The Effects of Consuming Fatty Acids from Different Sources on Atherosclerotic Development

by

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ABSTRACT

It is becoming increasingly evident that the development of atherosclerotic coronary heart disease (CHD) can largely be regulated by lifestyle and dietary choices. The type of fatty acids regularly consumed may promote or prevent atherogenesis. Flaxseed, the richest plant source of the ω-3 fatty acid alpha-linolenic acid (ALA) is thought to protect against atherosclerotic disease. However, the mechanism(s) by which flaxseed exerts these anti-atherogenic effects requires further investigation. Alternatively, there are dietary fatty acids that are thought to induce significant deleterious effects upon our cardiovascular health. Epidemiological evidence associates dietary *trans* fatty acids (TFAs) with atherosclerotic CHD. This evidence has largely focused on the main source of TFAs in the North American diet, industrially hydrogenated vegetable shortening (iTFAs). It is assumed that TFAs stimulate atherosclerosis but the only studies to date have shown no effect of TFAs on atherosclerosis. Even less is known of the impact of naturally occurring TFAs from dairy and meat products of ruminant animals (rTFAs) on atherosclerotic disease.

We investigated the effects of flaxseed supplementation on atherosclerosis and vascular function in two animal models, the hypercholesterolemic rabbit and the cholesterol fed, low density lipoprotein receptor ($LDLr^{-/-}$) deficient mouse. New Zealand White rabbits and $LDLr^{-/-}$ mice were fed a diet containing flaxseed in the absence or presence of dietary cholesterol for a period of 6 to 24 weeks. We found that dietary flaxseed inhibits the atherogenic effects of a high cholesterol diet in both animal models. The anti-atherogenic effect was achieved in the mouse model through a capacity to lower circulating cholesterol levels and at a cellular level by inhibiting cell proliferation and

inflammation. This reduction is also associated with an improved vascular relaxation response as demonstrated in the rabbit model.

We also investigated the effects of consuming TFAs from two sources, industrially hydrogenated iTFAs rich in elaidic TFA (C18:1*t*-9) or naturally-occurring ruminant rTFAs rich in vaccenic TFA (C18:1*t*-11), on atherosclerotic development in the $LDLr^{-/-}$ mouse in the presence or absence of elevated dietary cholesterol. Our results demonstrate that consuming iTFAs dose dependently initiates atherosclerotic development but not beyond the effects of dietary cholesterol alone. However, consuming rTFAs rich in vaccenic acid protects against hyperlipidemia and atherosclerosis in the presence or absence of dietary cholesterol.

The effects of combining dietary flaxseed and iTFAs in the diet were also examined in this model. Adding whole ground flaxseed or flaxseed oil (ALA) to diets containing low and high doses of iTFAs completely prevented atherosclerotic development in the absence of dietary cholesterol. Flaxseed was also able to partially prevent atherosclerosis caused by iTFAs and cholesterol. Our results suggest that the ω-3 ALA fatty acid rich content of flaxseed is mainly responsible for the anti-atherogenic effects of flaxseed. Our results highlight potential mechanisms for the beneficial effects of dietary flaxseed and the mixed effects of TFAs on cardiovascular health and underscore the need for further basic and clinical investigations.

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To Jefferson

love always,

Dr. Grumbles

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACh	acetylcholine
ApoB	apoprotein B
ALA	α-linolenic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	carbon
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHD	coronary heart disease
CLA	conjugated linoleic acid
COX	cyclooxygenase
CRP	C-reactive protein
CVD	cardiovascular disease
DAB	diaminobenzidine tetrahydrochloride dihydrate substrate
DBP	diastolic blood pressure
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EDRF	endothelium-derived relaxant factor
EDTA	ethylenediaminetetraacetic acid

E-C	excitation-contraction
EFA	essential fatty acids
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid
ET-1	endothelin-1
FA	fatty acid
FAME	fatty acid methyl ester
FBS	fetal bovine serum
FH	familial hypercholesterolemia
FMD	flow-mediated dilation
g/d	grams per day
GC-FID	gas chromatography coupled with flame-ionization detection
GLA	gamma-linolenic acid
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HRP	horseradish peroxidase
ICAM-1	intracellular adhesion molecule-1
IDL	intermediate density lipoprotein
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
iTFA	industrially produced hydrogenated *trans* fatty acids
KCl	potassium chloride
LA	linoleic acid

LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
$LDLr^{-/-}$	low density lipoprotein receptor deficient
LOX	lipoxygenase
$Lp_{(a)}$	$lipoprotein_{(a)}$
LXR	liver X receptor
mac-3	macrophage marker M3/84
MAP	mean arterial pressure
MCP-1	monocyte chemoattractant protein-1
MUFA	monounsaturated fatty acid
NE	norepinephrine
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
NS	not significant
NZW	New Zealand white rabbit
OA	oleic acid
ω	omega (miniscule)
Ω	omega (majuscule)
PAF	platelet activating factor
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen

PDFM partially defatted flaxseed meal

PDGF platelet derived growth factor

PGI_2 prostacyclin

PPAR-γ peroxisome proliferator-activated receptor gamma

PUFA polyunsaturated fatty acid

RB regular butter

RCT randomized controlled trial

rTFA ruminant *trans* fatty acids

SAA serum amyloid A

SBP systolic blood pressure

SDG secoisolariciresinol diglucoside

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM standard error of the mean

SFA saturated fatty acid

SMC smooth muscle cell

SNP sodium nitroprusside

SR sarcoplasmic reticulum

SREBP sterol regulatory element binding protein

TC total cholesterol

TFA *trans* fatty acid

TLC thin-layer chromatography

TNF-α tumor necrosis factor alpha

TG triglycerides

TxA_2	thromboxane A_2
VB	vaccenic acid rich butter
VCAM-1	vascular cell adhesion molecule-1
VF	ventricular fibrillation
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell

CHAPTER I: LITERATURE REVIEW

FATTY ACIDS

Fatty acid structure

Fatty acids (FA) are aliphatic monocarboxylic acids with a long unbranched carbon chain. The carbon chain of FAs can vary in length and in degree of saturation. Fatty acids commonly have an even number of carbon atoms (between 4 to 28), because the biosynthesis of FAs involves acetyl-CoA, a coenzyme which carries a two carbon atom group. The carbon chain can be saturated with hydrogen atoms or may be unsaturated to various degrees. FAs are, therefore, denoted by the degree of saturation. They can be saturated (SFA), monounsaturated (MUFA) or polyunsaturated fatty acids (PUFA). The degree of unsaturation of the FA chain determines its configuration. Unsaturated carbon atoms form double bonds which result in kinks in the configuration of the fatty acid chain. In general, SFAs have a linear structure, MUFAs have one kink in the FA chain, and PUFAs have two or more double bonds resulting in a curved, highly flexible, and fluid structure (Figure 1).

Unsaturated FAs can also be classified based on the location of the first double bond relative to the methyl terminus of the carbon chain (n-carbon), also known as the final or "omega" carbon (ω-carbon). Two common omega FA families, the ω-3 and the ω-6 PUFAs, possess their first double bond at either the third or sixth carbon from the chain terminus.

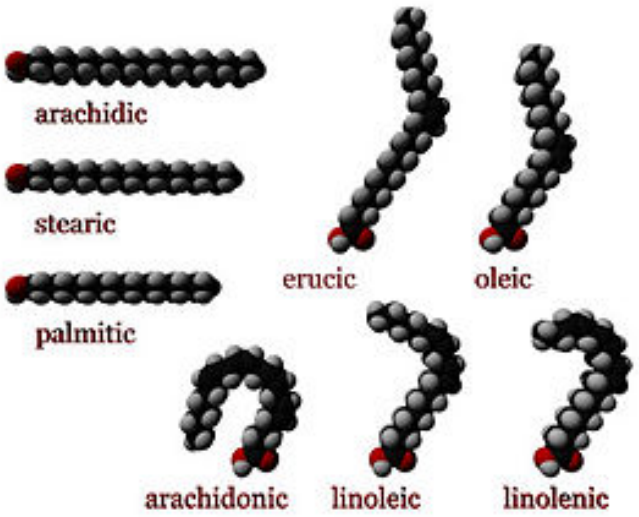


Figure 1. Three dimensional representations of several fatty acids

Saturated fatty acids (SFA): Arachidic, Stearic, and Palmitic; Monounsaturated fatty acids (MUFA): Erucic and Oleic; and Polyunsaturated fatty acids (PUFA): arachidonic, linoleic, and linolenic. *Image adapted from Wikipedia, The Free Encyclopedia* [1].

