrifuged at 5000 RPM for 10 minutes (Centrifuge 5804 R; Eppendorf) at room temperature to obtain plasma samples; total cholesterol, TG, and HDL-C were measured from the plasma samples at baseline, week 4, week 8, and week 16. Plasma samples from week 12 were used for cytokine analysis.

5.5.3 Plasma lipid assays

Enzymatic reagents and standards for total cholesterol and TG kits purchased from Diagnostic Chemicals Limited were used on plasma samples and absorbance at 500 nm was quantified using a microplate reader (autoreader EL311; Bio-tek instruments) according to kit instructions. For TG assay, 300 µl of TG reagent was added to 5 µl of each sample and standards (provided by the kit); samples were then incubated for 10 minutes at ambient temperature before measurement. The cholesterol assay was identical, except 250 µl cholesterol reagent was added to each 3 µl plasma sample and standards. For HDL-C assay, an HDL precipitant (50 µl) was added to plasma samples (20 µl) and centrifuged

for 20 minutes at 4°C, 5000 RPM (Centrifuge 5804 R; Eppendorf); the 30 µl supernatant was removed and 250 µl cholesterol reagent added. Absorbance was read by microplate reader at 500 nm. All samples were measured in duplicate and expressed in mmol/L. Plasma lipids were measured from all samples in all groups at each time point within 3 days following collection. From total and HDL-C, non-HDL-C was calculated by subtracting HDL-C from total cholesterol. The Friedewald equation cannot be used to calculate LDL-C because the blood samples were not taken in the fasting state (Krummel, 2004). Ratios of non-HDL-C/HDL-C and TC/HDL-C were also calculated.

5.5.4 Tissue lipid extraction and fatty acid analysis

Lipid extraction of liver and heart tissues was carried out according to Folch et al (1957). Total tissue weight, lipid weight, and percentage of lipid of total tissue weight were calculated. The tissue (approx. 0.7 g but varied with each sample) was homogenized in 4 ml 0.05% $CaCl_2$. To each sample, 20 ml chloroform:methanol (2:1) was added and vortexed to extract the lipid. The solvent (lower) phase was removed and dried down to obtain total lipid weight, which was then resuspended in 1 ml chloroform/methanol (2:1) for liver samples and 200 µl for heart samples, according to lipid content. Total lipid weight was divided by liver sample weight to calculate lipid weight as a percent total liver weight. Thin-layer chromatography (TLC) with G-silica gel was used to separate lipid classes: phospholipids, cholesteryl esters, free fatty acids, and TG. The developing solvent system was composed of: petroleum ether; diethyl ether;

acetic acid (80:20:1 by volume). TLC plates with H-silica gel were used to separate the individual phospholipids: SM, PS, PC, PS, lysoPC, PI, and PE. This solvent system is composed of: chloroform; 2-propanol; triethylamine; methanol; 0.25% KCl (30:25:18:9:6 by volume). Approximately 1-2 mg of lipid was spotted on each plate per sample. After allowing the plate to run to completion, spraying with aniline naphthalene sulphonic acid (ANSA), and viewing under UV light, the respective neutral and phospholipid bands were scraped for each sample.

Each individual lipid class was saponified (only CE, TG, SM) and then methylated with 14% BF_3 in methanol. The FA methyl esters were dried down under nitrogen gas and resuspended with hexane to prepare samples for FA analysis with gas chromatography. For FA analysis, an Agilent DB225 30 m x 0.25 mm internal diameter x 0.25 um filter thickness column was used, which was connected to a Shimadzu 17A gas chromatograph. The temperature schedule was: initial temperature, 70°C; increased to 180°C at 15°C/minute, held for 1 minute; increased to 210°C at 3°C/minute, held for 10 minute; and increased to 240°C at 20°C/minute, held for 5 minutes. FA analysis using gas chromatography was conducted according to Park et al. (2005). The percent of total FA was calculated by recording the amount of each FA and determining each percent from the total using Microsoft Excel.

5.5.5 Tissue protein, cholesterol, and triglyceride analysis

The proportion of total protein, cholesterol, and TG of heart and liver tissues was analyzed with standard enzymatic assays and quantified spectrometrically or

with a plate reader. Approximately 0.7 g tissue samples were homogenized with 4 ml 0.05% $CaCl_2$ and 10 µl were extracted for protein analysis. The 10 µl samples were combined with 240 µl DDH_2O; from those samples, 50 µl was added to 1450 µl Bradford reagent. Protein was measured with a standard curve using bovine serum albumin (BSA; 1 mg/ml; with standard curve: 10 µg, 20 µg, 30 µg, 40 µg, 50 µg, 100 µg). Absorbance was measured with a spectrometer (595 nm) according to Bradford (1976). Both cholesterol and TG from the extracted lipids were analyzed with enzymatic kits from Diagnostic Chemicals Limited (No. 225-26 for cholesterol and No. 210-75 for TG) and analyzed using a microplate reader, according to kit instructions. All lipid samples, suspended in chloroform:methanol (2:1), were dried down and diluted with isopropanol so as to fall within the range of the standard curve; standards were provided in the kit. To the 10 µl of each diluted sample and standard, 100 µl of TG reagent and 140 µl of deionized water was added for TG analysis; and 200 µl of cholesterol reagent was added to the 10 µl diluted samples and standards for cholesterol analysis. The samples were incubated for 5 minutes at 37º C and then read within 15 minutes with a microplate reader at 515 nm. The standards for TG assay were 0 mmol/L, 0.25 mmol/L, 0.5 mmol/L, 1 mmol/L, and 2 mmol/L. The standards for cholesterol assay were 0 mmol/L, 0.625 mmol/L, 1.25 mmol/L, 2.5 mmol/L, and 5 mmol/L, respectively. All samples and standards for cholesterol and TG were measured in duplicate.

5.5.6 Plasma bile acids

Due to the formation of gallstones in all groups, plasma bile acids were measured to determine if the different oil compositions in the diets influenced plasma bile acid concentrations. Plasma samples from week 16 (end point) were used for bile acid enzymatic assay. The bile acid kit was purchased from Diagnostic Chemicals Limited catalogue No. 702-13 (Charlottetown, PEI, Canada). 5 µl of plasma samples were combined with 270 µl of reagent 1 and 90 µl of reagent 2 and incubated for 10 minutes at room temperature. The samples were then analyzed in a microplate reader (Dynex Revelation 4.22) at 415 nm. A standard curve was produced with standards from the kit ranging from 1µmol/L to 50 µmol/L. All samples were measured in duplicate and expressed in µmol/L.

The bile acid assay is based on an enzymatic reaction between Thio-NAD and the bile acids (3-α-hydroxysteroid) found in the sample in the presence of the enzyme, 3-α-hydroxysteroid dehydrogenase. The bile acids become oxidized (3-ketosteroid) and the hydrogen molecule is now part of Thio-NADH. The rate of absorbance by Thio-NAD at 415 nm is proportional to the concentration of bile acids in the sample (Diagnostic Chemicals Limited).

5.5.7 Phospholipase expression in myocytes

Three hearts per group were used for analysis according to protocol from Invitrogen life technologies as previously described by McHowat et al. (2001). The first step was the isolation of RNA. TRIzol preserves the condition of the RNA while other cellular components are washed away and dissolved in the organic solution (chloroform). DNA can be precipitated by isopropanol and used

for electrophoresis. The detailed procedure is as follows: the hearts (approximately 100 mg) were first homogenized with TRIzol (1 ml) and incubated at room temperature for 5 minutes, followed by the addition of chloroform (0.2 ml/1 ml TRIzol). Samples were then shaken and separated by centrifugation at 10,000 x g for 15 minutes at 4˚C. The aqueous (upper) phase was transferred to a fresh tube. Isopropanol (500 µl) was added to the samples, which were then incubated for 10 minutes and centrifuged at 10.000 x g for 10 minutes at 4˚C. The supernatant was removed and 1 ml of ethanol was added; the samples were then vortexed and centrifuged again. The pellet was dried and redissolved in RNase-free water for 10 minutes at 60˚C; total RNA was calculated per mg of heart muscle.

RNA electrophoresis was conducted; 1% algarose gel was prepared, added to tray (up to 0.5 cm) and allowed to cool. The samples were prepared by adding 3 µl loading buffer, 0.1 µl Ethidium Bromide, 1 µl of RNA sample and 8 µl RNase free deionized water (total 12 µl). The gel in the tank should be cooled and the comb removed. The samples were then loaded onto the lanes in the gel. After running 30-40 minutes, the gel was photographed under UV light. Results were quantified using Real time PCR; the sequences for the respective phospholipases are in **Table 12**. Twenty-five cycles (forward and reverse) at 94ºC for 30 seconds, 62ºC for 1 minute, and 68ºC for 2 minutes were used. Gene expression of $cPLA_2$, $iPLA_2$, and $sPLA_2$ will be expressed as a ratio of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) to the respective PLA_2.

Table 11. Sequences used for phospholipase expression in cardiac myocytes

Phospholipase	Sequence
$cPLA_2$	
Forward	ATCGGATGCTAATGGCCTTG
Reverse	ATTCGGGGTCATCGAAAATG
$iPLA_2$	
Forward	CGCAGTTTCAAGGTGTTGGA
Reverse	CTTGATGCCDGGATGGCTTT
$sPLA_2$	
Forward	TTCTACGGTTGCCATTGTGG
Reverse	CTTGTTCCGGGCAAAACATT

T, thymine; C, cytosine; G, guanine; A, adenine

5.5.8 Cytokine gene expression in spleen

Spleens were collected at sacrifice and immediately placed in dry ice, then stored at -80° C until used. Two samples per group for IL-2, IL-4, and TNF-α, and 3 samples per group for IL-10 were used for analysis due to financial limitations. The principle of the assay was to extract and isolate cDNA from the tissue and target expression of certain cytokines and measure their expression by the intensity of their reaction.

The tissue (approximately 100 mg) was homogenized with TRIzol reagent (1 ml); lipid, protein, and DNA were separated from RNA using chloroform (0.2 ml). Samples were then centrifuged at 12,000 g for 15 minutes at 4°C (Centrifuge 5804 R; Eppendorf). The aqueous (top) phase, which contains total RNA, was then transferred to a clean tube. The sample was then precipitated using isopropanol and centrifuged a second time using the same protocol as the previous step. The RNA pellet was re-dissolved in sterile diethyl pyrocarbonate (DEPC) water. The RNA concentration was measured using absorbance at

260nm. cDNA was synthesized using 5 ug of total RNA; this cDNA sample was quantified with Real Time PCR TF7500 (Applied Biosystem Inc.) and 7500 System Software (Applied Biosystem Inc.) using Power SYBRr Green PCR Master Mix P/N: 4367659 (Applied Biosystem Inc.). Samples were analyzed for gene expression of cytokines, IL-2, IL-4, IL-10, and TNF-α, according to kit protocol; all samples were measured in triplicate. Results from each group were averaged and expressed in optical density.

5.6 Statistical analysis

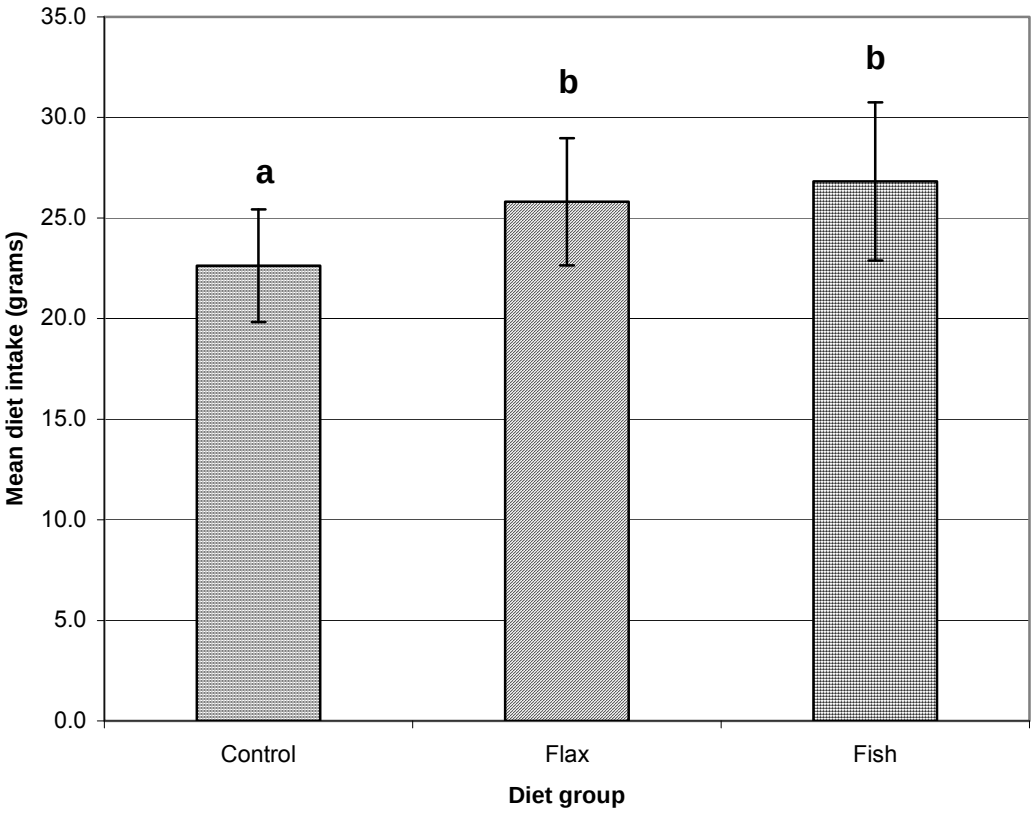
Results are presented as mean +/- standard deviation and analyzed with one-way ANOVA and Tukey test to determine differences among groups using SPSS for Windows version 11.5 (SPSS Inc, Chicago, IL). Results are considered significant at $p<0.05$. 'Repeated measures' analysis was used to detect an effect of time on blood lipid measurements.

6. Results

6.1 Food intake

Mean weekly food intake per group per mouse was significantly higher in both fish and flax groups compared to control, as seen in **Figure 6**. Mean food intake per mouse per group over the entire experimental course was 18% and 23% higher in the flax and fish group, respectively, compared to control. There was not a statistically significant difference between the two treatment groups.