Polyunsaturated fatty acids

Polyunsaturated fatty acid metabolism

Mammals, including humans, can produce most fatty acids by *de novo* synthesis in the liver by the action of fatty acid synthases from acetyl-CoA precursors. However, only plants are capable of *de novo* production of all PUFAs. Mammals lack the ability to introduce double bonds in fatty acids at the omega-3 (ω-3) or omega-6 (ω-6) position, as they do not have the enzymes necessary to do so. Hence, humans cannot produce two fatty acids: the ω-6 FA linoleic acid (LA) and the ω-3 FA alpha-linolenic acid (ALA) (Figure 1). These FA are termed essential fatty acids (EFAs), since they cannot be made in the body from other substrates and must be supplied in the diet. Once consumed, LA and ALA can be elongated and desaturated in mammals to produce longer chain PUFAs and physiologically active eicosanoids by enzymes known as elongases and desaturases.

LA is the parent FA for all long chain ω-6 PUFAs and ALA is the parent FA for all long chain ω-3 PUFAs. These two FA families share and, therefore, compete for the same enzymes during the elongation and desaturation processes to produce hormone-like substances termed eicosanoids. The delta-5 desaturase enzyme is the rate limiting step for the metabolism of PUFAs. The ratio of ω-6 to ω-3 PUFA consumed is very important to human health, since the ω-3 and ω-6 pathways compete with one another for enzyme activity and mammals lack the ability to convert between these two PUFA families. A dietary imbalance of the two PUFA families can lead to an overabundance or a deficiency in either class of PUFA. An overabundance of one family will limit the metabolic production of the longer chain products of the other. The typical Western diet provides ω-6 and ω-3 PUFAs in a ratio ranging from 8:1 to 25:1 [2], well above the recommendations of national health agencies of a 4:1 ratio [3]. Excessive amounts of ω-6 PUFAs and a very high ω-6/ω-3 ratio, as is found in today's Western diets, has been associated in promoting the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases [4]. Increasing levels of ω-3 PUFA, resulting in a lower ω-6/ω-3 ratio, would reduce the competition for these enzymes and encourage the metabolism of downstream products of the ω-3 PUFA ALA.

Polyunsaturated fatty acids in the food supply

Since most Western diets are already very rich in ω-6 PUFAs, greater focus needs to be placed on incorporating ω-3 PUFAs in the diet. Dietary sources of ω-3 PUFAs are readily available, but in limited quantities. Examples of foods containing ALA include certain vegetable oils, dairy products, flaxseed, walnuts and vegetables [5, 6]. Fatty fish,

such as mackerel, herring and salmon, provide an excellent source of the long chain derivatives of ALA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [3].

Eicosanoids

Eicosanoids are biologically active lipid mediators that regulate a wide range of functions, including blood pressure, blood clotting, circulating lipid levels and immune and inflammatory responses in the body. They are produced primarily by cyclooxygenases (COX-1 and COX-2) or lipoxygenases (LOX) from 20-carbon PUFAs and their metabolites. The ω-3 and ω-6 PUFA families compete for these enzymes to produce prostaglandins, thromboxanes, leukotrienes and other oxygenated derivatives from different series. Figure 2 demonstrates the conversion of EFAs to their downstream products through multiple elongation and desaturation steps. The 20-carbon ω-6 PUFA, arachidonic acid (AA; C20:4 ω-6), derived from the parent EFA linoleic acid (LA; C18:2 ω-6), produces eicosanoids from the 2- and 4-series. These eicosanoids are more biologically active than the 3- and 5-series eicosanoids derived from the ω-3 PUFA eicosapentaenoic acid (EPA; C20:5 ω-3). For example, thromboxane A_2 (TxA_2), a 2-series metabolite of the ω-6 AA, is a pro-inflammatory mediator and a potent vasoconstrictor and platelet aggregator. By competing for the COX and LOX enzymes, ω-3 PUFAs inhibit the conversion of AA to pro-inflammatory eicosanoids, such as TxA_2. Derivatives of the ω-3 PUFA family can also limit the effects of TxA_2, by decreasing the affinity of the TxA_2 receptor for TxA_2 [7]. Increasing the consumption of ω-3 PUFAs results in a greater incorporation of ω-3 PUFAs into cell membrane phospholipids, thus altering membrane fluidity, cell and nuclear transport, and ultimately leading to the generation of more ω-3 derived eicosanoids and a simultaneous reduction of ω-6

eicosanoid inflammatory mediators through competitive inhibition. Ω-3 PUFAs have a similar action on the metabolic pathway of ω-6 PUFAs as anti-inflammatory drugs, such as aspirin and other NSAIDs, which act by downregulating eicosanoid synthesis by inhibiting the COX-2 enzyme [8].

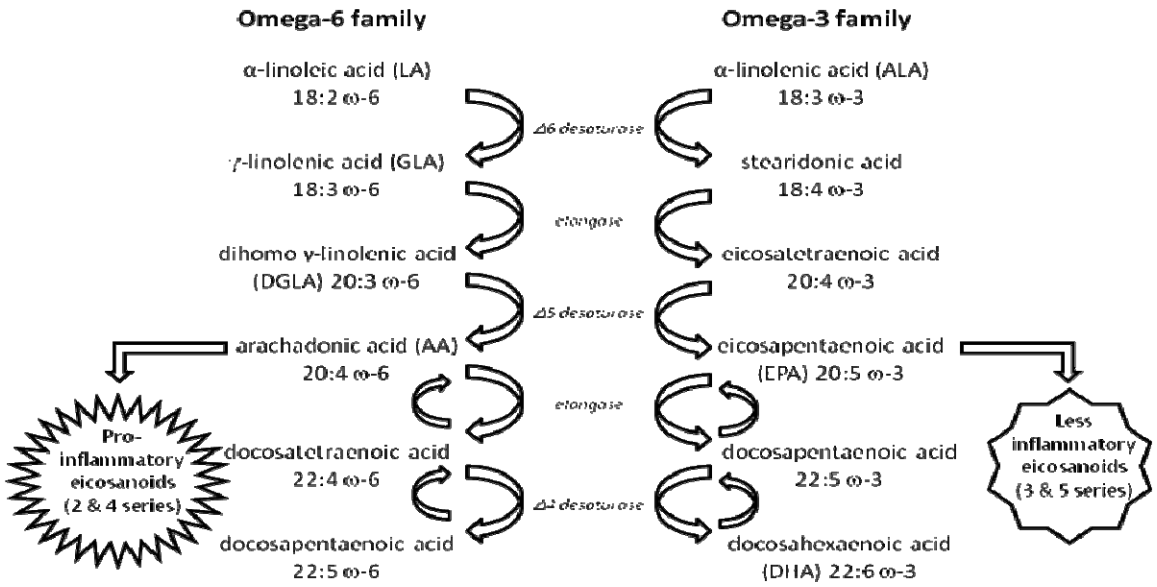


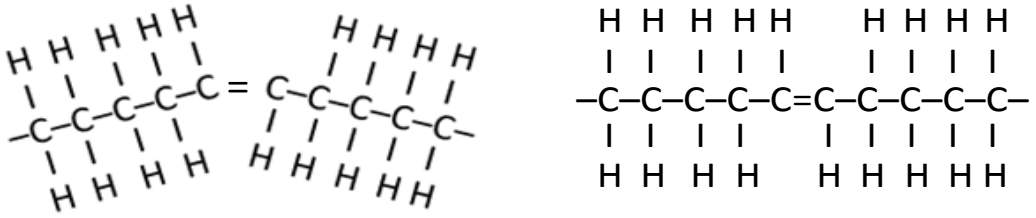
Figure 2. Metabolism of ω-6 and ω-3 polyunsaturated fatty acids

Metabolism of the two fatty acid families requires competition for the same elongation and desaturation enzymes. The desaturation steps tend to be slow and rate limiting compared to the more rapid elongation steps. Eicosanoids, such as prostaglandins, leukotrienes and thromboxanes, can be derived from AA and EPA and can mediate different physiological actions. AA produces pro-inflammatory eicosanoids of the 2 and 4 series, such as thromboxane A_2. EPA produces eicosanoids of the 3 and 5 series which are less inflammatory [9].

Trans fatty acids

The double bond between unsaturated carbon atoms in MUFAs and PUFAs are predominantly in a *cis* configuration (Figure 3). Thus, the single hydrogen atoms surrounding the double bond are on the same side of the fatty acid chain, resulting in a bent FA chain. However, fatty acid chains can also exist in a *trans* configuration. These

trans double bonds can either be naturally produced during the biohydrogenation of unsaturated FAs during bacterial fermentation in the stomach of ruminant animals, such as cows and sheep, or they can be commercially produced during the partial hydrogenation of vegetable oils into semi-solids, such as margarines and shortening. *Trans* fatty acids (TFA) are unsaturated FAs that contain one or more double bond in a *trans* configuration. TFAs are naturally present in low levels in meat and dairy products, or they are found in higher levels in commercially manufactured foods containing or fried in hydrogenated vegetable shortening, such as cookies, crackers, and donuts. The hydrogenation process changes the naturally bent *cis* configuration of MUFA and PUFA into the more linear *trans* configuration, as the single hydrogen atoms surrounding the *trans* double bond are on opposite sides of the FA chain. TFAs are, therefore, similar in conformation and behavior to SFA. The process of hydrogenating vegetable oils increases the shelf and frying stability of vegetable oils and produces a fat source with improved texture for bakery products.



Cis configuration
bent molecule

Trans configuration
straight molecule

Figure 3. *cis* and *trans* configuration of fatty acid chains

Trans fatty acids in the food supply

Intake of TFAs worldwide ranges from 0.5–12% of total fat intake, which represents 1–4% of caloric energy [10]. TFA intake in North America is in the upper end of that range with the average daily TFA intake estimated to be 5.8 grams or 2.6% of calories [11]. The typical Western diet includes many foods that are made with or fried in hydrogenated vegetable shortening. Tables 1 and 2 lists major food sources of *trans* fats and the TFA content of typical foods in the Western diet. Several epidemiological and interventional investigations have found a significant positive association between the consumption of TFAs and coronary heart disease (CHD) [12-23]. A high TFA intake is also associated with elevated atherogenic risk factors, including high levels of cholesterol, triglycerides and inflammatory markers in the blood [13-18, 24]. According to the National Heart, Lung, and Blood Institute of the National Institutes of Health, more than 12.5 million Americans have CHD, and more than 500,000 die each year. That makes CHD one of the leading causes of death in the United States [25]. As a result of the multiple adverse effects reported due to elevated TFA intake, numerous agencies and governing bodies recommend limiting TFAs in the diet and reducing TFAs in the food supply. A listing of the *trans* fat content of manufactured food products is now mandatory in North America [26, 27]. Listing the saturated fat, *trans* fat, and cholesterol content on food labels provides consumers with the information required to make food choices that may help reduce the risk of CHD.

Table 1. Major food sources of *trans* fats in the typical Western Diet

TABLE 1	
Major food sources of *trans* fats in the typical Western Diet	
40%	cakes, cookies, crackers, pies, bread, etc.
21%	animal products
17%	margarine
8%	fried potatoes
5%	potato chips, corn chips, popcorn
4%	household shortening
3%	salad dressing
1%	breakfast cereal
1%	candy

Table adapted from FDA Revealing trans fats [11].

Table 2. Total, saturated and *trans* fat content of common foods containing *trans* fats

TABLE 2

Total fat, saturated fat, and *trans* fat content (per serving) of common foods containing *trans* fats

Product	*Common serving size*	*Total fat (g)*	*Saturated fat (g)*	*Trans Fat (g)*	*Trans/Total Fat (%)*
French Fries*	Medium (147 g)	27	7	8	30
Margarine, stick	1 tbsp	11	2	3	27
Margarine, tub	1 tbsp	7	1	0.5	7
Shortening	1 tbsp	13	3.5	4	31
Doughnut	1	18	4.5	5	28
Candy bar	1 (40 g)	10	4	3	30
Cake, pound	1 slice (80 g)	16	3.5	4.5	28

* fast food (fried in hydrogenated vegetable shortening)

Table adapted from USDA National Nutrient Database for Standard Reference [26].

Fatty acid digestion, catabolism and transport

Fatty acids have many key biological functions, such as acting as structural components of cell membranes, serving as energy sources and participating in signaling pathways. Fatty acids are usually consumed as triglycerides, which cannot be absorbed by the intestine. They are emulsified by bile salts and broken down into free fatty acids and monoglycerides by pancreatic lipase. Once across the intestinal barrier, they are

reformed into triglycerides, which complex with apoproteins and are released into the lymph system and then into the circulation as chylomicrons. Eventually, they bind to the membranes of adipocytes, muscle fibers, or hepatocytes where they are either stored or oxidized for energy. Chylomicron remnants are processed into the various lipoproteins, namely very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) in the liver. Hepatocytes secrete fatty acids, either recycled or newly synthesized, into the circulation in the form of VLDL. In peripheral tissues, lipoprotein lipase digests part of the VLDL into LDL and free fatty acids, which are taken up for metabolism. LDL contains only the B apoprotein (ApoB) and is cholesterol ester rich as compared to VLDL. LDL cholesterol normally circulates in the body for 2.5 days, after which time, LDL receptors (LDLr) on cell membranes, mainly in hepatocytes, recognize the apo-B protein on LDL and allow LDL cholesterol to enter the tissue through endocytosis in clathrin-coated pits and be degraded to provide cholesterol for basic cellular functions (Figure 4) [28]. Intracellular cholesterol levels are highly regulated in hepatocytes. When low cholesterol levels are detected, endogenous production of cholesterol by the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase pathway is stimulated, and lipoprotein uptake is increased by up-regulating the expression of LDL receptors [29]. Finally, free cholesterol and phospholipids from the chylomicron remnants are transferred to high density lipoprotein (HDL). HDL is deemed the “healthy” cholesterol, as it is involved in reverse cholesterol transport.

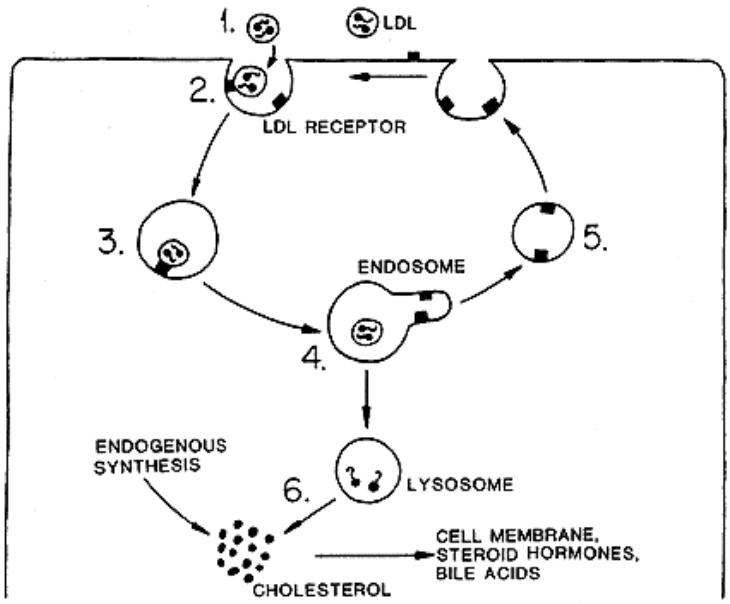


Figure 4. Cellular uptake of LDL particles

(1) LDL particles circulating in the blood bind to LDL-receptors in clathrin coated pits (2) on the cell surface. LDL receptors are transmembrane proteins that have a site that recognizes and binds to the apolipoprotein B-100 on the surface of the LDL. These pits invaginate and are internalized as vesicles by endocytosis (3). Fusion of vesicles gives rise to an endosome (4) in which the LDL dissociates from the receptor, which is recycled (5) to the surface. The LDL is delivered to a lysosome where the cholesteryl ester is cleaved to yield free cholesterol to be utilized for membrane synthesis or is converted to steroid hormones and bile acids. Cholesterol taken up by the cell inhibits the cell's own cholesterol synthesis [28].

CARDIOVASCULAR SYSTEM

Aortic structure and function

The aorta is the largest artery in all mammals. It originates from the left ventricle of the heart and branches into smaller arteries to conduct oxygenated blood to all tissues of the body. Arteries contain connective tissue and elastic smooth muscle cells, allowing the aorta to be rigid and distensible. The main constituent of connective tissue, collagen, provides the vessel with elasticity and tensile strength that provides the ability of the aorta to withstand the high intraluminal pressures exerted by the blood following ejection from the left ventricle. By stretching, the aorta can store the kinetic energy of the ejected

blood to maintain blood pressure and transfer it slowly to the smaller branching vessels during diastole.