Figure 6. Mean (± standard deviation) weekly food intake per mouse per group

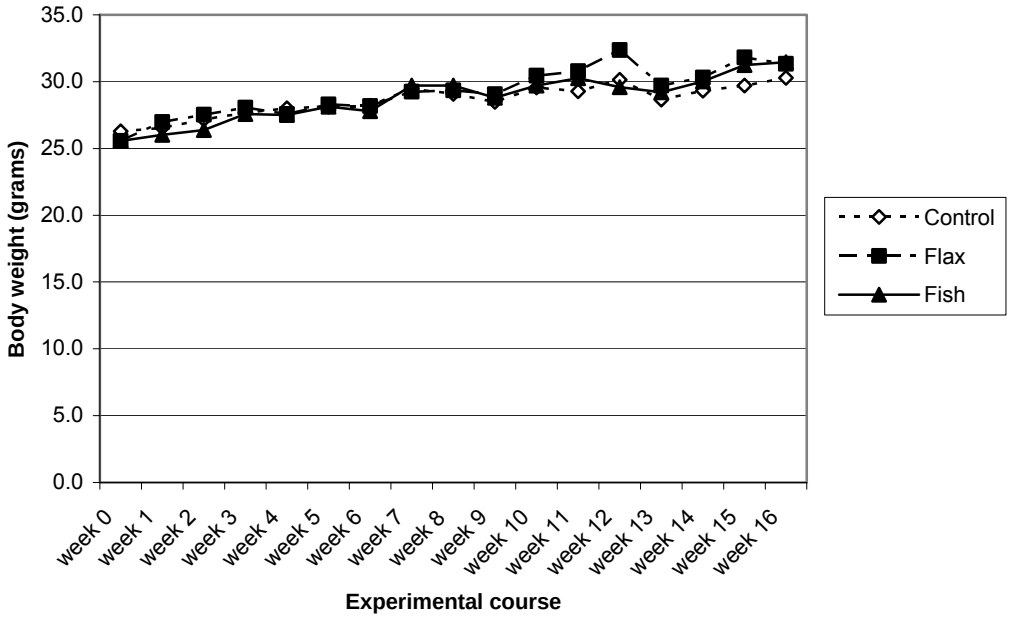


* $p<0.05$ compared to control group

6.2 Body weight

A steady increase in body weight was observed in all groups throughout the experimental course, which indicates tolerance of the diets. However, body weight did not significantly differ between the groups at any point throughout the study, as illustrated in **Figure 7**.

Figure 7. Mean body weight per group

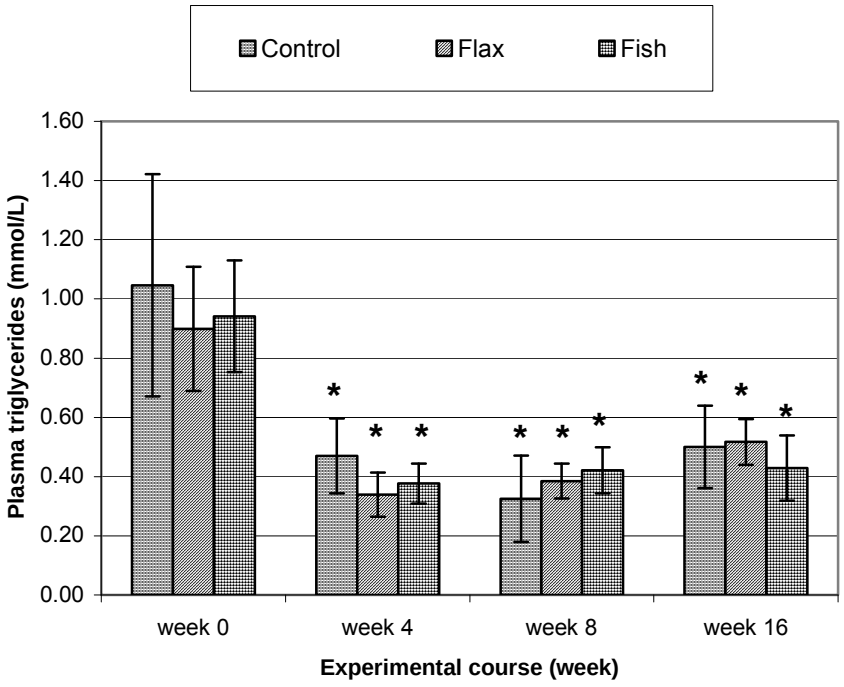


6.3 Plasma triglycerides

No significant differences in plasma TG concentrations were observed between groups at any of the four time points throughout the experimental course, as illustrated in **Figure 8**. At week 4, all groups had a significant reduction compared to baseline, with 55%, 62%, and 60% reductions in the control, flax, and fish group, respectively. TG concentrations in all groups remained

significantly lower than baseline within their respective groups throughout the study and values at weeks 4, 8, and 16 did not differ from each other within groups.

Figure 8. Mean plasma triglycerides by groups and experimental course



Data are presented as mean ± standard deviation (control, n=6; flax, n=6; fish, n=7)
Effect of time differences were analyzed by repeated measures
*Plasma TG decreased ($p<0.05$) from baseline in all groups and remained significantly lower compared to baseline

6.4 Plasma total cholesterol (TC)

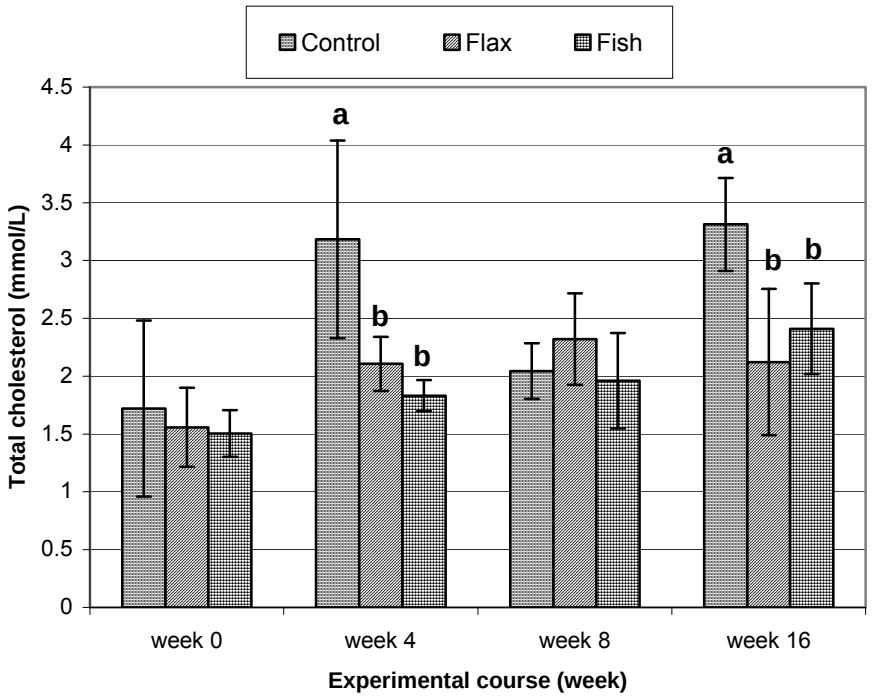
At baseline plasma TC concentrations were comparable among groups (**Figure 9**). At week 4, both treatment groups had significantly lower TC

compared to control; the flax group had a 34% and the fish group had a 42% lower plasma total cholesterol concentrations than the control group. TC concentrations at week 8 did not significantly differ among the groups. At week 16, plasma TC concentrations were similar to those observed at week 4. TC plasma concentrations were 36% and 27% lower in the flax group and fish group, respectively, compared to the control group at week 16. Statistical analysis with repeated measures detected a significant effect of time on plasma TC.

6.5 Other plasma lipids

Due to the limitations on maximum blood samples taken, HDL-C could only be measured in most samples at weeks 4 and 16. Data on HDL-C, non-HDL-C, and blood lipid ratios are presented in **Table 12**. At week 16, mean HDL-C in the fish group was significantly higher compared to the control group. At weeks 4 and 16, both flax and fish groups had significantly lower mean non-HDL-C concentrations compared to the control group. The fish group had a significantly lower non-HDL-C/HDL-C ratio at week 16 compared to week 4 and also compared to control at week 16. At week 16, the fish group had a significantly lower TC/HDL-C ratio compared to both the control and flax group.

Figure 9. Mean plasma total cholesterol by group and experimental course



Values with different superscript letter at same week are significantly ($p<0.05$) different

Data are presented as mean ± standard deviation (control, n=7; flax, n=7; fish, n=7)

A significant effect of time was noted by repeated measures analysis

Table 12. Blood lipid levels (mean +/- SD) at week 4 and week 16 of the study

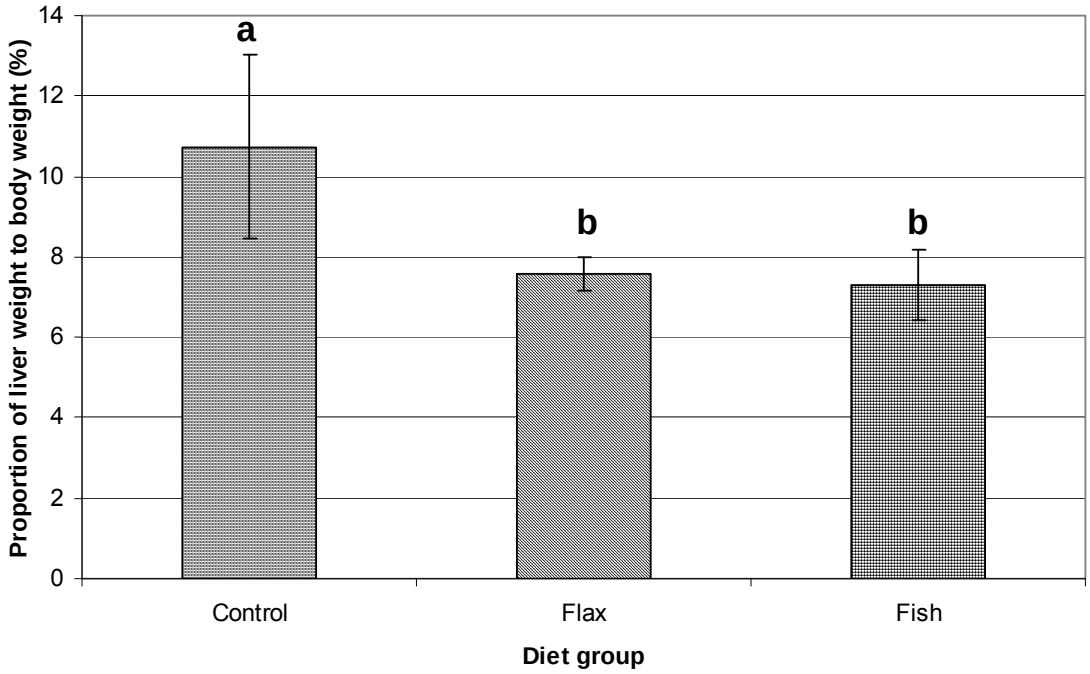
Measurement	Control (n=5-6)	Flax (n=7)	Fish (n=4-6)
HDL-C (mmol/L)			
Week 4	1.45±0.81	0.85±0.15	0.96±0.16
Week 16	1.33±0.18[a]	0.94±0.06[a]	1.69±0.56[b]
non-HDL-C (mmol/L)			
Week 4	1.73±0.24[a]	1.26±0.22[b]	1.06±0.47[b]
Week 16	1.98±0.38[a]	1.18±0.59[b]	0.98±0.24[b]
Non-HDL-C/HDL-C ratio			
Week 4	1.59±0.90	1.54±0.39	1.04±0.65
Week 16	1.51±0.37[a]	1.23±0.54[a]	0.69±0.19[b]
TC/HDL-C ratio			
Week 4	2.59±0.90	2.54±0.39	2.04±0.55
Week 16	2.51±0.37[a]	2.23±0.54[a]	1.52±0.40[b]

Values with different superscript letter in a row are significantly different
HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol

6.6 Liver weight

The liver weight in the control, flax and fish groups is 2.1g ± 0.25g, 2.00g ± 0.17g, 1.78g ± 0.19g, respectively. Liver weight was significantly lower in the fish group compared to control; liver weight in the flax group was non-significantly (p=0.366) lower compared to control. The reduced liver weight in the fish group may be reflective of reduced lipid weight. Liver to body weight ratio was significantly lower in both treatment groups compared to control, as shown in **Figure 10**.

Figure 10. Proportion liver weight of total body weight

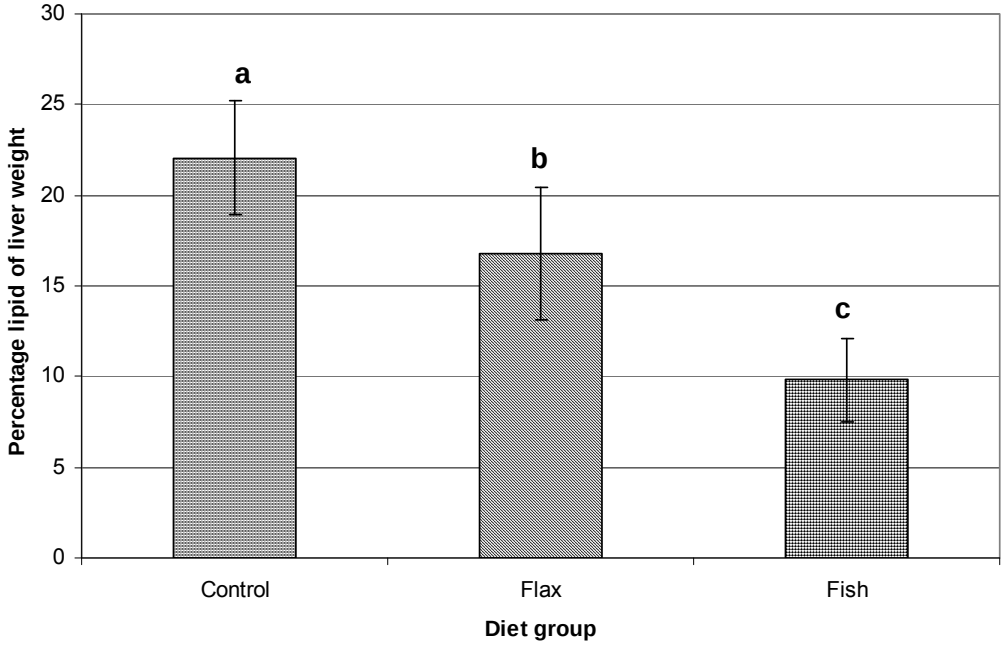


Data are presented as mean ± standard deviation (control, n=5; flax, n=5; fish, n=6)
Values with different superscript letter are significantly different ($p<0.05$)

6.7 Total liver lipid concentrations

Percentage lipid of total liver sample weight was significantly reduced in both the treatment groups compared to control at 24% and 56% for the flax group and fish group, respectively. Furthermore, the lipid content was significantly lower (42%) in the fish group compared to flaxseed, 9.8% versus 16.8% respectively, as presented in **Figure 11**.