The aorta is comprised of three distinct histological layers (Figure 5): the tunica intima, the tunica media, and the tunica adventitia [30]. The tunica intima is the thinnest and innermost layer of the aorta. It consists of a single layer of endothelial cells mounted on a basement membrane. The endothelial cells are in direct contact with the circulating blood and they play a critical role in the homeostasis and function of the aorta. The endothelium regulates vascular tone via regulation by the peripheral nervous system and is normally non-adhesive. Endothelial cells secrete chemicals involved in vascular tone, thrombolysis, coagulation, inflammation and immune function [30]. The tunica intima also consists of a subendothelial space with fibro-elastic connective tissue and circularly arranged elastic bands called the internal elastic lamina [30]. The middle layer of the vessel is the tunica media or muscular layer. It consists predominantly of vascular smooth muscle cells (VSMCs), with a matrix of elastic fibers and connective tissue [30]. The vascular smooth muscle cells of this layer can regulate the lumen size and, therefore, blood flow, by contracting and relaxing to narrow or widen the diameter of the arterial wall. The matrix contains elastin and collagen fibers and functions to support and orientate the vascular smooth muscle cells. The tunica media is separated from the tunica adventitia by another thick layer of elastic bands called the external elastic lamina. The adventitia is the external covering of the artery. This layer is predominately composed of connective tissue (fibroblast cells, collagen and elastin), and provides the artery with strength and protection [31]. This layer also contains the *vaso vasorum* or nutrient capillaries which supply oxygen, blood, lymph or the nerve supply to the artery [30].

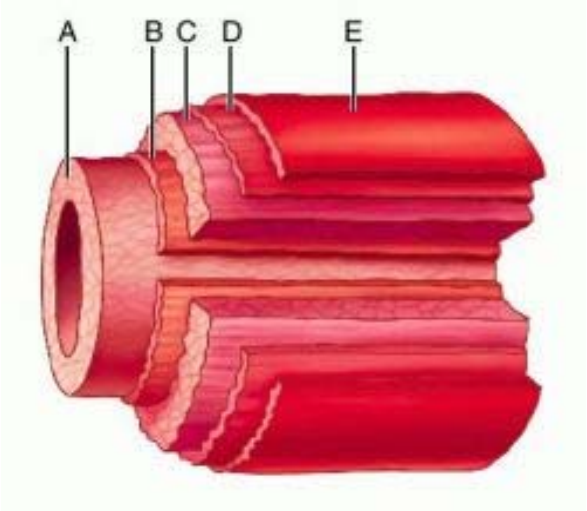


Figure 5. Representation of arterial layers

(A), tunica intima; *(B),* internal elastic lamina; *(C),* tunica media; *(D),* external elastic lamina; *(E),* tunica externa. *© 2007 Saunders, an imprint of Elsevier. Dorland's Medical Dictionary for Health Consumers Inc. Image used with permission from Elsevier on August 13, 2009* [32, 33].

Endothelium: structure and function

Since its anatomic discovery in the 19th century, the endothelium was considered to fulfill no other purpose than that of a selective barrier to the diffusion of macromolecules from the blood to the interstitial space. It was not until the late 1970s when Furchgott and colleagues postulated the role of the endothelium in vascular tone [34, 35]. They demonstrated that the intact endothelium releases a factor, endothelium-derived relaxant factor (EDRF), now commonly known as nitric oxide (NO), which induces acetylcholine-dependent vasorelaxation in VSMC. In contrast, acetylcholine-dependent contraction of the VSMC is induced when the endothelium is injured or removed, demonstrating the pivotal role the endothelium plays in vascular tone [36]. Henceforth, a functional paradigm defined endothelial integrity as a balance in the bioavailability of endothelium-derived relaxing factors and endothelium-derived contracting factors. Any deviation from this state, most notably the impairment of

vasorelaxation in response to pharmacologic (ie acetylcholine) or nonpharmacologic (ie shear stress) stimuli, is referred to as endothelial dysfunction [35].

Including regulation of vascular tone, it is clear today that the endothelium plays numerous additional roles in many important regulatory processes of the vascular wall, such as cell signalling, modulation of inflammation, lipid metabolism, and the immune response, promotion/inhibition of vascular growth, and modulation of platelet aggregation, coagulation, thrombosis and thrombolysis [35, 37]. Central to this multifunctional role of the endothelium is the metabolism and secretion of a number of vasoactive substances and growth factors involved in coagulation, platelet activation and aggregation, vascular permeability and inflammation, and VSMC proliferation and migration (Figure 6) [35]. The endothelium can sense changes in hemodynamic forces via mechanoreceptors or blood borne signals via ligand receptors located in the cell membrane of the endothelial cells [37]. In response to such physical or chemical stimuli, endothelial cells release substances either into the lumen or toward the vascular smooth muscle cell layer to exert important regulatory effects [37]. Such substances include the vasoactive substances nitric oxide, endothelin (ET-1), and eicosanoids such as prostacyclin (PGI_2) and thromboxane (TXA_2), adhesion molecules like intracellular and vascular adhesion molecule (ICAM-1, VCAM-1), growth factors such as platelet derived growth factor (PDGF) or immune modulators such as cytokines (MCP-1), interleukins (IL-6) or tumor necrosis factor (TNF-α).

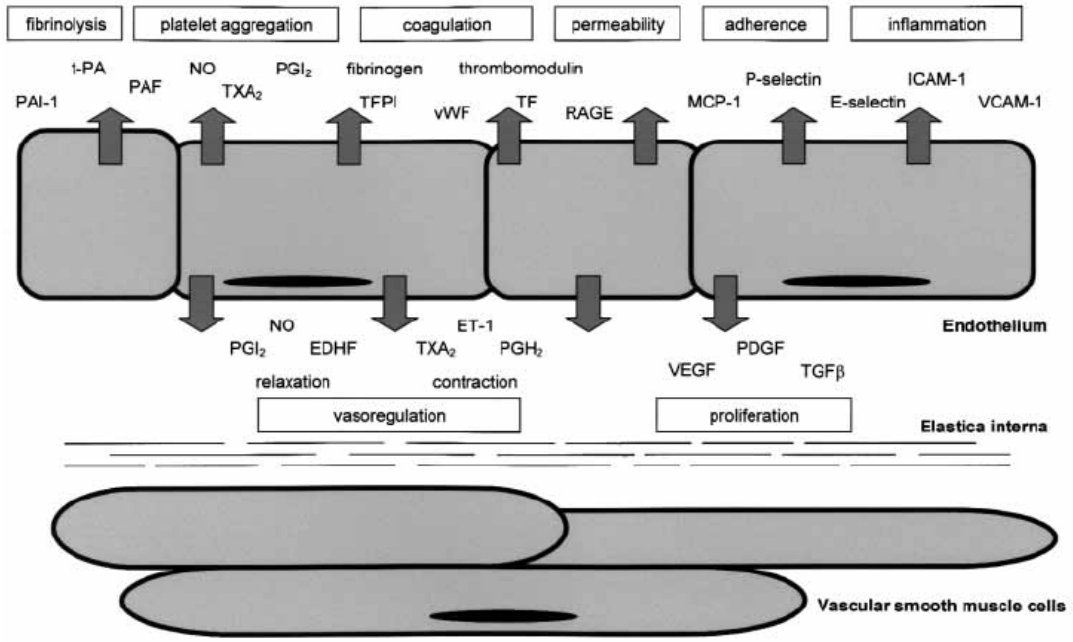


Figure 6. Multifunctional roles of the endothelium

The endothelium secretes various mediators that regulate *fibrinolysis*, including tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1); *platelet activation and aggregation*, including nitric oxide (NO), platelet activating factor (PAF), thromboxane (TXA_2), prostacyclin (PGI_2), fibrinogen, tissue factor pathway inhibitor (TFPI), von Willebrand factor (vWF), thrombomodulin, and tissue factor (TF); *inflammation, cell adhesion, and endothelial permeability*, including receptors for advanced glycosylated end products (RAGE), monocyte chemoattractant protein 1 (MCP-1), P-selectin, E-selectin, intracellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1); *vasorelaxation*, including NO, PGI_2, and endothelium-derived hyperpolarizing factor (EDHF); *vasoconstriction*, including TXA_2, endothelin (ET-1), and prostacyclin (PGH_2); and *smooth muscle cell proliferation and angiogenesis*, including platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor β (TGFβ). *© 2001 Herrmann and Lerman. The endothelium: dysfunction and beyond. Image used with permission from Elsevier on August 7, 2009* [35].