Figure 11. Total liver lipid expressed as percentage of total liver weight



Total lipid with different letter above is significantly ($p<0.05$) different from each other
Data are presented as mean ± standard deviation (control, n=5; flax, n=7; fish, n=5)

6.8 Liver cholesterol, triglyceride and protein concentrations

Results for liver cholesterol and TG were comparable among groups. Observed cholesterol concentrations were elevated as a reflection of the high cholesterol content of the diet. When analyzing the cholesterol/TG ratio, it was found that this value was significantly reduced in the fish group (1.34±0.68) compared to control (2.48±0.56) and flax (2.32±0.62). Total protein did not differ between groups; control displayed the lowest percent protein of total liver weight with 7.99±0.95, flax with 9.00±2.00, and fish with 10.74±5.33.

6.9 Fatty acid composition of liver total phospholipid and neutral lipids

The FA composition of the liver PL is outlined in **Table 13.** The fatty acid composition in PL is most relevant of all lipids classes as it more closely reflects the heart PL composition. The levels of EPA and DHA were significantly higher in the fish group as compared to the flax and control groups in PL. In addition, the levels of EPA and DHA were significantly higher in the flax group compared to control. DPA is also significantly different in all groups, with concentrations being highest in the fish group, followed by flax, and the control group with the lowest amount of DPA. ALA was significantly higher in the flax group as compared to both control and flax groups. Furthermore, consumption of both 'designer oils' were comparably associated with a significant reduction in AA levels in total PL as compared to control. Other n-6 fatty acids, 20:2 (n-6) and 20:3 (n-6), were also significantly reduced in the fish group compared to control.

Table 13. Fatty acid composition of liver total phospholipids

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.04±0.08[a]	0.03±0.07[a]	0.16±0.05[b]
15:0	0.35±0.09	0.31±0.10	0.23±0.05
16:0	26.57±1.13	25.48±3.67	23.14±0.97
16:1 (5 + 7)	1.99±0.22[a]	2.00±0.25[a]	1.50±0.39[b]
17:0	0.50±0.10[a]	0.46±0.08[a]	0.61±0.12[b]
18:0	14.05±1.42	14.24±2.60	16.17±2.23
18:1 (7 + 9)	18.97±-1.37	19.25±1.58	17.01±1.86
18:2 (6)	19.92±1.08	18.49±3.07	17.53±0.69
18:3 (6)	ND[1]	0.03±0.07	0.03±0.05
18:3 (3)	ND[a]	0.67±0.33[b]	0.11±0.06[a]
20:0	0.45±0.09	0.67±0.85	0.59±0.14
20:1	0.67±0.07	0.60±0.09	0.71±0.10
20:2 (6)	0.71±0.09[a]	0.47±0.13[b]	0.45±0.19[b]
20:3 (6)	2.60±0.24[a]	2.41±0.61[a]	1.21±0.31[b]
20:4 (6)	6.96±1.43[a]	4.59±1.51[b]	3.88±0.26[b]
20:5 (3)	0.21±0.29[a]	1.35±0.59[b]	3.48±0.55[c]
22:0	0.46±0.04	0.43±0.07	0.42±0.05
22:5 (3)	ND[a]	0.67±0.21[b]	1.12±0.14[c]
22:6 (3)	3.39±0.51[a]	5.99±2.02[b]	9.98±1.00[c]
24:0	0.64±0.10	0.60±0.08	0.57±0.07
24:1	1.47±0.17[a]	1.27±0.20[ab]	1.09±0.20[b]

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation
[1]Not detected

Fatty acid composition in free fatty acid, TG, and cholesteryl ester are respectively found in **Table 14**, **Table 15**, and **Table 16**. These lipid fractions are relevant because they provide the fatty acids that eventually become incorporated into phospholipids. Levels of DHA were significantly higher in the fish group compared to control and flax in FFA, TG and CE; furthermore, DHA in the flax group was significantly higher than control in all lipid fractions. A similar pattern amongst the groups was seen with EPA in TG. However in FFA and CE, EPA was significantly higher in fish compared to both flax and control with no

significant differences between flax and control. Concentrations of ALA were significantly increased in the flax group compared to both control and fish groups in FFA, TG, and CE. Both linoleic acid (18:2 n-6) and AA (20:4 n-6) were comparably and significantly reduced in the flax and fish group compared to control in FFA, TG, and CE.

Table 14. Fatty acid composition of liver free fatty acids

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.10±0.23	0.20±0.19	0.27±0.17
15:0	ND[1]	0.03±0.08	0.08±0.18
16:0	14.95±1.20[a]	14.75±0.59[a]	17.47±2.32[b]
16:1 (5 + 7)	3.57±1.10	4.03±0.63	2.93±0.81
17:0	ND[a]	ND[a]	0.29±0.18[b]
18:0	2.98±1.82[a]	3.66±0.62[a]	5.40±0.69[b]
18:1 (7 + 9)	28.21±2.37[ab]	30.17±2.48[a]	26.22±1.44[b]
18:2 (6)	35.65±2.74[a]	29.47±2.00[b]	28.66±2.37[b]
18:3 (6)	0.12±0.27	0.15±0.19	0.09±0.13
18:3 (3)	ND[a]	4.33±1.09[b]	0.49±0.27[a]
20:0	ND[a]	ND[a]	0.22±0.23[b]
20:1	1.02±0.24	1.00±0.17	0.86±0.12
20:2 (6)	1.38±0.40[a]	0.93±0.54[ab]	0.31±0.18[b]
20:3 (6)	2.86±0.08[a]	1.83±0.27[b]	0.80±0.25[c]
20:4 (6)	5.65±0.48[a]	3.72±0.77[b]	2.86±0.37[b]
20:5 (3)	1.45±1.72[a]	1.64±0.50[a]	4.69±0.69[b]
22:5 (3)	ND[a]	0.35±0.45[ab]	1.17±1.11[b]
22:6 (3)	2.06±0.37[a]	3.73±0.71[b]	7.21±1.23[c]

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation
[1]Not detected

Table 15. Fatty acid composition of liver triglycerides

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.40±0.04	0.39±0.27	0.34±0.08
16:0	7.76±1.22	8.52±2.35	5.82±1.28
16:1 (5 + 7)	3.26±1.09	3.37±0.77	2.09±0.73
17:0	ND[1]	0.05±0.14	0.02±0.05
18:0	1.57±0.32	2.72±2.34	2.21±0.57
18:1 (7 + 9)	38.38±2.44[ab]	41.33±2.10[a]	35.14±2.03[b]
18:2 (6)	37.02±1.25[a]	26.53±2.23[b]	26.89±1.58[b]
18:3 (6)	0.59±0.10[a]	0.25±0.17[b]	0.15±0.09[b]
18:3 (3)	0.16±0.23[a]	5.00±0.92[b]	0.68±0.06[a]
20:0	0.37±0.28[a]	0.32±0.24[b]	0.73±0.29[a]
20:1	2.18±0.39	2.06±0.37	2.34±0.31
20:2 (6)	1.65±0.38	1.24±1.29	0.48±0.08
20:3 (6)	3.48±0.27[a]	1.52±0.39[b]	0.64±0.16[c]
20:4 (6)	1.18±0.16[a]	0.61±0.46[b]	0.49±0.13[b]
20:5 (3)	0.04±0.10[a]	0.51±0.24[b]	2.36+±0.20[c]
22:0	ND	ND	0.05±0.10
22:5 (3)	0.15±0.21[a]	1.10±0.78[a]	4.13±0/94[b]
22:6 (3)	1.81±0.11[a]	4.41±1.07[b]	14.96±2.53[c]
24:0	ND	0.06±0.15	0.10±0.14
24:1	ND[a]	ND[a]	0.36±0.16[b]

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1]Not detected

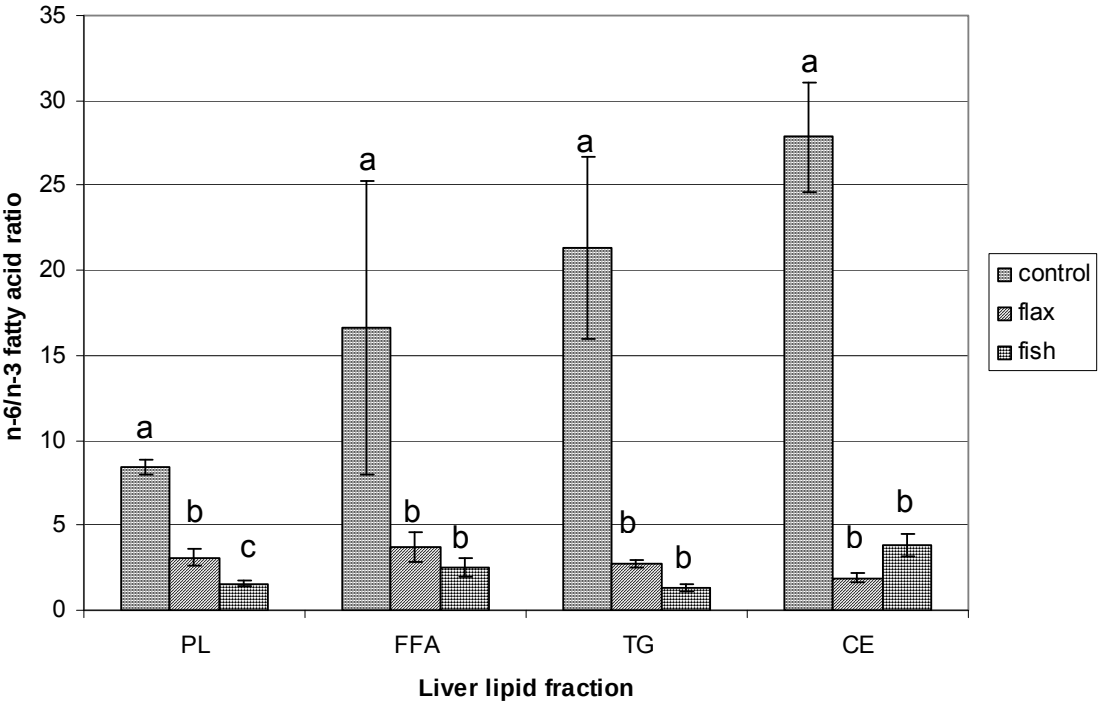
Table 16. Fatty acid composition of liver cholesteryl esters

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.22±0.02	0.25±0.03	0.26±0.15
14:1	0.04±0.6	0.05±0.07	0.03±0.06
15:0	0.10±0.01[ab]	0.10±0.05[a]	0.03±0.06[b]
16:0	5.25±0.37[a]	6.15±1.00[a]	11.34±5.67[b]
16:1 (5 + 7)	11.31±1.27[ab]	12.17±0.80[a]	9.21±2.47[b]
17:0	0.11±0.01	0.14±0.03	0.17±0.13
17:1	0.81±0.5[a]	0.87±0.11[ab]	0.98±0.08[b]
18:0	0.51±0.08[a]	0.67±0.13[a]	1.69±0.93[b]
18:1 (7 + 9)	50.94±0.64	53.50±1.88	51.54±5.22
18:2 (6)	28.66±1.43[a]	16.53±1.03[b]	19.08±2.11[c]
18:3 (3)	0.73±0.06[a]	8.43±1.67[b]	1.53±0.23[a]
20:0	0.21±0.01	0.22±0.01	0.16±0.15
20:3 (6)	0.43±0.06[a]	0.13±0.07[b]	0.12±0.14[b]
20:4 (6)	0.36±0.7[a]	0.10±0.07[b]	0.11±0.15[b]
20:5 (3)	ND[1a]	0.16±0.12[a]	0.99±0.29[b]
22:5 (3)	ND[a]	ND[a]	0.17±0.18[b]
22:6 (3)	0.33±0.07[a]	0.53±0.14[a]	2.60±0.99[b]

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation
[1]Not detected

Both treatment groups, flax and fish, displayed significantly lower n-6:n-3 FA ratios in PL, TG, FFA, and CE compared to control. The n-6:n-3 FA ratios in total liver PL are 8.40±0.44, 3.10±0.49, and 1.58±0.14 for the control, flax, and fish groups, respectively. Ratios did not differ between flax and fish groups in TG, FFA, and CE. **Figure 12** illustrates the n-6:n-3 ratios of the neutral lipids plus total PL, respectively.

Figure 12. Mean ratio of total n-6:n-3 fatty acids in liver neutral lipid fractions



Groups within a particular lipid fraction with different superscript letter are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation (control, n=5; flax, n=7; fish, n=5)
PL, phospholipid; FFA, free fatty acid; TG, triglyceride; CE, cholesteryl ester

6.10 Fatty acid composition of liver individual phospholipids

Individual phospholipid FA compositions are found in **Table 17** for PC, **Table 18** for PE, **Table 19** for lysoPC, **Table 20** for SM, **Table 21** for PS, and **Table 22** for PI. DHA was significantly higher in the fish group compared to control and flax in PC, PE, lysoPC, PS, and PI. Furthermore, DHA was significantly higher in flax compared to control in PC, PS, and PI. Similarly, EPA was significantly increased in the fish group compared to control and flax groups

in PC, PE, lysoPC, PI, and SM. In PC, PE, lysoPC, and PI, EPA was also significantly increased in the flax group compared to control. AA was similarly and significantly reduced in fish and flax compared to control in all fractions except PS (AA was significantly reduced only in fish compared to control). Linoleic acid was significantly reduced in fish compared to control in PC, PE, and lysoPC. There were no significant differences between control and flax with respect to linoleic acid in any of the fractions.

Table 17. Fatty acid composition of liver phosphatidylcholine

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.15±0.02	0.17±0.03	0.17±0.03
14:1	0.01±0.02	0.03±0.02	0.02±0.01
15:0	0.25±0.06	0.26±0.06	0.23±0.04
16:0	25.99±1.92	26.94±1.49	24.40±2.04
16:1 (7 + 5)	1.95±0.23^{ab}	2.16±0.21^{a}	1.61±0.38^{b}
17:0	0.31±0.05^{a}	0.31±0.04^{a}	0.44±0.09^{b}
17:1	0.19±0.04^{a}	0.20±0.03^{a}	0.26±0.02^{b}
18:0	6.18±1.10^{a}	6.44±0.83^{a}	8.04±0.84^{b}
18:1 (7 + 9)	15.40±1.17	17.19±1.44	15.79±1.45
18:2 (6)	27.11±1.61^{a}	25.40±1.91^{a}	21.83±1.52^{b}
18:3 (6)	0.33±0.03^{a}	0.28±0.02^{b}	0.20±0.02^{c}
18:3 (3)	0.04±0.05^{a}	1.12±0.27^{b}	0.18±0.01^{a}
20:0	0.23±0.05^{a}	0.18±0.05^{a}	0.36±0.09^{b}
20:1	0.44±0.03^{a}	0.44±0.06^{a}	0.61±0.11^{b}
20:2 (6)	0.92±0.14^{a}	0.56±0.07^{b}	0.47±0.09^{b}
20:3 (6)	3.82±0.22^{a}	2.88±0.25^{b}	1.34±0.37^{c}
20:4 (6)	9.01±1.54^{a}	4.36±0.74^{b}	3.94±0.38^{b}
20:3 (3)	0.08±0.05^{a}	0.22±0.02^{b}	0.07±0.03^{a}
20:5 (3)	0.18±0.04^{a}	1.72±0.28^{b}	5.04±0.98^{c}
22:0	0.02±0.02^{a}	0.02±0.02^{a}	0.07±0.3^{b}
22:1	0.01±0.01	0.02±0.02	0.03±0.01
22:2	0.06±0.01^{a}	0.02±0.02^{b}	0.02±0.01^{b}
22:4 (6)	0.15±0.03^{a}	0.06±0.03^{b}	0.05±0.03^{b}
22:5 (3)	0.21±0.02^{a}	0.63±0.09^{b}	1.26±0.17^{c}
22:6 (3)	5.44±0.64^{a}	7.56±1.30^{b}	12.53±0.70^{c}
24:0	0.03±0.03^{a}	0.03±0.02^{a}	0.08±0.03^{b}
24:1	0.06±0.02^{a}	0.05±0.01^{a}	0.10±0.02^{b}

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

Table 18. Fatty acid composition of liver phosphatidylethanolamine

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=4)
14:0	0.03±0.07[a]	0.21±0.11[b]	0.09±0.02[ab]
14:1	ND	0.02±0.04	0.02±0.04
15:0	0.04±0.09	0.14±0.06	0.12±0.02
16:0	15.00±1.45	16.94±2.18	14.68±1.35
16:1 (5 + 7)	0.85±0.09[a]	1.91±0.90[b]	0.63±0.21[a]
17:0	0.58±0.12[ab]	0.48±0.10[a]	0.77±0.10[b]
17:1	ND[a]	0.06±0.08[ab]	0.11±0.01[b]
18:0	18.84±1.95[a]	15.91±2.53[a]	23.09±2.36[b]
18:1 (7 + 9)	17.30±1.27[ab]	17.86±2.22[a]	14.03±1.99[b]
18:2 (6)	12.08±1.01[a]	14.07±1.73[a]	7.84±0.21[b]
18:3 (6)	ND	0.07±0.09	ND
18:3 (3)	ND[a]	1.38±0.57[b]	ND[a]
20:0	0.24±0.15[a]	0.25±0.07[a]	0.58±0.17[b]
20:1	0.58±0.05[a]	0.53±0.09[a]	0.76±0.10[b]
20:2 (6)	0.74±0.11[a]	0.42±0.12[b]	0.34±0.11[b]
20:3 (6)	2.37±0.09[a]	1.82±0.15[b]	0.76±0.28[c]
20:4 (6)	16.28±2.67[a]	8.25±1.83[b]	5.10±0.52[b]
20:3 (3)	ND[a]	0.19±0.04[b]	0.03±0.06[a]
20:5 (3)	0.25±0.06[a]	2.35±0.71[b]	5.33±0.50[c]
22:4 (6)	0.65±0.24[a]	0.25±0.17[b]	0.04±0.05[b]
22:5 (3)	0.53±0.09[a]	1.30±0.18[b]	1.82±0.36[c]
22:6 (3)	11.09±1.83[a]	13.16±2.52[a]	21.09±0.95[b]

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

Table 19. Fatty acid composition of liver lysophosphatidylcholine

Fatty acid (%, w/w)	Control (n=4)	Flax (n=5)	Fish (n=3)
14:0	0.12±0.08	0.18±0.04	0.22±0.09
15:0	0.24±0.16	0.26±0.04	0.24±0.04
16:0	31.62±2.99	32.67±2.36	29.85±3.55
16:1 (5 + 7)	1.91±0.16[ab]	2.27±0.18[a]	1.74±0.35[b]
17:0	0.54±0.05	0.49±0.05	0.63±0.14
17:1	0.11±0.12	0.11±0.11	0.25±0.05
18:0	10.85±2.42	12.04±1.33	13.54±1.39
18:1 (7 + 9)	16.76±2.59	17.92±1.57	16.76±2.06
18:2 (6)	18.92±2.18[a]	17.09±1.89[ab]	13.95±1.28[b]
18:3 (6)	0.33±0.01[a]	0.27±0.03[b]	0.22±0.01[c]
18:3 (3)	0.09±0.12	0.03±0.06	ND[1]
20:0	0.44±0.14	0.39±0.11	0.64±0.16
20:1	0.65±0.07[a]	0.69±0.10[a]	1.01±0.13[b]
20:2	1.20±0.18[a]	0.62±0.11[b]	0.65±0.11[b]
20:3 (6)	2.87±0.23[a]	2.17±0.27[b]	0.96±0.30[c]
20:4 (6)	5.58±1.29[a]	2.91±0.83[b]	2.17±0.33[b]
20:5 (3)	0.03±0.06[a]	1.20±0.11[b]	3.33±0.35[c]
22:0	0.20±0.07	0.16±0.06	0.14-±0.04
22:1	0.05±0.06	0.03±0.04	ND
22:2	0.03±0.06	0.02±0.04	0.02±0.03
22:4 (6)	0.39±0.42	ND	ND
22:5 (3)	0.25±0.19[a]	0.52±0.03[a]	1.36±0.20[b]
22:6 (3)	4.98±0.70[a]	6.66±1.22[a]	10.79±1.11[b]
24:0	0.22±0.03	0.19±0.07	0.16±0.05
24:1	0.18±0.13	0.19±0.18	0.16±0.05

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

Table 20. Fatty acid composition of liver sphingomyelin

Fatty acid (%, w/w)	Control (n=5)	Flax (n=7)	Fish (n=5)
14:0	0.32±0.05	0.34±0.06	0.29±0.07
15:0	0.22±0.14	0.19±0.14	0.14±0.13
16:0	20.31±3.61[a]	17.56±2.76[a]	14.50±1.92[b]
16:1 (5 + 7)	0.60±0.27[a]	0.23±0.23[b]	0.26±0.11[ab]
17:0	0.43±0.09	0.44±0.05	0.36±0.06
18:0	5.35±0.96	5.25±1.03	4.42±0.52
18:1 (7 + 9)	4.75±1.51[a]	2.85±0.98[b]	2.83±0.58[b]
18:2 (6)	7.28±4.02	3.35±2.71	3.53±1.02
18:3 (3)	0.19±0.17	0.16±0.16	0.04±0.10
20:0	1.07±0.19[a]	1.36±0.23[b]	1.27±0.08[ab]
20:4 (6)	0.99±0.23[a]	0.36±0.17[b]	0.35±0.08[b]
20:5 (3)	0.10±0.21[a]	ND[1a]	0.37±0.10[b]
22:0	6.24±1.20[a]	8.56±1.14[b]	9.97±1.61[b]
22:4 (6)	4.33±1.15[a]	6.01±1.54[a]	7.40±1.32[b]
22:6 (3)	1.38±0.24	1.41±0.48	1.96±0.56
24:0	10.59±3.07	13.81±3.08	14.74±2.79
24:1	17.67±3.72[a]	22.55±3.69[ab]	24.91±1.94[b]

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

Table 21. Fatty acid composition of liver phosphatidylserine

Fatty acid (%, w/w)	Control (n=4)	Flax (n=7)	Fish (n=4)
15:0	0.08±0.09	0.03±0.05	0.03±0.04
16:0	14.57±1.45[a]	12.55±1.79[ab]	10.81±1.12[b]
17:0	0.79±0.19	0.71±0.10	0.82±0.10
18:0	29.32±4.32	30.61±2.22	33.92±2.31
18:1 (7 + 9)	17.62±5.10	18.84±1.22	14.43±2.10
18:2 (6)	7.19±2.42[ab]	8.35±1.09[a]	5.61±0.36[b]
20:2	1.07±0.17[a]	0.53±0.05[b]	0.44±0.12[b]
20:3 (6)	2.36±0.76[a]	2.75±0.42[a]	1.09±0.38[c]
20:4 (6)	8.66±3.41[a]	6.14±1.22[ab]	3.53±0.19[b]
22:0	0.40±0.11	0.38±0.10	0.36±0.03
22:1	0.23±0.38	0.15±0.03	0.09±0.11
22:5 (3)	1.11±0.27[a]	1.64±0.16[b]	2.29±0.38[c]
22:6 (3)	8.96±3.20[a]	14.82±2.68[b]	24.13±1.44[c]
24:0	0.64±0.23	0.53±0.06	0.44±0.04
24:1	0.13±0.25	ND[1]	ND

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

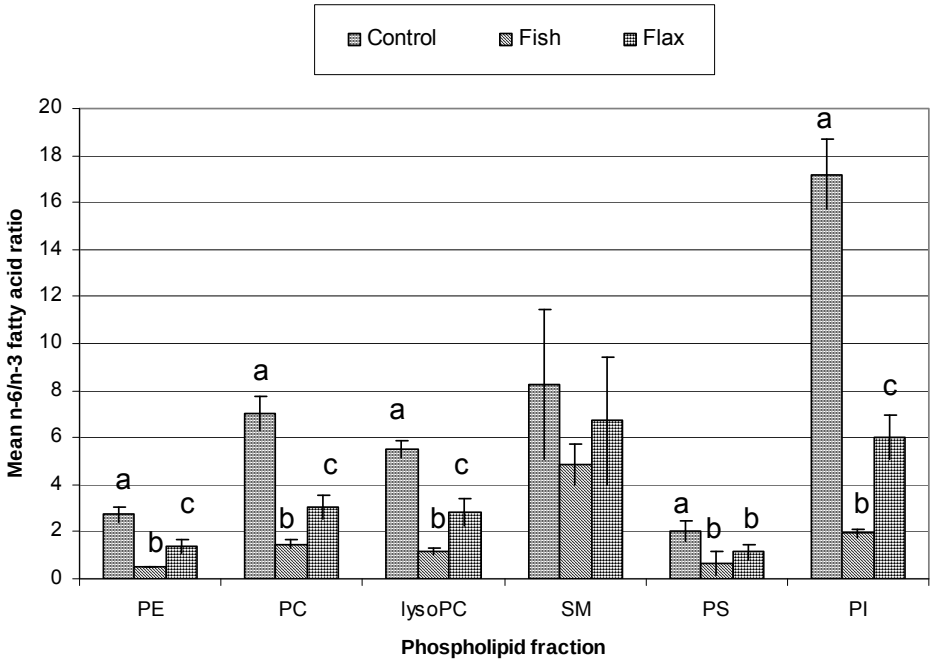
Table 22. Fatty acid composition of liver phosphatidylinositol

Fatty acid (%, w/w)	Control (n=5)	Flax (n=6)	Fish (n=4)
14:0	0.01±0.03	0.04±0.05	0.05±0.04
16:0	5.90±0.48	5.63±1.21	5.16±0.33
16:1 (5 + 7)	0.32±0.01	0.45±0.15	0.34±0.08
17:0	0.75±0.14	0.65±0.13	0.78±0.11
18:0	29.84±1.74	31.63±3.90	31.99±2.50
18:1 (7 + 9)	13.69±1.31	14.40±3.91	12.39±2.31
18:2 (6)	12.27±1.35	10.82±1.34	10.83±1.04
18:3 (6)	0.13±0.07	0.15±0.02	0.11±0.00
18:3 (3)	0.20±0.12	0.12±0.09	0.18±0.07
20:0	0.42±0.07^{ab}	0.36±0.09^{a}	0.53±0.07^{b}
20:1	0.27±0.02^{a}	0.26±0.02^{a}	0.37±0.04^{b}
20:2	0.41±0.09	0.44±0.13	0.29±0.03
20:3 (6)	9.47±0.91^{a}	9.58±1.03^{a}	4.81±0.99^{b}
20:4 (6)	21.90±1.89^{a}	16.19±1.26^{b}	14.37±1.27^{b}
20:3 (3)	ND[1a]	0.20±0.13^{b}	0.20±0.06^{b}
20:5 (3)	0.09±0.15^{a}	1.53±0.29^{b}	4.89±0.91^{c}
22:0	0.11±0.07	0.14±0.08	0.07±0.03
22:2	0.03±0.07	0.11±0.23	0.11±0.13
22:4 (6)	0.34±0.10	0.22±0.13	0.19±0.10
22:5 (3)	0.24±0.03^{a}	0.94±0.13^{b}	2.38±0.38^{c}
22:6 (3)	2.08±0.21^{a}	3.60±0.77^{b}	8.37±0.66^{c}
24:1	0.13±0.08	0.29±0.41	0.06±0.01

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation
[1] Not detected

The n-6:n-3 FA ratio of the individual phospholipid fractions are illustrated in **Figure 13**. The treatment groups had significantly lower n-6:n-3 FA ratios compared to control in all individual PL fractions tested except SM. The n-6:n-3 FA ratio in the SM fraction did not differ between groups. Furthermore, the flax group had significantly higher ratios compared to the fish group in PE, PC, lysoPC, and PI.