Vascular smooth muscle cells

Vascular smooth muscle cells (VSMC) lie in the tunica media of arteries. They control blood flow by regulating contraction and relaxation of the vessel. VSMCs have a single nucleus and are arranged in sheets connected by gap junctions. They are termed "smooth muscle" as they lack the characteristic striations found in skeletal muscle [38]. The contractile proteins, including actin and myosin, are not arranged into distinct

sarcomeres that form orderly bands throughout the muscle cell. They instead run in oblique directions. Therefore, contraction occurs along multiple axes. SMCs contain less protein than a typical striated muscle cell and much less myosin. SMCs have specific smooth muscle isoforms of actin and myosin and contain some different regulatory proteins, including calmodulin, caldesmon and calponin. The contractile apparatus of SMCs consists of a thin filament of actin and tropomyosin and a thick filament of myosin. Actin filaments attach to the sarcolemma by focal adhesions dispersed throughout the cytoplasm and attach to other actin filaments via dense bodies. These dense bodies are supported by an organized cytoskeleton consisting of intermediate filament proteins vimentin and desmin. SMCs are fastened to one another by adherens and gap junctions. SMCs are, therefore, mechanically, chemically and electrically coupled to one another such that contraction of one cell facilitates the spread of chemicals or action potentials and invoke some degree of contraction in adjoining SMCs. During contraction, actin filaments radiating from a dense body reach toward another group of actin filaments radiating from a second dense body. The actin filaments crosslink with a bundle of myosin filaments located midway between two dense bodies [39]. The force of contraction, therefore, is transmitted from one dense body to another within the cell and as they are also attached to the cell membrane, the force of contraction is also transmitted between adjacent cells [39].

Vasoconstrictor mechanisms

Excitation-contraction coupling in vascular smooth muscle cells

Contraction in VSMC can be initiated by mechanical (ie stretch), electrical (ie cellular depolarization), and chemical (ie hormones, drugs, neurotransmitters) stimuli. VSMC undergo slow, sustained, tonic contractions caused by the sliding action of actin and myosin filaments over each other. This action is fueled by the hydrolysis of ATP. The mechanism of contraction involves different signal transduction pathways, all of which increase intracellular calcium. Figure 7 describes the mechanisms involved in VSMC contraction. Stimulation of cross bridge formation is initiated by a calcium-regulated phosphorylation of myosin caused by a release of calcium from sequestered stores in the sarcoplasmic reticulum (SR) via one of two excitation-contraction (E-C) coupling mechanisms; calcium-induced calcium release or the G-protein coupled second messenger system IP_3 [40].

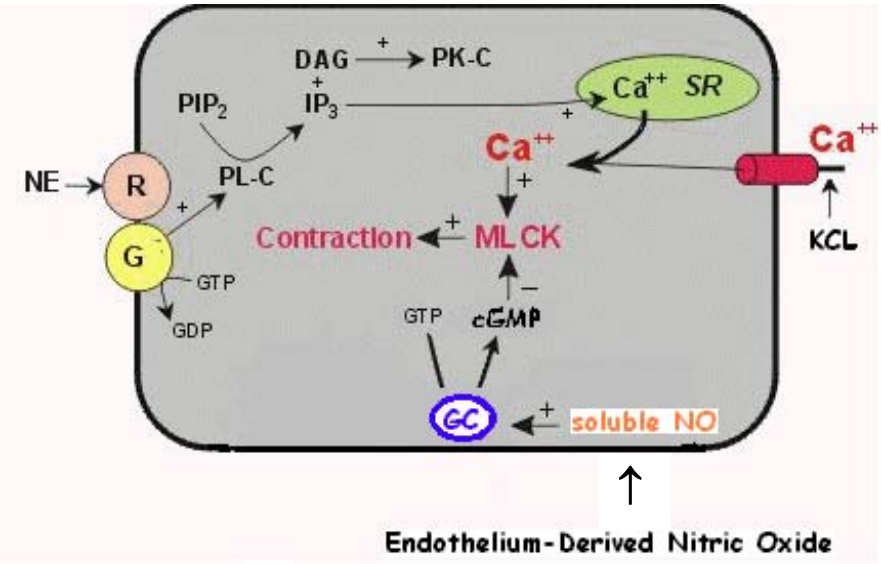


Figure 7. Mechanisms of vasoconstriction and vasodilation in VSMCs

An increase in free intracellular calcium in vascular smooth muscle cells (VSMCs) can result from either increased flux of calcium into the cell through calcium channels or by the release of calcium from the sarcoplasmic reticulum (SR) internal stores. The free calcium binds to calmodulin and this complex activates myosin light chain kinase (MLCK), an enzyme that is capable of phosphorylating myosin light chain subunits found on myosin heads in the presence of ATP. MLC phosphorylation leads to cross-bridge formation between the myosin heads and the actin filaments, and hence, smooth muscle contraction. A release of calcium from internal stores can also result from stimulation by norepinephrine (NE) of a receptor (R) in the cell membrane coupled to a G-protein (G) which leads to an increase in the activity of phospholipase C (PL-C), which splits phosphatidyl inositol diphosphate (PIP_2) into inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 promotes the release of calcium from the SR. DAG may also play a role in VSMC contraction by activating protein kinase C (PKC). Transmembrane receptor operated or voltage operated calcium channels can also induce a contraction when stimulated by the appropriate receptor or by depolarization of the cell as would occur with an increased concentration of extracellular potassium ions (KCl). The increased intracellular calcium concentration results in the activation of the myosin light chain kinase triggering contraction of the vessel. Vasodilatory cGMP is formed by the stimulation of guanylate cyclase (GC) in response to the diffusion of nitric oxide (NO) from adjacent endothelial cells. cGMP inhibits the action of the MLCK therefore promoting relaxation of the vessel. © *1998-2009 Richard E. Klabunde. Cardiovascular Physiology Concept. Image used with permission from Richard E. Klabunde on August 7, 2009* [41, 42].

Vasodilator mechanisms

Intracellular calcium concentrations, therefore, are very important in regulating smooth muscle tone. The concentration of intracellular calcium depends upon the balance between the calcium that enters the cells, the calcium that is released from intracellular storage sites (SR), and the removal of calcium either back into storage sites or out of the cell. Relaxation of the vascular smooth muscle cell is induced by an efflux of calcium

from the cytosol. Calcium is released, sequestered, and removed from the cytosol via receptor-mediated channels, the ATP-dependent calcium pump or by the sodium-calcium exchanger [42]. This outflux results in the disassembly of the calcium-calmodulin complex and the deactivation of myosin light-chain kinase. The myosin light-chain phosphatase is also activated which removes the phosphate from the myosin ATPase and, therefore, terminates cross bridge formation [43].

Most vasodilatory agents, such as nitric oxide (NO), adenosine, or prostacyclins, initiate vasodilation of VSMCs by activating enzymes (either adenylate or guanylate cyclase) which produce cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP), respectively [44]. cAMP and cGMP both cause vasodilation by inhibiting the myosin light-chain kinase and by stimulating calcium pumps or the L-type calcium channels to remove calcium from the cell resulting in relaxation of the vascular smooth muscle cell [44].

Nitric oxide: a key player in the regulation of the vessel wall

Nitric oxide (NO), a gas synthesized by the vascular endothelium, plays a key role in the healthy maintenance as well as the pathology of arteries. NO, also known as endothelium-derived relaxing factor (EDRF), is synthesized from the amino acid L-arginine and oxygen by various nitric oxide synthase (NOS) enzymes, of which two forms are present in the endothelium. Endothelial (e)NOS generates NO in response to shear stress and other physiological stimuli whereas inducible (i)NOS is expressed in response to immunological stimuli and, once activated, generates large amounts of NO that contribute to pathological conditions [45]. NO synthesis and release is triggered by the binding of vasoactive substances, such as ACh or bradykinin, to mechanoreceptors

sensitive to stretch or pressure on the endothelial cell surface. NO release is, therefore, triggered by changes in shear force of blood flow [46]. NO acts locally and acutely by diffusion through cell membranes to adjacent VSMC in the tunica media. There it activates guanylate cyclase triggering the generation of cGMP. This protein kinase decreases intracellular calcium levels thereby causing vascular relaxation [44]. NO can also be synthesized, although to a lesser extent, in VSMCs.

CARDIOVASCULAR DISEASE

Coronary artery atherosclerosis is the principle cause of cardiovascular morbidity and mortality in North America [47, 48]. Atherosclerosis is a progressive degenerative disease which involves a chronic inflammatory response and an accumulation of macrophages and LDL cholesterol in the walls of arteries. This results in the narrowing and stenosis of blood vessels. Atheromatous plaques develop in the subendothelial space of arteries and consist of soft, yellowish differentiated macrophages that have engulfed cholesterol and lipids, referred to as foam cells, and areas of cholesterol crystal formation and/or calcification in advanced fibrous plaques. Atherosclerosis induces two significant pathological processes: an ischemic event due to blood flow obstruction and vascular contractile dysfunction.

Elevated circulating cholesterol levels have long been associated with the development of atherosclerosis and cardiovascular risk. The lipid hypothesis, first proposed in the mid 19th century by the German pathologist Virchow, suggested that the accumulation of blood lipids causes atherosclerosis [49]. This belief was supported by several studies demonstrating that feeding rabbits cholesterol induces atherosclerosis [50-

52]. Further evidence demonstrating that cardiovascular risk can be reduced by either limiting dietary cholesterol intake or inhibiting endogenous cholesterol production by cholesterol-lowering drugs has contributed to our current understanding of the role of cholesterol and lipids in atherosclerotic disease.