Figure 13. **Mean n-6:n-3 fatty acid ratio in individual liver phospholipid fractions**



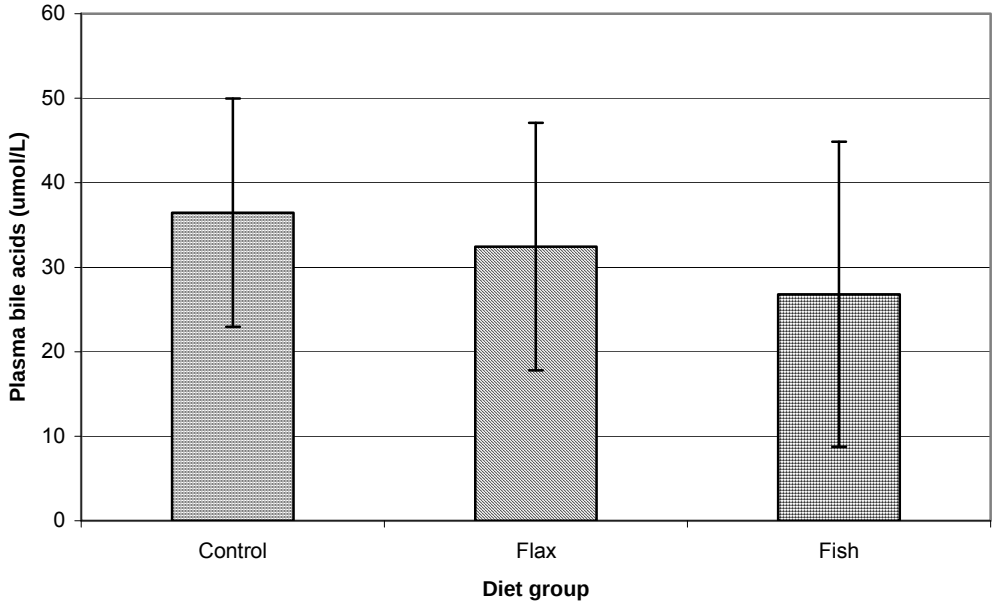
Groups with different superscript letters in a particular phospholipids fraction are significantly ($p<0.05$) different from each other
Data are presented as mean ± standard deviation (n=3-7/group)
PE, phosphatidylethanolamine; PC, phosphatidylcholine; lysoPC, lysophosphotidylcholine; SM, sphingomyelin; PS, phospatidylserine; PI, phosphtidylinositol

6.11 Plasma Bile Acids

As previously mentioned, there were several mice that had to be euthanized throughout the study due to weight loss, dehydration, and jaundice. At sacrifice, these mice presented with an engorged gallbladder, with cholesterol gallstones, and a pale liver – likely indicating steatosis. It was apparent that the stones had probably obstructed bile flow. This was likely caused by the cholic acid in the diet in addition to this mouse model being genetically susceptible to gallstones (Khanuja et al, 1995). Plasma bile acid levels were measured to

determine if there was a difference between the groups. Plasma bile acid concentrations were lowest in the fish group followed by the flax group and then control, as illustrated in **Figure 14**. Differences between groups were not statistically significant.

Figure 14. Plasma bile acids at week 16

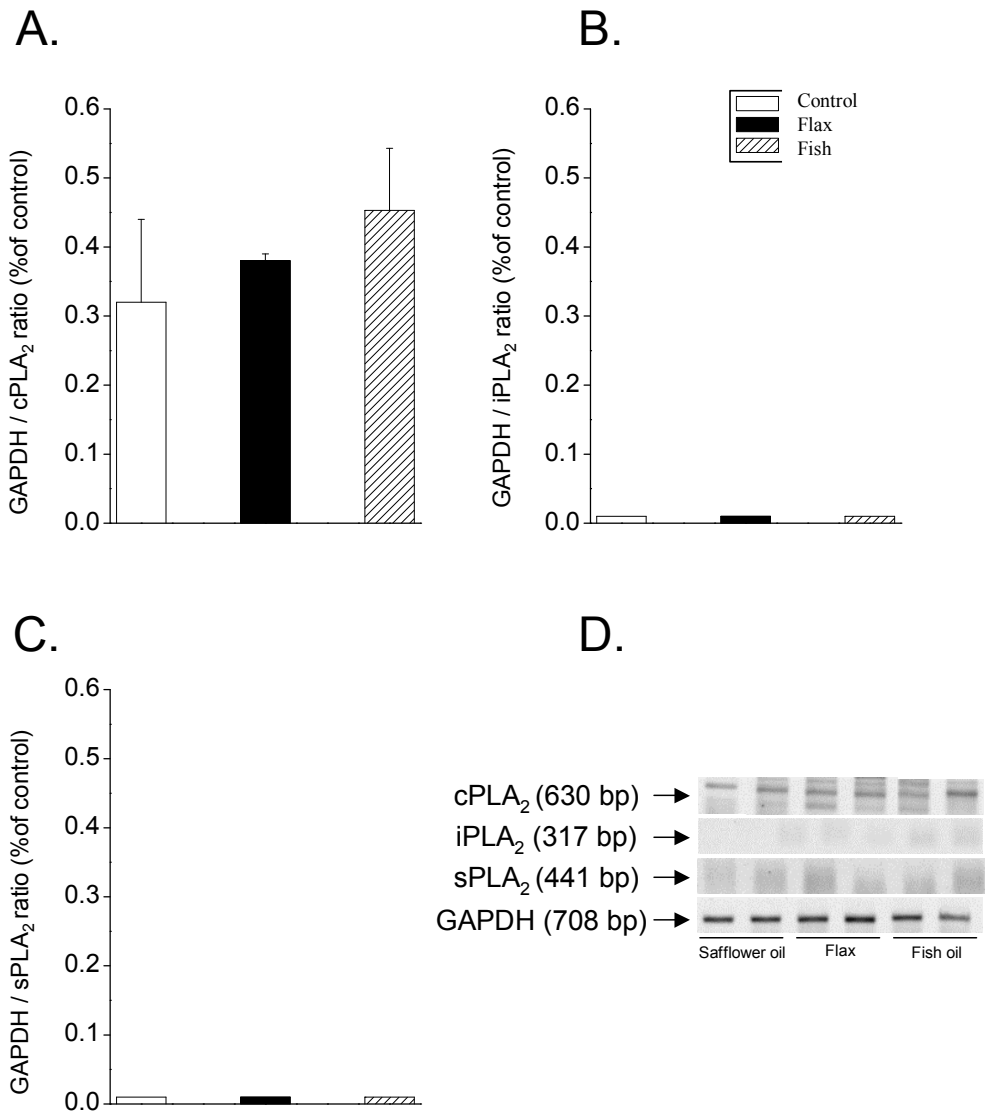


Data are presented as mean ± standard deviation (control, n=6; flax, n=6; fish, n=5)

6.12 Phospholipase gene expression in hearts

Gene expression of $cPLA_2$, $iPLA_2$, and $sPLA_2$ in hearts did not significantly differ among the groups. In fact, expression of $iPLA_2$ and $sPLA_2$ were undetectable, which may have been caused by contamination of the samples. All results are illustrated in **Figure 15**, panels A to D.

Figure 15. Gene expression of selected phospholipases in hearts

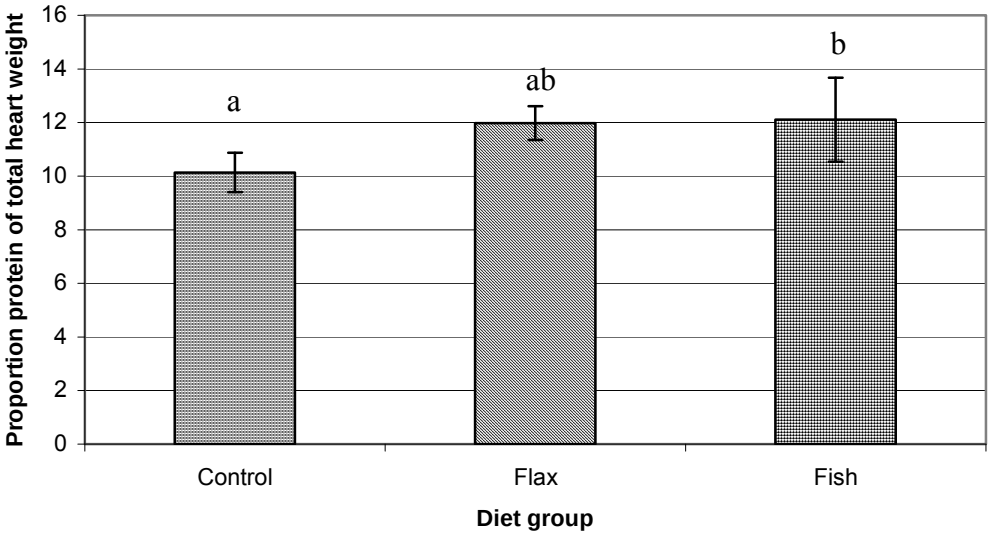


Panel A: $cPLA_2$; Panel B: $iPLA_2$; Panel C: $sPLA_2$; Panel D: Results of band intensity under UV light.
Data are presented as mean ± standard deviation (n= 3/group)
$cPLA_2$, cytoplasmic phospholipase A_2 ; $iPLA_2$, Ca-independent phospholipase A_2; $sPLA_2$, secratory phospholipase A_2

6.13 Total lipid, cholesterol, triglyceride, and protein composition of heart

Total heart lipid could not be accurately determined due to the miniscule amounts of lipid present in the heart. Total protein was higher in both treatment groups; however, only the fish group had a significantly higher percent of protein per gram heart weight compared to control, as shown in **Figure 16**; a similar pattern was observed in liver tissues as presented earlier.

Figure 16. Mean protein as a percentage of total heart weight

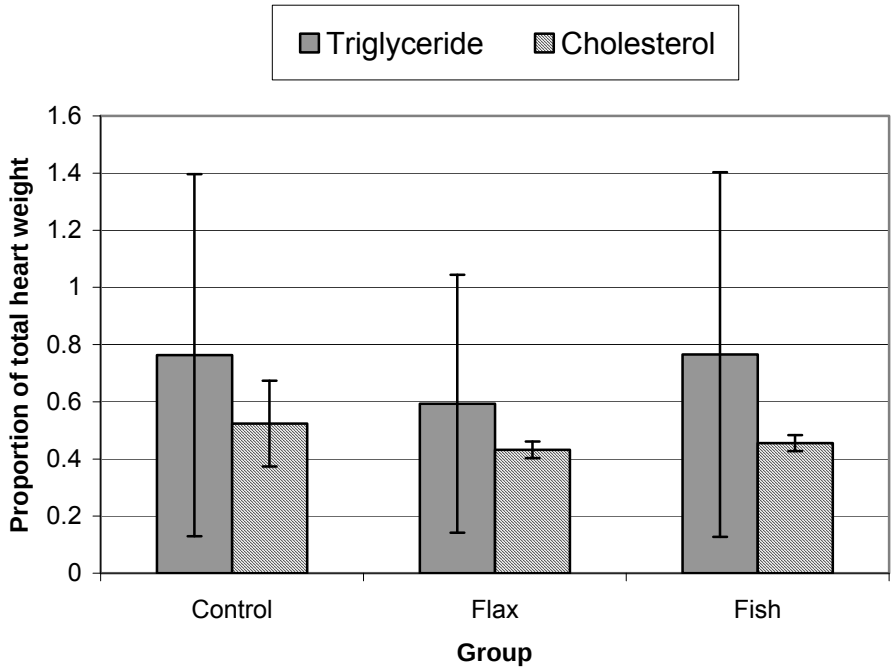


Data are presented as mean ± standard deviation (control, n=5; flax, n=4; fish, n=5)
*$p<0.05$ compared to control

There were also no significant differences among groups with respect to both heart total TG and cholesterol levels. Proportions of cholesterol and TG of total heart weight are displayed in **Figure 17**. Cholesterol/TG ratio in the heart tissues followed a similar trend as liver tissues did, with the fish group displaying

a lower ratio; however, there were no statistically significant differences between the groups.

Figure 17. Mean triglyceride and cholesterol content as a percentage of total heart weight



Data are presented as mean ± standard deviation (control, n=6; flax, n=5; fish, n=6)

6.14 Fatty acid composition of heart total phospholipids and heart neutral lipids

The effect of dietary treatments on heart FA composition was studied in total PL, FFA, TG and CE. Results of PL FA composition in heart tissues are summarized in **Table 23.** Levels of DHA were significantly higher in the fish group compared to control in PL; DHA in the flax group was not significantly different from either control or fish groups. EPA was significantly different in all

groups in PL, where concentrations were highest in fish, followed by flax and lastly control. The fish group had significantly lower levels of 20:3 n-6, linoleic acid (18:2 n-6) and AA compared to both flax and control groups.

Table 23. Fatty acid composition of heart phospholipid

Fatty acid (%, w/w)	Control (n=6)	Flax (n=5)	Fish (n=6)
14:0	0.10±0.03	0.08±0.02	0.09±0.03
14:1	0.04±0.02	0.04±0.02	0.04±0.02
15:0	0.11±0.04	0.09±0.02	0.10±0.03
16:0	15.53±3.49	14.01±1.43	16.37±3.41
16:1 (5 + 7)	0.28±0.04	0.24±0.03	0.29±0.08
17:0	0.35±0.10	0.032±0.03	0.41±0.09
18:0	27.02±5.76	25.59±3.26	28.08±5.89
18:1 (7 + 9)	6.55±1.23	6.97±0.69	6.60±1.64
18:2 (6)	15.51±4.77[a]	14.32±1.71[a]	9.12±1.83[b]
20:0	0.80±0.42	0.55±0.11	0.57±0.10
20:1	0.25±0.03	0.27±0.05	0.32±0.08
20:2 (6)	1.02±1.07	0.43±0.05	0.44±0.08
20:3 (6)	0.79±0.15[a]	0.85±0.04[a]	0.40±0.04[b]
20:4 (6)	5.06±1.51[a]	4.18±0.74[a]	2.44±0.17[b]
20:3 (3)	0.03±0.02[a]	0.11±0.03[b]	0.07±0.01[a]
20:5 (3)	0.04±0.03[a]	0.17±0.03[b]	0.61±0.11[c]
22:0	0.66±0.34	0.50±0.10	0.47±0.08
22:1	0.03±0.02[a]	0.06±0.01[b]	0.03±0.01[a]
22:4 (6)	0.27±0.07[a]	0.11±0.01[b]	0.05±0.01[b]
22:5 (3)	0.57±0.10	0.88±0.12	0.61±0.48
22:6 (3)	16.34±7.25[a]	22.84±6.56[ab]	29.44±7.46[b]
24:0	0.32±0.13	0.24±0.25	0.23±0.02
24:1	0.29±0.08	0.03±0.05	0.30±0.04

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation

FA composition in heart tissues are outlined in **Table 24** for TG, **Table 25** for FFA, and **Table 26** for CE. The levels of DHA and EPA in the TG, FFA, and CE were comparable between the flax and control groups. However, the hearts

from the fish group had significantly higher EPA and DHA concentrations in TG, and FFA as compared to those in the control group. FFA from the hearts in the control group had significantly higher levels of AA as compared to the fish group. Levels of AA were not different among the flax and control groups in FFA, TG and CE. Linoleic acid was significantly reduced in the fish group compared to control in TG, FFA, and CE, with no significant differences in linoleic acid between fish and flax in TG and CE.

Table 24. Fatty acid composition of heart triglycerides

Fatty acid (%, w/w)	Control (n=6)	Flax (n=5)	Fish (n=6)
14:0	1.12±-0.33	1.21±0.24	1.30±0.19
14:1	0.07±0.04	0.03±0.04	0.04±0.04
15:0	0.17±0.05	0.26±0.17	0.25±0.19
16:0	15.32±5.01	18.54±1.61	19.69±2.65
16:1 (7 + 5)	3.61±2.03	2.22±0.79	2.37±0.72
17:0	0.24±0.07	0.57±0.63	0.39±0.15
17:1	0.13±0.07	0.51±0.68	0.42±0.49
18:0	6.58±2.68	10.32±6.61	9.70±4.00
18:1 (9 + 7)	29.80±4.71	30.08±6.51	25.82±4.50
18:2 (6)	35.80±5.26[a]	26.92±4.58[b]	25.82±3.61[b]
18:3 (6)	0.09±0.04[ab]	0.02±0.03[a]	0.09±0.05[b]
18:3 (3)	0.06±0.06	0.05±0.06	0.09±0.09
20:0	0.84±0.75	1.36±1.31	0.71±0.70
20:1	1.18±0.55	1.24±0.33	1.29±0.27
20:2 (6)	0.53±0.18	0.42±0.27	0.36±0.12
20:3 (6)	0.54±0.27	0.66±0.53	0.34±0.24
20:4 (6)	0.54±0.27	0.34±0.18	0.53±0.44
20:3 (3)	ND[1]	0.03±0.04	0.02±0.02
20:5 (3)	0.01±0.02[a]	0.04±0.04[a]	0.64±0.25[b]
22:0	0.19±0.17	0.09±0.05	0.08±0.05
22:1	0.23±0.18	0.51±0.36	0.24±0.12
22:4 (6)	0.17±0.09	0.55±0.71	0.16±0.15
22:5 (3)	0.15±0.07[a]	0.21±0.20[a]	1.08±0.58[b]
22:6 (3)	0.62±0.25[a]	0.87±0.75[ab]	4.29±3.48[b]
24:0	0.10±0.08[a]	0.04±0.04[ab]	0.02±0.02[b]
24:1	0.11±0.08	0.03±0.03	0.10±0.06

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

Table 25. Fatty acid composition of heart free fatty acids

Fatty acid (%, w/w)	Control (n=6)	Flax (n=4)	Fish (n=5)
14:0	0.67±0.37	0.57±0.05	0.63±0.10
14:1	0.07±0.03	0.07±0.03	0.05±0.05
15:0	0.15±0.04	0.13±0.01	0.16±0.01
16:0	14.68±3.58[a]	15.78±0.61[ab]	18.87±1.20[b]
16:1 (7 + 5)	1.57±0.71	1.31±0.15	1.33±0.26
17:0	0.28±0.08	0.26±0.01	0.33±0.02
17:1	0.13±0.02[a]	0.13±0.01[a]	0.03±0.00[b]
18:0	12.00±3.59	10.02±0.59	10.95±0.94
18:1 (9 + 7)	17.51±2.58	19.22±0.63	15.47±2.58
18:2 (6)	37.62±3.57[a]	35.16±1.03[a]	25.49±1.85[b]
18:3 (6)	0.11±0.02[a]	0.11±0.04[a]	0.17±0.01[b]
18:3 (3)	0.04±0.04[ab]	0.06±0.00[a]	ND[1b]
20:0	0.36±0.18[a]	0.20±0.03[ab]	0.16±0.02[b]
20:1	0.69±0.22	0.82±0.14	0.68±0.10
20:2 (6)	0.85±0.50	0.49±0.06	0.45±0.06
20:3 (6)	0.98±0.13[a]	1.02±0.15[a]	0.59±0.06[b]
20:4 (6)	4.60±0.69[a]	4.29±0.39[ab]	3.50±0.54[b]
20:3 (3)	0.02±0.03[a]	0.12±0.00[b]	0.07±0.01[c]
20:5 (3)	0.10±0.04[a]	0.36±0.05[a]	2.19±0.39[b]
22:0	0.10±0.07	0.08±0.01	0.08±0.02
22:1	0.16±0.04[a]	0.39±0.08[b]	0.16±0.04[a]
22:4 (6)	0.40±0.07[a]	0.21±0.05[b]	0.19±0.06[b]
22:5 (3)	0.46±0.08[a]	1.16±0.25[a]	2.47±0.70[b]
22:6 (3)	4.13±0.33[a]	6.61±0.55[a]	13.75±3.05[b]
24:0	0.05±0.03	0.06±0.01	0.04±0.04
24:1	0.05±0.03	0.06±0.01	0.07±0.01

Values with different superscript letter in a row are significantly different ($p<0.05$)

Data are presented as mean ± standard deviation

[1] Not detected

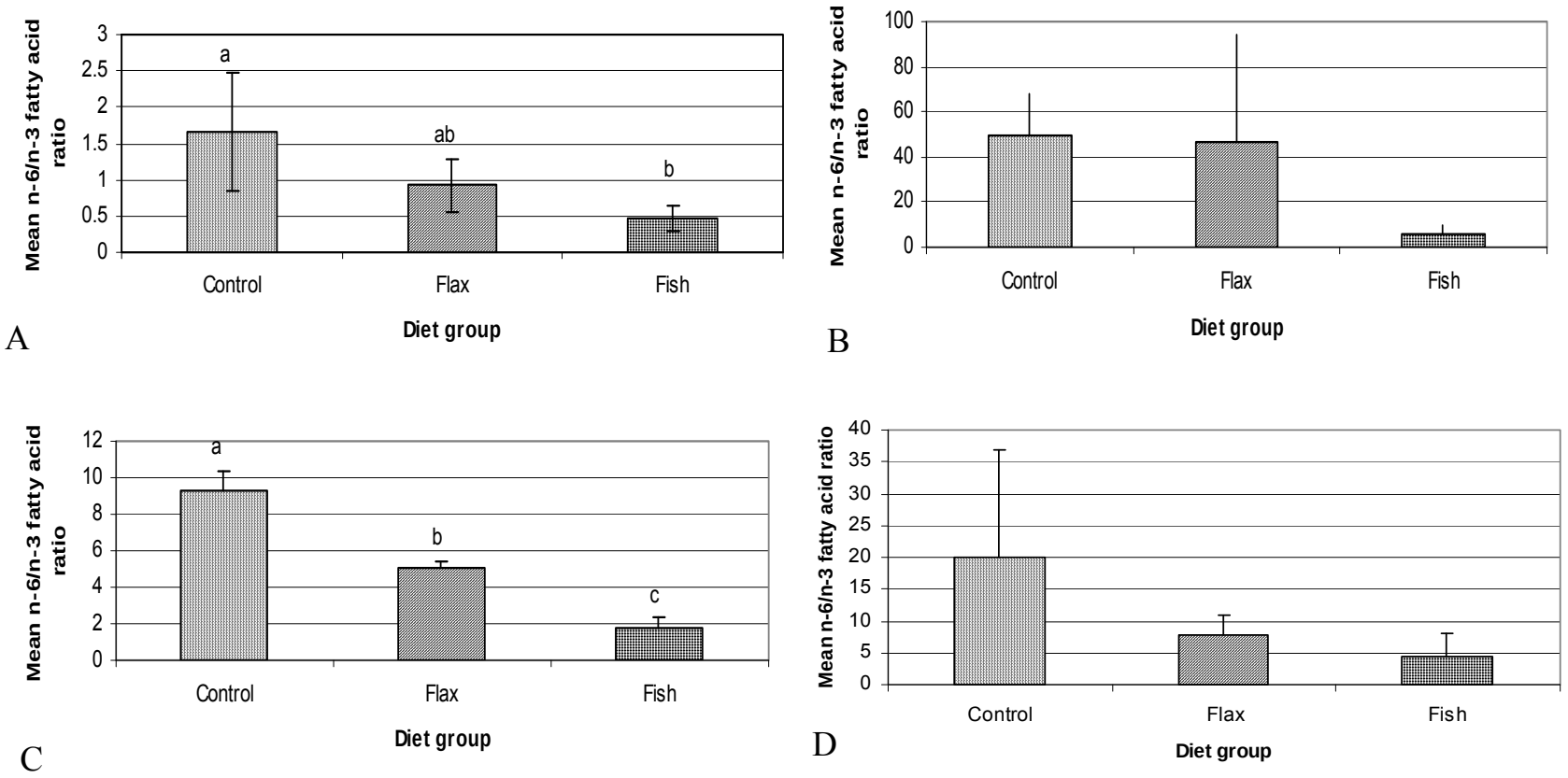
Table 26. Fatty acid composition of heart cholesteryl esters

Fatty acid (%, w/w)	Control (n=6)	Flax (n=5)	Fish (n=5)
14:0	0.91±0.92	0.59±0.21	0.70±-0.47
14:1	0.18±0.22	0.71±0.44	0.64±0.59
15:0	0.79±0.78	0.52±0.08	0.52±0.22
16:0	10.45±7.41	4.12±1.78	5.16±3.22
16:1 (7 + 5)	2.45±1.20[a]	0.98±0.33[b]	1.13±0.67[b]
17:0	1.25±1.65	0.31±0.08	0.47±0.42
17:1	0.46±0.43	0.09±0.10	0.29±0.34
18:0	5.13±5.08	2.14±0.89	3.05±3.02
18:1 (9 + 7)	14.49±8.28[a]	5.48±3.23[b]	4.19±2.24[b]
18:2 (6)	11.90±7.39[a]	5.45±3.00[ab]	3.76±1.38[b]
18:3 (3)	0.30±0.60	0.06±0.09	0.12±0.21
20:0	0.80±1.00	0.23±0.11	0.22±0.16
20:2 (6)	0.16±0.15	0.05±0.05	ND[1]
20:3 (6)	0.21±0.22	0.09±0.10	0.09±0.20
20:4 (6)	0.49±0.29	0.48±0.27	0.40±0.29
22:1	0.41±0.68	0.15±0.09	0.25±0.29
22:5 (3)	0.02±0.05	0.05±0.07	0.09±0.08
22:6 (3)	0.40±0.45	0.76±0.60	1.21±1.18
24:0	0.05±0.09	ND	0.05±0.07
24:1	3.66±3.13	1.79±2.08	3.74±1.13

Values with different superscript letter in a row are significantly different ($p<0.05$)
Data are presented as mean ± standard deviation
[1] Not detected

The fish group showed significantly lowered n-6:n-3 ratio in the PL and FFA fractions as compared to those in the control but not flax group. No significant differences in n-6:n-3 FA ratio were observed in TG and CE fractions among the groups. The heart n-6:n-3 FA ratio in the total PL and neutral lipids are illustrated in **Figure 18**.

Figure 18. Mean ratio of n-6:n-3 fatty acids in the heart phospholipid (A), triglyceride (B), free fatty acid (C), and cholesteryl ester (D)

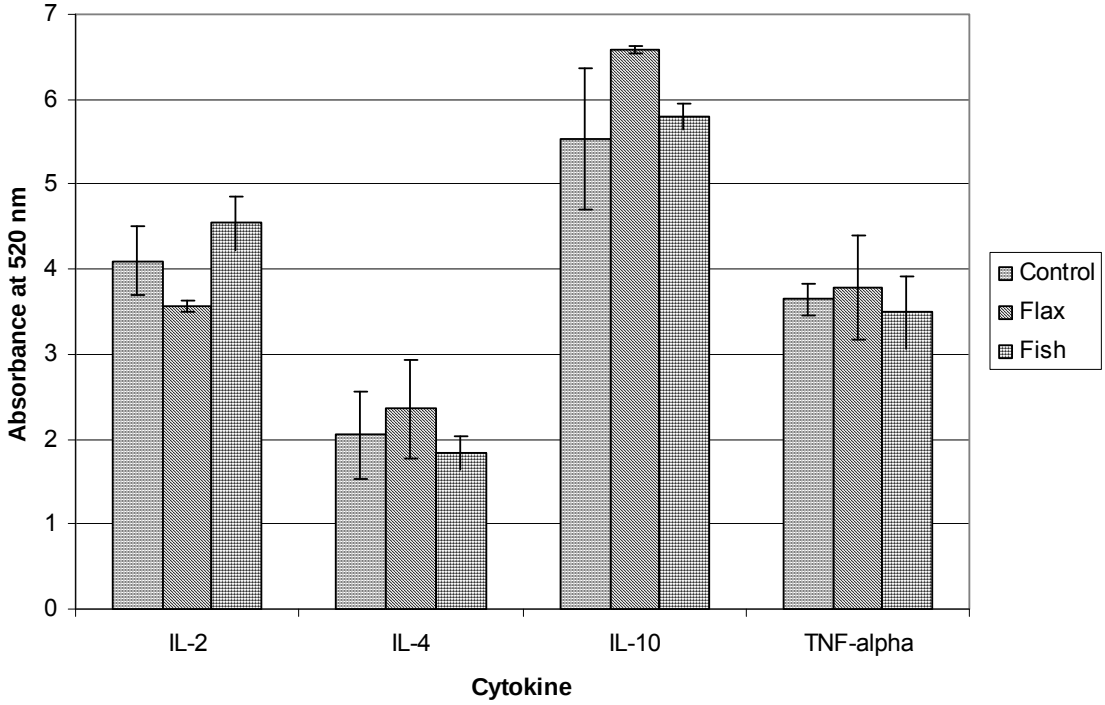


Data are presented as mean ± standard deviation (n=4-6/group)
Different letters indicate significant ($p<0.05$) differences between the groups