LDL receptor

LDL cholesterol levels can be elevated in the blood due to a high intake of dietary cholesterol or due to a lack or loss of function of LDL receptors (LDLr) on cell membranes. If cholesterol cannot be taken up into cells, mainly hepatocytes, it cannot be metabolized and it remains in the circulation where it can damage the cardiovascular system. Familial hypercholesterolemia (FH) is a genetic disorder characterized by elevated LDL cholesterol levels in the blood and premature and advanced cardiovascular disease. Many patients with FH have mutations in the *LDLR* gene that encodes the LDL receptor protein which normally removes particles containing apolipoprotein B (ApoB) from the circulation. Patients who have one abnormal copy of the *LDLR* gene have heterozygous FH, a common genetic disorder, occurring in 1 in 500 people. These patients have prolonged hypercholesterolemia and often develop premature cardiovascular disease [53]. Homozygous FH, occurs in one in a million births with two abnormal copies of the *LDLR* gene, and may cause severe cardiovascular disease in childhood [53]. Although atherosclerosis occurs to a certain extent in all humans, FH patients may develop accelerated atherosclerosis due to the excess LDL cholesterol. The degree of atherosclerotic development can depend upon the number of LDLr still expressed and the functionality of these receptors. LDLr activity is less than 2% in

patients with homozygous FH, while heterozygotes have LDLr activity ranging from 2–25%, depending on the nature of the *LDLR* gene mutation [53].

Several transgenic mouse models have been developed to study atherosclerotic disease, including a mouse lacking cellular LDL receptors ($LDLr^{-/-}$). $LDLr^{-/-}$ mice, a model of human familial hypercholesterolemia, develop severe hypercholesterolemia and atherogenesis when fed an atherogenic diet. The atherosclerotic lesions have characteristic features of human atheromas. The lesions progress over time to form advanced lesions that have a necrotic core, abundant cholesterol clefts, extracellular fat, and a fibrous cap [54]. The $LDLr^{-/-}$ mouse is an excellent model for use in dietary intervention trials because it only develops diet-induced atherosclerotic lesions, unlike the spontaneous atherosclerotic development observed in, for example, the ApoE receptor deficient mouse [55].

However, cardiovascular risk is not entirely dependent on circulating cholesterol levels. Many cardiovascular events occur in individuals with cholesterol concentrations below the National Cholesterol Education Program thresholds of 200 mg/dL for total cholesterol and 130 mg/dL for low-density lipoprotein (LDL) cholesterol [56]. Therefore, other contributing factors have been implicated in atherosclerotic development.

Pathogenesis of atherosclerosis

According to the "Response to Injury" model, Ross postulates that atherosclerosis is an inflammatory condition preceded by endothelial cell dysfunction [57, 58]. This "injury" to the arterial endothelium results from the alteration in cell attachment to the arterial lining, so that shear blood flow causes desquamation of the endothelium.

Adherence and aggregation of platelets ensues at the site of injury. The endothelium becomes dysfunctional and susceptible to atherogenesis in response to stress or injury. The following key processes are involved during atherogenesis: 1) Endothelial injury, 2) accumulation of cholesterol and lipids and the infiltration of monocytes into the subendothelial space, 3) macrophage differentiation and foam cell formation, 4) migration and proliferation of smooth muscle cells, 5) local thrombus formation, 6) calcification and/or plaque rupture, and 7) final occlusion due to plaque rupture/thrombus formation (Figures 8-10) [59].

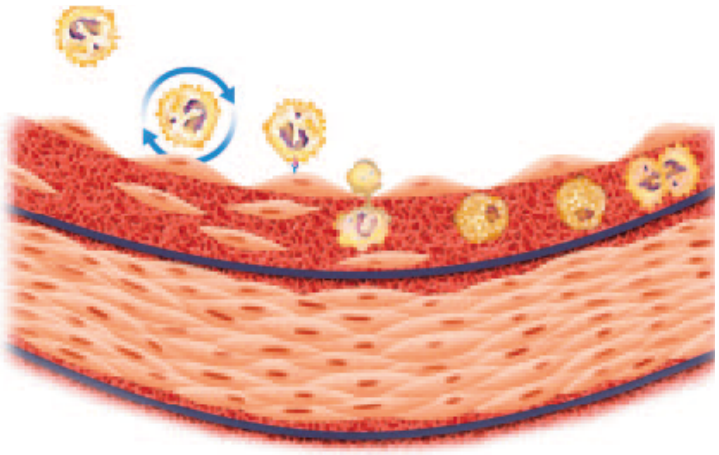


Figure 8. Initiation of atherosclerosis

The diagram represents a cross-section through an artery. The intima of normal arteries is composed of a single layer of endothelial cells overlying a subendothelial matrix of connective tissue and resident smooth muscle cells. The underlying tunica media, separated from the intima by the internal elastic lamina, contains multiple layers of vascular smooth muscle cells. The adventitia, the outermost layer of the blood vessel, separated from the media by the external elastic lamina, is not depicted in this diagram. Circulating monocytes adhere poorly to the endothelium under normal conditions. When the endothelium becomes inflamed, however, it expresses adhesion molecules that bind ligands on leukocytes. Chemokines expressed within the atheroma provide a chemotactic stimulus to the adherent monocytes, directing their differentiation into macrophages and migration into the intima. *© 2008 RRS Packard and P Libby. Inflammation in Atherosclerosis: From Vascular Biology to Biomarker Discovery and Risk Prediction. Clin Chem. Image used with permission from the American Association for Clinical Chemistry on August 23, 2009* [56].

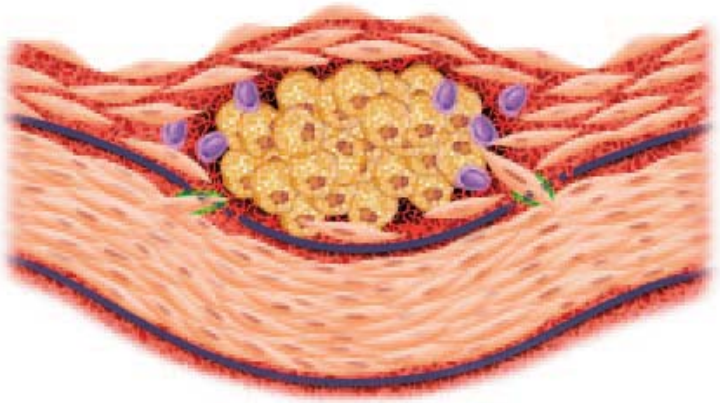


Figure 9. Progression of atherosclerosis

Macrophages express scavenger receptors in response to inflammatory mediators and endocytose modified LDL particles to become lipid-laden foam cells. These foam cells further contribute to lesion progression by secreting proinflammatory cytokines and growth factors that promote monocyte cell adhesion and infiltration, in addition to promoting the proliferation and migration of smooth muscle cells (SMC) into the lesion area. In response to inflammatory stimulation, vascular SMCs express enzymes (collagenases and matrix metalloproteinases) that degrade elastin and collagen in the extracellular matrix, allowing their penetration into the expanding lesion. *© 2008 RRS Packard and P Libby. Inflammation in Atherosclerosis: From Vascular Biology to Biomarker Discovery and Risk Prediction. Clin Chem. Image used with permission from the American Association for Clinical Chemistry on August 23, 2009* [56].

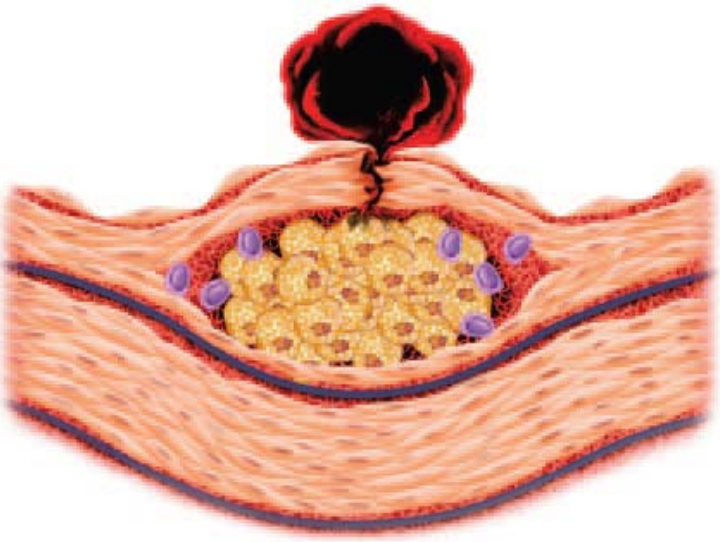


Figure 10. Thrombotic complication of atherosclerosis

Inflammatory mediators can eventually diminish the collagen content of the fibrous cap of the atheroma, rendering it weak and rupture-prone. The fibrous cap can ultimately rupture, as illustrated in this diagram, and inflammatory mediators induce potent procoagulant tissue factor which triggers a thrombus, which causes most acute complications of atherosclerotic disease. The thrombus can either block the vessel at the site of injury, or it can travel into the circulation and cause a blockage in an extremity, the brain (stroke), or the heart (myocardial infarction). *© 2008 RRS Packard and P Libby. Inflammation in Atherosclerosis: From Vascular Biology to Biomarker Discovery and Risk Prediction. Clin Chem. Image used with permission from the American Association for Clinical Chemistry on August 23, 2009* [56].

Endothelial activation

Following endothelial injury, the endothelial lining of arteries loses its ability to normally act as a barrier to blood constituents, control vascular responses, and inhibit platelet aggregation. The endothelium becomes pro-adhesive through cytokine-induced endothelial activation, which is important for the initiation and progression of atherosclerosis since it enables surface expression of endothelial leukocyte adhesion molecules and secretion of soluble pro-inflammatory products, such as interleukins-1 and -6 (IL-1 and IL-6), tumour necrosis factor alpha (TNF-α), vascular cell adhesion molecule-1 (VCAM-1), platelet-derived growth factors (PDGF) and monocyte chemoattractant proteins (MCP) [60]. Because most adhesion molecules and inflammatory products are not expressed under basal conditions, cytokine-induced endothelial activation requires initiation of gene transcription. Nuclear factor NF-κB, a gene regulatory protein implicated in the development of atherosclerosis, can activate gene transcription and expression of adhesion molecules [60, 61]. Thus, damage to the endothelial lining, due to factors such as hypertension, hypercholesterolemia, and systemic inflammation causes an infiltration of monocytes and lipoproteins, including LDL and oxidized LDL (oxLDL), into the subendothelial space of the arterial wall. Two adhesion molecules expressed following endothelial activation aid in the infiltration of substrates into the subendothelial space. Selectins cause monocytes to loosely adhere and roll along the inflammation-activated endothelial cells and integrins mediate firm attachment which enables monocytes to infiltrate into the subendothelial space [56]. There, monocytes differentiate into macrophages which engulf lipids and cholesterol to become foam cells. Macrophages secrete cytokines that encourage further infiltration of

monocytes into the subendothelial space and promote the differentiation and migration of smooth muscles cells into the lesion area. As the lesion progresses, the foam cells accumulate and apoptose to form the necrotic lipid core of the atherosclerotic lesion.

Atherosclerotic lesions can eventually become large, calcified, fibrous plaques that limit or occlude blood flow, or unstable plaques that rupture to form a thrombus, resulting in clinical symptoms (angina) and cardiovascular complications, such as arrhythmias, ischemic heart disease, peripheral vascular disease, myocardial infarction, and cerebrovascular disease (stroke). Atherosclerosis involves multiple processes including inflammation, oxidation, vascular proliferation and migration, and endothelial dysfunction.

Inflammation

Recent research demonstrates that inflammation participates in all stages of atherosclerosis, including the initiation and progression of lesions as well as thrombosis and its resulting complications. During the initiation of atherogenesis, proinflammatory risk factors elicit the expression of primary inflammatory cytokines that stimulate endothelial adhesion molecules and other inflammatory mediators. These primary cytokines also induce the production of interleukin-6 (IL-6), a messenger cytokine, which stimulates platelets, the liver, and adipose tissue to increase production of acute-phase reactants, such as C-reactive protein, fibrinogen, plasminogen activator inhibitor-1 (PAI-1), adiponectin, and matrix metalloproteinase-9 (MMP-9) [62]. Several inflammatory molecules have been implicated in atherothrombosis including adhesion molecules (ie VCAM-1) which cause binding of monocytes to the endothelium; chemoattractants (ie MCP-1) which cause the directed migration of leukocytes into the intima; and activators

(ie macrophage colony-stimulating factor, M-CSF) which cause the expression of scavenger receptors and further release of cytokines, and serve as co-mitogens stimulating the division and survival of macrophages in the evolving atheroma [56]. Many of these inflammatory markers and mediators are released and enter the circulation in soluble form at different stages in the development of atherosclerosis. They can be measured in the blood and used as inflammatory biomarkers for cardiovascular risk prediction [56].

Oxidation

Atherosclerosis has long been regarded as a state of heightened oxidative stress. Numerous studies report elevated levels of oxidized LDL (oxLDL) in the plasma of patients with demonstrated atherosclerotic disease, and that these high levels can be used to predict the patients' risk of myocardial infarction [63-66]. In addition, autoantibodies to oxLDL have also been reported in atherosclerotic lesions of cardiac patients [64]. Oxidized LDL demonstrates many atherogenic actions; oxLDL is involved in foam cell formation, it is cytotoxic for endothelial cells in culture, it acts as a chemoattractant for monocyte-macrophages and it inhibits the vasodilatative effect of nitric oxide (NO) [67-69]. LDL oxidation is a free radical driven chain reaction where polyunsaturated fatty acids are converted to lipid peroxides, which decompose to biologically active products, such as aldehydes [67]. Oxidation of LDL enhances the accumulation of cholesterol in macrophages, leading to foam cell formation and initiation of the atherosclerotic process. Unlike native LDL, oxLDL binds to receptors on the cellular membrane of macrophages, such as scavenger receptor A and CD36, which are not down-regulated by intracellular cholesterol concentration [70]. There is, therefore, no negative-feedback loop for the

accumulation of oxLDL in lipid-laden foam cells. The evidence that oxidants contribute to atherosclerotic disease is further supported by research in experimental animals that report that various antioxidant compounds (probucol, vitamin E) can prevent atherosclerosis [67]. Additionally, oxidized lipids may act as early mediators of a chronic inflammatory reaction through activation of transcription factors, such as nuclear factor-κB (NF-κB) or alternate pathways, that induce the expression of genes which instigates fatty streak development [71]. oxLDL initiates a chain of molecular events leading to the transmigration of monocytes into the subendothelial space and their differentiation into macrophages. Mature macrophages in the subendothelial space further potentiate the degree of LDL oxidation and its subsequent uptake. Mature macrophages also participate in the progressive inflammatory response in the atheroma [72].

Proliferation

Another key atherogenic process involves the focal accumulation and proliferation of vascular smooth muscle cells (VSMCs) into the neointimal space of arteries. Vascular proliferation is associated with other cellular processes such as inflammation, apoptosis and matrix alterations [73]. VSMCs contribute to the development of atherosclerotic lesions via the production of pro-inflammatory mediators such as VCAM-1 and MCP-1 and matrix molecules required for the retention of lipoproteins [73]. In turn, inflammatory mediators, growth factors, and both LDL and mildly oxidized LDL further induce VSMC proliferation [74]. VSMC proliferation and migration is also important in maintaining the stability of the atherosclerotic plaque. VSMCs and fibroblasts with extracellular calcification are the main constituents of the fibrocalcific cap enclosing advanced atherosclerotic lesions [73]. The fibrous cap

surrounding lipid-laden lesions is often thin and weak, due to inflammatory processes which induce the expression of collagenase and proteolytic inhibitors resulting in extracellular matrix degradation and VSMC apoptosis [62, 73]. VSMC proliferation and migration is an ongoing process during atherogenesis that provides an essential fibrous cap that could prevent plaque rupture. However, excessive and uncontrolled VSMC proliferation and migration can prove detrimental. Endothelial dysfunction can decrease the secretion of nitric oxide (NO) by the endothelial cell and result in a loss of growth inhibition produced by the NO. In addition, the inactivation of NO by reactive oxygen species may also reduce growth inhibition. These effects may contribute to excessive migration and proliferation of VSMCs and to an increased inflammatory response, resulting in unstable plaques and increased risk of myocardial infarctions and stroke [73].

Endothelial dysfunction

Damage to the endothelial lining of arteries upsets the balance between vasoconstriction and vasodilation and initiates a number of processes that promote or exacerbate atherosclerosis. These include increased endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines [75, 76]. The hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation, which is mediated by nitric oxide (NO). Decreased production or activity of NO, manifested as impaired vasodilation, may be one of the earliest signs of atherosclerosis [76]. Under normal healthy conditions, eNOS generates low concentrations of NO which protects against atherosclerosis and contributes to vessel homeostasis by regulating vascular tone and blood pressure, preventing platelet aggregation and adherence, and by inhibiting VSMC contraction and proliferation [37, 46]. Paradoxically, higher concentrations of NO

generated by iNOS in VSMCs and activated inflammatory cells, including macrophages and neutrophils, contributes to atherosclerosis by further activating macrophages, oxidizing LDL cholesterol and by causing cellular damage [45, 77]. The excessive NO is inactivated and converted to pro-oxidant adjuncts, including $ONOO^-$, during the activation of radical oxygen species formation, which overwhelms the anti-oxidant defense of the vascular endothelium [45].