6.15 Cytokine gene expression in spleen

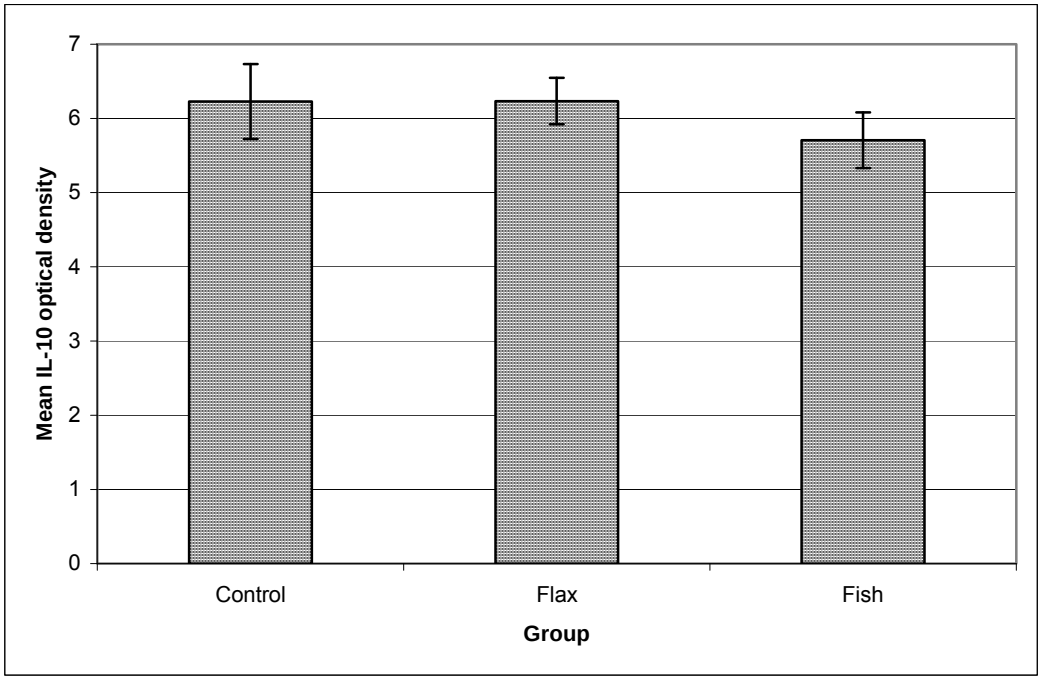
Means of two samples per group were conducted for preliminary data collection and did not reveal any obvious differences between groups with respect to IL-2, IL-4, IL-10, and TNF-α, as illustrated in **Figure 19**. IL-10 gene expression was selected for further analysis with n=3 per group and is illustrated in **Figure 20**. Statistical analysis did not reveal any significant differences among groups in IL-10 gene expression; this finding may be related to lower number of samples.

Figure 19. Cytokine gene expression in the spleens of experimental animals



Data are presented as mean ± standard deviation (n=2/group)

Figure 20. Mean IL-10 gene expression in the spleens of experimental animals



Data are presented as mean ± standard deviation (n=3/group)

7.0 Discussion

7.1 Summary of current knowledge and hypothesis

Several cardiovascular benefits are attributed to n-3 FAs, which may be influenced by the n-6 FA content of the diet. The literature has consistently shown that the very long-chain n-3 FAs are more effective in their cardiovascular benefit compared to their shorter chain counterparts (ALA). Current research has shown that a low n-6:n-3 ratio may exert several benefits. However, research comparing low n-6:n-3 ratio between two different sources, EPA/DHA and ALA, under well-controlled conditions has been scarce. We hypothesized that a low n-6:n-3 ratio, regardless of source, will reduce cardiovascular risk in C57BL/6 mice.

7.2 Effects of low dietary n-6:n-3 fatty acid ratio from two sources, EPA/DHA and ALA on cardiovascular risk factors

The effect of low ratios of n-6:n-3 FA, from different sources, on plasma lipids and tissue FA has been characterized. 'Designer oils' with a low n-6:n-3 ratio, from both plant and animal sources, displayed beneficial effects on cardiovascular risk factors and tissue composition in C57BL/6 mice. Overall, beneficial effects particularly with regard to DHA in tissues were improved in the fish group compared to both flax and control groups. However, overall improvements in blood lipids (TC, TG, and non-HDL cholesterol) were comparable among both flax and fish groups with the exception of HDL, which was significantly higher in the fish group.

7.2.1 Food intake and body weight

A significantly increased food intake as a result of flaxseed- and fish oil consumption has been observed in the present study. Barcelli et al. (1988) reported that rats fed either a high n-6 FA (from safflower oil) or high n-3 FA (from fish oil) diet gained more weight than rats fed a high saturated fat diet, with all diets containing identical amounts of total fat; however, food intake was not recorded in that study. Conversely, another study showed no difference in food intake or body weight between groups fed a high ALA, EPA, or DHA diet in Sprague-Dawley rats (Yamada et al., 1998). In the present study body weight did not significantly differ between groups. Furthermore, others have shown that n-3 FA may beneficially modify body composition (Garaulet et al., 2006; Ruzickova et al., 2004). Also, Cunnane et al. (1986) reported that high n-3 FA intake from cod liver oil reduced weight gain in genetically obese mice compared to those mice receiving evening primrose oil, high in n-6 FA. Yet, Valencak & Ruf (2006) failed to find an association between muscle phospholipid composition and basal metabolic rate. Nevertheless, it is possible that n-3 FA may have an effect on the hormone ghrelin, a hormone which stimulates appetite and subsequently increases food intake and body weight (Wren et al., 2001). The relationship between n-3 FA and appetite requires further investigation as animal model and species may play an important role. An alternate explanation to the increased food intake may also be the increased loss of chow pellets into the cage bedding material, which would have led to the 'missing food' being mistakenly recorded as intake. Another speculation is that these 'designer oils' may have some weight management properties which need further investigation.

7.2.2 Blood lipids

Plasma TG-lowering properties were observed in both experimental groups compared to baseline; beneficial effects of fish-oil on plasma TG have been consistently shown (Harris & Bulchandi, 2006). However, effects of ALA on plasma TG have been inconsistent (Goyens & Mensink, 2005). Interestingly, reductions in plasma TG were also observed in the control group. This may be explained by the introduction of the high fat diet (40% energy from fat) in the present study compared to the standard diet given during acclimation. The reduction in plasma TG may also be inversely correlated with the increase in total liver lipid levels.

In the present study, both experimental groups significantly reduced plasma total cholesterol and non-HDL cholesterol compared to control. At present, we cannot explain the lack of difference between groups in TC at week 8; perhaps it was a result of human error. Effects of both short and long-chain n-3 FA have not been consistently found to reduce plasma cholesterol concentrations and may increase LDL-C (Maki et al., 2003). Their potency to reduce cholesterol may be enhanced by the low ratio of n-6:n-3 and in addition, n-3 FA have shown to modulate both LDL and HDL particle size (Mori et al., 2000). Therefore, we speculate that an alteration in both LDL and HDL particle size may have occurred. Recently, a low dietary n-6:n-3 FA ratio (4:1 and 1:1) in LDL-deficient mice was also shown to reduce total cholesterol compared to a 20:1 n-6:n-3 FA ratio (Wang et al., 2007). The cholesterol/TG ratio in the liver was also reduced in

the fish group, which may be related to the reduced plasma cholesterol. A similar trend was also observed in the heart tissues. This observation on TC concentrations may also be exclusive to this animal model. In the present study, plasma HDL-C was also elevated in the fish group compared to control at week 16. This beneficial effect has been sporadically observed in other studies investigating the effect of fish oil on blood lipids (Calabresi et al., 2004). This increase in HDL may be a result of changes in the cholesterol distribution of HDL subfractions, which has been previously observed following fish oil supplementation in patients with familial combined hyperlipidemia (Calabresi et al., 2004).

7.2.3 Liver and heart fatty acid compositions

Overall, both experimental diets displayed alterations in the liver FA composition compared to controls. Both experimental diets were able to reduce liver steatosis compared to control. Fish oil has previously been shown to prevent fatty liver induced by perfluorooctanoic acid in mice (Kudo & Kawashima, 1997) and reverse the condition (Alwayn et al., 2005). The reduction in liver total lipid may indicate beneficial cardiovascular implications. Low n-6:n-3 FA ratio or n-3 FA in general, affect several metabolic receptors in the liver, including peroxisome proliferator-activated receptors (PPARs), which consist of several sub-types and possess various functions related to lipid metabolism. This includes modification of FA metabolism by reducing TG synthesis/storage and increasing FA oxidation (Davidson, 2006). This theory may explain plasma TG-lowering effects of the ‘designer oils’ observed in this study compared to baseline. Because

of the association of fatty liver with insulin resistance and metabolic syndrome (Chavez-Tapia et al., 2006; Hamaguchi et al., 2005; Neuschwander-Tetri, 2007), it would also be interesting to investigate the effects of the 'designer oils' on plasma glucose and insulin. Finally, the FA composition of liver lipid fractions is of unknown physiological importance but does reflect the FA composition in the heart and provides further evidence for the ability of ALA to increase tissue EPA and DHA.

Lower n-6:n-3 FA ratios and higher EPA/DHA concentrations in heart and liver of the flax group compared to control suggests a successful conversion of some of the dietary ALA to EPA/DHA. The literature has consistently shown that a small amount of ALA can be converted to EPA/DHA endogenously (Harper et al, 2006), however the efficiency is often questioned. The significant difference between all groups in n-6:n-3 FA ratio of liver phospholipids indicates, that even in the presence of the low dietary n-6:n-3 FA ratio, ALA cannot be converted to its longer-chain derivatives; nor can it be incorporated into phospholipids at an identical rate to that of direct EPA/DHA intake. Nevertheless, ALA significantly reduced tissue AA in most lipid fractions to a level comparable to that achieved by fish oil (EPA/DHA). These results are consistent with previous findings (Gerster, 1998). Furthermore, linoleic acid, the parent n-6 FA, was also significantly reduced in both fish and flax compared to control. This implies that there may be less conversion to AA and thus less competition with n-3 FA for the desaturase and elongase enzymes.

Comparing FA profiles of the heart phospholipids from the fish and flax groups, one may speculate that the flax group increased EPA/DHA in the heart tissue at a rate that was more comparable to that of dietary EPA/DHA (fish group); especially when compared to the significant differences in FA profile, particularly EPA/DHA, of fish and flax groups in liver phospholipids. Therefore, this may further support the notion of tissue-dependent conversion of ALA to EPA/DHA (Barcelo-Coblijn et al., 2005). The incorporation of these FA in cardiac cell membrane aid in rationalizing the anti-arrhythmic effects of flaxseed oils (Ander et al., 2004) and fish oils (Chrysohoou et al., 2007). Many animal studies (Culp et al., 1980; McLennan et al., 1993; McLennan, 1993; McLennan et al., 1988; Xiao et al., 2008) have strongly suggested that EPA and DHA have direct protective effects on the heart. We speculate that although flaxseed oils have exhibited an anti-arrhythmic effect on the heart, this is due to the conversion of ALA to DHA. Infused ALA has not demonstrated the same anti-arrhythmic effect as DHA, as shown by Dhein et al. (2005) in isolated rabbit heart. These effects may be as a result of alterations in the function of sodium and calcium channels, which influence membrane fluidity. These alterations may be a consequence of ischemia-activated phospholipase A2, which releases EPA and DHA from myocardial membrane PL. However, we did not note significant differences in gene expression of 3 types of phospholipase A_2; unfortunately we were limited by the small sample sizes for this analysis.

7.3 Summary of main findings and conclusion

Two new formulations of dietary oils containing a n-6:n-3 FA ratio of approximately 2:1 in mice were tested. Incorporation of n-3 FA was observed in various lipid fractions tested. Although the levels of EPA and DHA in tissues from the fish oil–based treated group were statistically higher than those in the flaxseed oil-based treated animals in some of the lipid fractions tested, the overall modifications in the tissue lipid profile are deemed beneficial, regardless of source. This change in lipid composition is deemed beneficial because of the association of increased DHA in heart phospholipids with reduced risk of cardiac arrhythmia (Xiao et al., 2008). However, fish oil may be more potent in enhancing tissue DHA levels, but not in reducing the tissue AA concentrations. Further studies warrant investigation of clinical implications of reduced n-6:n-3 FA ratio, from both flaxseed- and fish oil, in heart tissue and its impact on cardiac function as well as possible mechanism(s) of action. In addition, lowering dietary ratio of n-6:n-3 FA can further lower CVD risk when combined with an overall healthy diet and lifestyle (Lichtenstein et al., 2006).

7.4 Strengths and limitations

A major strength of this study is the high level of control with respect to the diet; all fatty acids were controlled for, in addition to other macro- and micronutrients. Secondly, the experimental course was more than sufficient to observe any effects of both time and treatment in any risk factors measured. Thirdly, the animal model used was one of the most extensively used animal models for human disease (Bedell et al., 1997).

There were also several limitations in the present study. Firstly, the cholic acid content might have been high for the chosen animal model, which led to increased incidence of gallstones and premature sacrifice of some animals. This contributed to a decreased sample size, which subsequently may have lead to an inability to observe significant changes in several lipid parameters as well as bile acids. Secondly, the size of the animal model did not allow for sufficient blood collection; thus we were unable to assess all blood lipids at every desired time point. Thirdly, another limitation of the animal model is that it is resistant to atherosclerosis and hypercholesterolemia and thus required a high fat, high cholesterol diet, which is not reflective of human metabolism. Some may consider the use of PicoLab mouse chow added to the diet as opposed to a purified diet a limitation. Finally, the pooling of samples for cytokine quantification is considered a limitation, as we were unable to conduct statistical analysis and observe variation. Nevertheless, the pooled samples give some preliminary indication as to the effects of these diets on inflammatory profile.

7.5 Future Research

This project will lead the way for future studies to continue to assess the effects of 'designer oils' with low n-6:n-3 ratio on cardiovascular risk factors. One potential area includes the investigation of the effect of n-3 FA, both ALA and EPA/DHA, on food intake and appetite particularly assessing the effects on hormones such as ghrelin. Determining the effect of n-3 FA on body weight as result of food intake also requires further investigation.

Another area of potential research includes assessing cardiac function, via echocardiograph, as a likely reflection of lipid/FA composition of the heart tissues. We speculate both fish and flax oil will improve heart function, reducing arrhythmias.

We have observed a reduction in both plasma total cholesterol and liver cholesterol/TG ratio as a result of low dietary n-6:n-3 FA ratio, regardless of source. The relationship between these two factors is unknown as well as whether this low dietary ratio reduces cholesterol synthesis. Therefore, the mechanism of how low dietary n-6:n-3 FA ratio affects cholesterol metabolism should be investigated. Assessing HMG-CoA reductase, lipase, and LDL receptor activities may be an initial step.

A more complete assessment of plasma cytokines is also an area of future investigation. Our results merely give an indication as to how different sources of n-3 FA may influence inflammatory profile. A greater number of samples require analysis so statistical analysis can be used.

7.6 Implications in the field of nutrition

Results from this study contribute to the body of research of both fish and flaxseed oil on cardiovascular risk. More importantly the results add to the limited knowledge of the optimal ratio of n-6:n-3 FA, and provide support for a dietary n-6:n-3 FA ratio of 2:1. Furthermore, these results provide evidence for other factors limiting conversion of ALA to DHA/EPA other than competition for enzymes with n-6 FA. Our data has additional impact for research on vegetarian and vegan diets (Davis & Kris-Etherton, 2003), which contains little or no DHA

and EPA as well as diets in the future due to the decline in global fish supply, and consequently a decline in global dietary EPA/DHA (Pauly et al., 2002).

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9. Appendix

9.1 Plasma cytokine identification

Plasma taken at week 12 was used for cytokine identification. Mice were injected with 200 µl (100 µg lipopolysaccharide/ml (LPS)) from *Escherichia coli* intraperitonealy two hours prior to blood collection. LPS is a major virulence factor of gram-negative pathogens, which stimulates the release of AA metabolites from the LOX and COX pathways and cytokine production to a detectable concentration (Vardar-Sengül, 2006). Plasma samples were analyzed with RayBio^{TM} Cytokine Antibody Arrays – Mouse Cytokine Array I & 1.1 (RayBiotech Inc., Norcross, GA, USA) (Watanabe et al., 2005). Due to both plasma sample size and financial limitations, pooled samples were analyzed (n=1 for each group). The following cytokines were measured: 1) pro-inflammatory cytokines: IL-2, IL-6, IL-12 p40p70, IL-12p70, IL-17, MCP-1, MCP-5, RANTES, sTNF RI, and TNF-alpha; 2) anti-inflammatory cytokines: IL-5, IL-13, IL-10; and 3) cytokines with unknown or controversial effect on cardiovascular outcomes: VEGF, thrombopoietin, GM-CSF, G-CSF, IFN- γ, IL-3, IL-4, IL-9 and SCF. Concentrations are expressed in optical density.

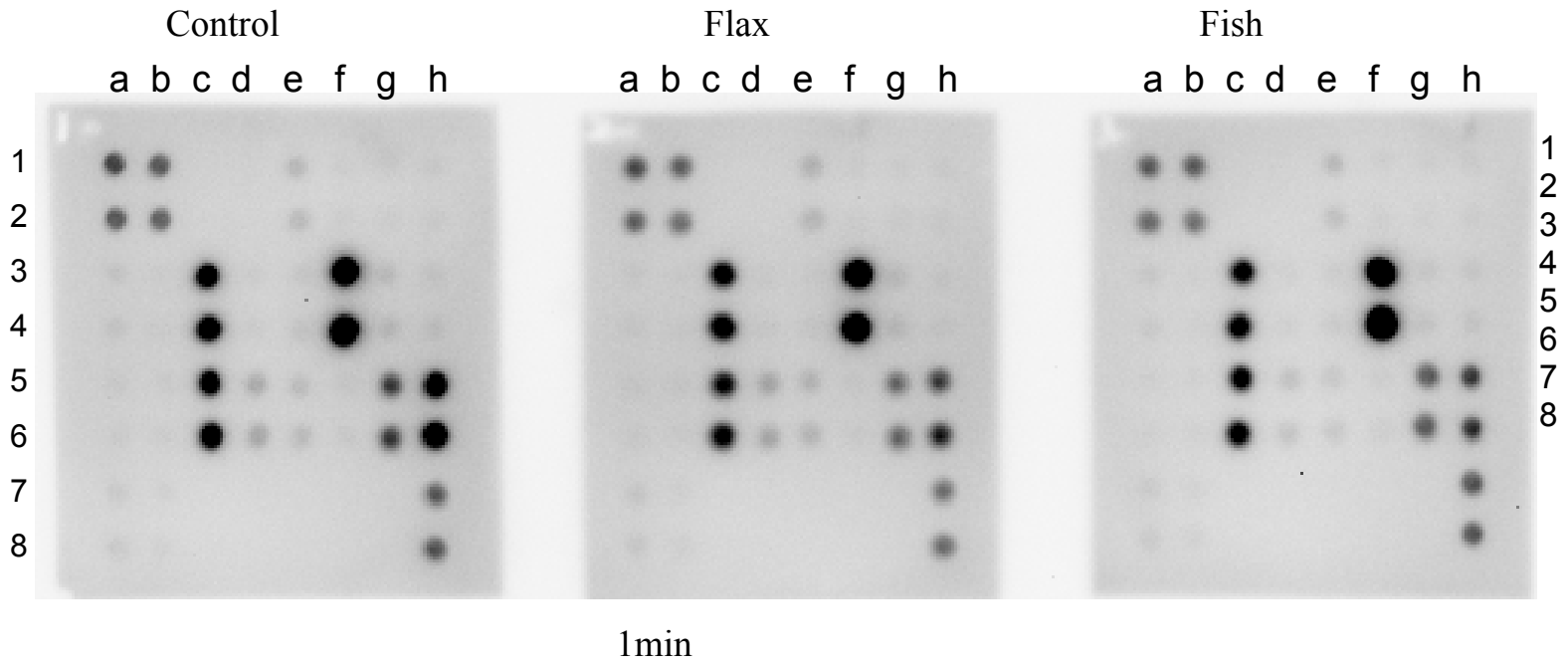
9.2 Plasma cytokine concentrations

According to the protocol described in the methods, the samples were arranged on the plate as illustrated in **Appendix A**. The results of the antibody array blots are shown in **Appendix B**. The results were assessed from pooled samples (n=1), thus statistical analysis could not be completed. The expression was then quantified and presented as a percent change from control in **Appendix**

C. The fish group appeared to display a more consistent reduction in noxious cytokines compared to control than the flax group did. The most notable reduction in both flax and fish compared to control was TNF-alpha. More apparent differences between flax and fish occurred in the concentration of RANTES and MCP-5. Overall, it appears as though a low n-6:n-3 ratio, regardless of source, did not increase beneficial cytokines compared to control, particularly IL-10 and IL-13. Varied results were observed for cytokines without any known relationship with atherosclerosis. One notable change occurred in VEGF concentration in the flax group, which was approximately 100% reduced compared to control. These results are only preliminary and require further investigation to confirm an influence of diet.

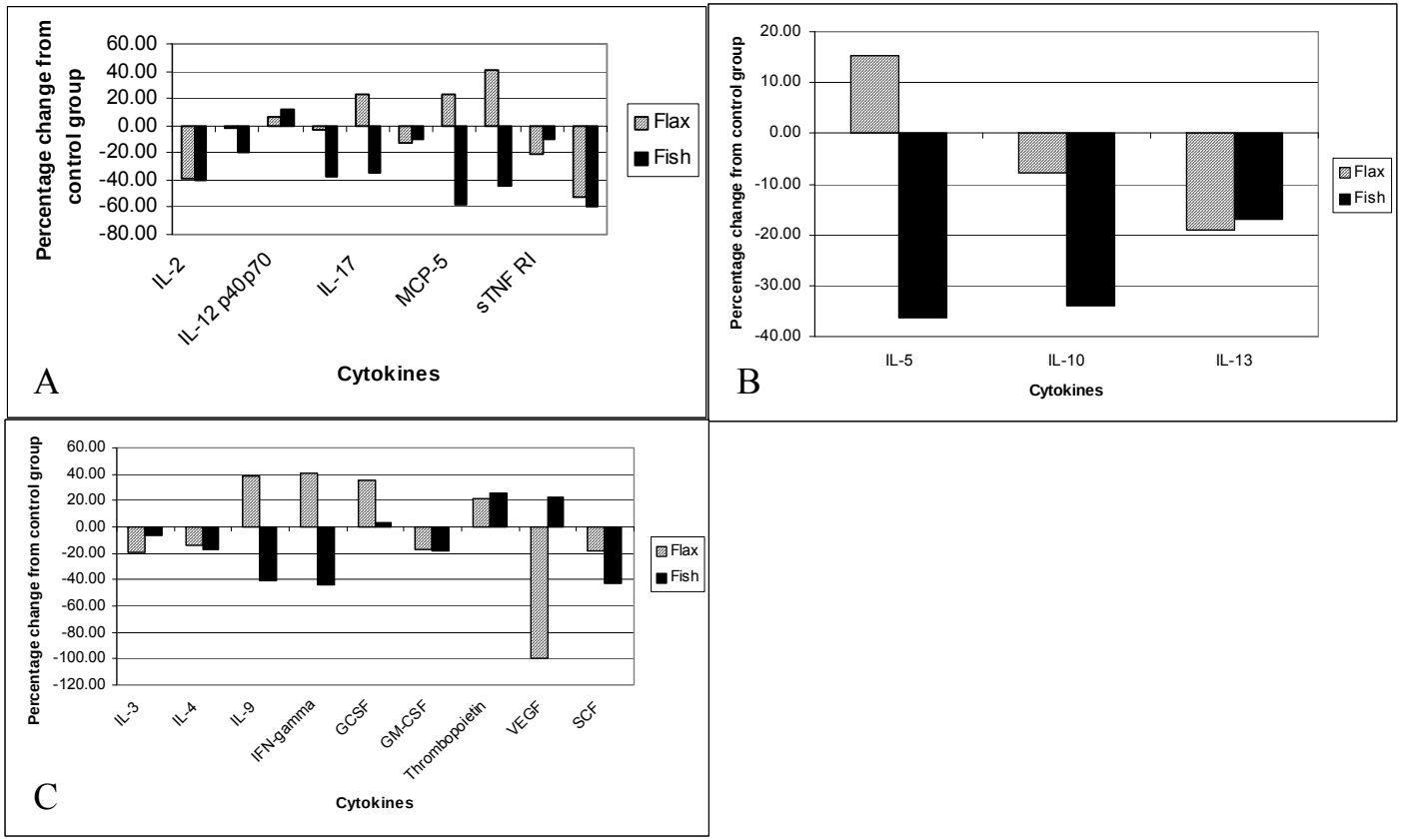
	a	b	c	d	e	f	g	h	
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3	1
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3	2
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13	3
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13	4
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF-a	5
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNF RI	TNF-a	6
7	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos	7
8	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos	8

Appendix A. The arrangement of 22 cytokines on the mouse cytokine antibody array
IL, interleukin; MCP, monocyte chemoattractant protein; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon gamma; RANTES, regulated on activation normally T-cell expressed and secreted; SCF, stem cell factor; TNF-α, tumor necrosis factor alpha; sTNF RI, soluble tumor necrosis factor receptor I; VEGF, vascular endothelial growth factor.



Appendix B. Examples of mouse cytokine antibody array blots probed with the isolated protein samples. Arrangement and identification of cytokines are displayed in Figure 13.

Appendix C. Effect of diet on cytokines as a percentage change of control known to be harmful with respect to atherosclerosis (A), beneficial with respect to atherosclerosis (B), and with unknown activities with respect to atherosclerosis (C) in week 12 plasma



9.3 Discussion of plasma cytokines

Both experimental groups displayed improvements in inflammatory protein profile; the reduction was more apparent in the fish group however. Fish oil has previously been shown to reduce TNF-α, IL-1, IL-6, CRP, IL-12, and interferon-δ (Caughey et al., 1996; Mori & Beilin, 2004; Phillips et al., 2003, Endres et al., 1989; James et al., 2000; Fritsche et al., 1999); flaxseed oil has also been shown to reduce indexes of inflammation (Babu et al., 2003; Zhao et al., 2004). Thus these effects are consistent with the literature. In addition, these effects are postulated due to the production of anti-inflammatory eicosanoids, PGI_3, TXA_3, and LTB_5, from EPA via the COX and LOX enzymes. In contrast, no effects from diets were seen on plasma concentration or spleen gene expression of anti-inflammatory cytokines. It appears as though n-3 FA have the ability to reduce noxious cytokines but are unable to increase the beneficial cytokines. This may be an area of future research to combine the effects of treatments that can reduce the negative effects while simultaneously increase the positive effects on cytokine profile.

9.4 Fatty acid composition of individual heart phospholipids

Individual PL, including PE, PC, lysoPC, PI, and SM were analyzed in pooled heart lipid samples (n=1 per group) because of limited sample quantities. Thus statistical analysis on these fractions (individual PLs) could not be conducted. Similar patterns of FA distribution were observed in individual phospholipids fractions (PI, lysoPC, PE, and SM) that were seen in total PL. The

order of the n-6:n-3 ratio in the individual heart PL fractions PE and PI from highest to lowest were found in the control, flax and fish groups. Other individual PL fractions varied in their order of n-6:n-3 ratio; unfortunately results are limited by the small sample size.

Appendix D. Fatty acid composition of individual heart phospholipids (from pooled samples; n=1 per group)
[1] Not detected

Phospholipid fraction	Total n-6 (%, w/w)	Total n-3 (%, w/w)	n-6:n-3 ratio
Spingomyelin			
Control	6.39	2.02	3.16
Flax	7.28	8.34	0.87
Fish	6.07	5.06	1.20
Lysophosphatidylcholine			
Control	1.15	0.28	4.16
Flax	0.87	ND[1]	-
Fish	6.77	31.37	0.22
Phosphatidylcholine			
Control	10.68	14.23	0.75
Flax	1.70	0.66	2.57
Fish	1.71	1.87	0.92
Phophatidylinositol			
Control	2.38	0.39	6.09
Flax	28.12	9.88	2.85
Fish	4.12	1.61	2.56
Phosphatidylethanolamine			
Control	1.40	2.90	0.48
Flax	12.58	39.98	0.31
Fish	6.48	44.98	0.14