Prevention and regression of atherosclerosis

Diet and atherosclerosis

It is becoming increasingly evident that atherosclerotic heart disease is largely attributable to factors that can be altered or prevented by lifestyle modification. Yusuf and co-workers recently reported that greater than 90% of heart disease can be influenced through behaviour modifications like nutritional interventions, cessation of smoking and regular exercise [48]. Dietary choices, including the amount and type of fatty acids consumed can, therefore, heavily impact on the development of atherosclerosis. Increasing the consumption of ω-3 fatty acids has been suggested as one dietary strategy to provide cardioprotection against ischemic heart disease and significantly reduce the incidence of myocardial infarcts and stroke [78, 79]. The most common way to consume ω-3 fatty acids has been in the form of marine oils like fish.

POLYUNSATURATED FATTY ACIDS (PUFAs) & CARDIOVASCULAR DISEASE

Epidemiological studies & clinical trials

Consumption of long chain ω-3 PUFA provides anti-atherogenic effects in experimental and epidemiological studies [80-82]. The recent Study on Prevention of

Coronary Atherosclerosis by Intervention with Marine Omega-3 fatty acids (SCIMO) demonstrated that consumption of 1.65 g/day of a fish oil supplement by patients with coronary artery disease (CAD) for 2 years resulted in less progression and more regression of coronary atherosclerotic plaques [82]. This effect, however, was not observed in the carotid arteries, suggesting that ω-3 fatty acids may have different effects in different vascular beds [82]. Results from the Seven Countries Study showed an inverse relationship between fish consumption and CAD [83], while the Health Professionals study found no relationship [84]. Conversely, the Health Professionals study found a beneficial effect on CAD from dietary ALA, specifically [84], but the Dutch cohort of the Seven Countries Study did not [3]. The only study to show a negative effect of fish consumption on CAD was conducted in Finland, but it may have been influenced by mercury contamination of the fish [84].

Cardiovascular effects of ω-3 PUFAs

Ω-3 PUFA provide their anti-atherogenic effects through one or a combination of several potential mechanisms. They may exert their effect on atherogenesis by: a) altering the circulating lipid profile; b) changing the physicochemical function of cell membranes, whereby affecting eicosanoid biosynthesis, cell signaling and gene expression, and c) modulating vascular smooth muscle cell proliferation and migration.

Effects of ω-3 PUFAs on circulating lipid profile

Many epidemiological and dietary interventions have shown that consumption of ω-3 PUFA significantly alters serum lipid profile. A strong inverse relationship exists between ω-3 PUFA consumption and circulating plasma triacylglycerol (TG)

concentrations [85]. Dutch men who consumed approximately 30 g/day of fish over a long period of time had lowered serum TG compared to a control group [86]. A health survey of the Inuit of Nunavik, Canada, who traditionally consume large amounts of marine foods rich in ω-3 PUFA, revealed abnormally high plasma phospholipid concentrations of ω-3 PUFA [87]. A negative correlation between ω-3 PUFA and plasma TG levels, and a positive relationship with high-density lipoprotein (HDL) cholesterol levels may account for the low mortality rate due to cardiovascular disease observed in this population [87]. Interestingly, an increase in the levels of low density lipoprotein (LDL) cholesterol and total cholesterol levels was also discovered [87]. Elevated plasma cholesterol levels have long been associated with an increased risk of atherosclerosis. This may help to explain the cardioprotective effects of PUFA. However, the anti-atherogenic effect of ω-3 PUFA supplementation is not always linked to a change in total plasma cholesterol levels [85, 88, 89]. Dietary intervention trials suggest LDL cholesterol levels increase with ω-3 PUFA supplementation in a dose dependent manner [87, 88, 90, 91]. HDL levels may be altered by fish oil as well. Nilsen et al. showed a significant decrease in total cholesterol and a significant increase in HDL cholesterol following fish oil supplementation [92]. The concomitant increase in HDL cholesterol levels relative to increasing LDL cholesterol often leaves the total cholesterol to HDL cholesterol ratio, a common measure of atherogenic risk, unchanged [84, 87, 88]. This evidence suggests ω-3 PUFA reduce atherosclerotic development via mechanisms other than lowering LDL cholesterol.

The hypotriacylglycerolemic effect and the consequent increase in LDL cholesterol observed with ω-3 PUFA supplementation may be due to altered very-low-

density lipoprotein (VLDL) metabolism. A recent dietary intervention trial confirmed that ω-3 PUFA decrease plasma TG and VLDL apo B [89]. The pool size of VLDL is decreased due to a reduction in hepatic secretion of VLDL and increased conversion of VLDL to LDL [89]. Further evidence suggests that the increase in LDL cholesterol levels due to ω-3 PUFA supplementation appears to be in LDL particle size rather than the number of LDL molecules. Ω-3 PUFAs increase levels of LDL-2 particles, which carry more cholesterol ester molecules per LDL particle, resulting in an increase in total circulating cholesterol levels [93]. However, the intermediate LDL-2 subfraction facilitates the removal of LDL cholesterol from the circulation as it has a higher affinity to the LDL receptor than its larger (LDL-1) and smaller (LDL-3) isoforms [93]. Thus, the increase in LDL cholesterol levels caused by ω-3 PUFA supplementation reflects changes in LDL composition, favouring the efficient removal of LDL cholesterol and should therefore not necessarily be viewed as an adverse effect of ω-3 PUFAs [93].

Effects of ω-3 PUFAs on cell membranes

Increased intake of EPA and DHA inevitably results in greater incorporation of these FA into circulating lipids and tissues. Ω-3 PUFA may replace ω-6 PUFA in cell membrane phospholipids, thus altering the physicochemical properties of the membrane [3]. The physicochemical alterations in membrane properties may directly or indirectly influence the function of membrane-bound receptors, ion channels and enzymes, and affect downstream signaling pathways that will have a direct effect on vascular endothelial and smooth muscle cell function [94]. Eicosanoid production is also affected by FA composition of the membrane.

Alteration of the eicosanoid profile may have important effects on inflammation [95]. Eicosanoids, 20-carbon FA derived from ω-6 and ω-3 EFA by the addition of oxygen atoms into the FA chains, confer a wide variety of potent, hormone-like actions on various tissues. The eicosanoid families, including the prostaglandins, thromboxanes and leukotrienes, influence many biological activities including platelet aggregation, smooth muscle contraction, and inflammatory responses. The 20-carbon ω-3 and ω-6 PUFA compete for the cyclooxygenase (COX) and lipooxygenase (LOX) enzymes. The 2- and 4-series eicosanoids derived from ω-6 PUFA are more biologically active than the 3- and 5-series eicosanoids derived from ω-3 PUFA. Thromboxane A_2 (TxA_2), a metabolite of arachidonic acid (AA), is a potent vasoconstrictor and platelet aggregator. Fish oils inhibit TxA_2 in vitro and in vivo [96]. Ω-3 derivatives also decrease the affinity of the TxA_2 receptor for TxA_2, thus further inhibiting TxA_2-induced platelet aggregation [7]. Increased consumption of ω-3 PUFAs results in greater incorporation of ω-3 PUFAs into cell membrane phospholipids, ultimately leading to the generation of more ω-3 derived eicosanoids. This results in simultaneous reduction of ω-6 PUFA-derived pro-inflammatory eicosanoids because ω-3 PUFA can competitively inhibit the conversion of AA to pro-inflammatory eicosanoids. Ω-3 PUFA act as potential COX substrates, decrease the affinity of the COX enzyme for ω-6 PUFA, and suppress production of ω-6 eicosanoid inflammatory mediators [3, 97].

The vascular endothelium is also modified by the ingestion of ω-3 PUFA. Vasoactive substances and growth factors are released by the vascular endothelium, which activate immune cells, gene transcription, and functions involved in the regulation

of monocyte adhesion, inflammation, vascular cell growth, cell migration, and vascular tone [37]. In response to stress or injury, the endothelium can become dysfunctional and be susceptible to atherogenesis.

Ω-3 PUFA may modulate atherogenesis by inhibiting signaling events related to endothelial activation. Expression of endothelial leukocyte adhesion molecules and soluble pro-inflammatory proteins is inhibited when ω-3 PUFA alter expression and production of macrophage cytokines [60, 61, 81, 98-100]. This modulatory effect of ω-3 PUFA on gene expression is associated with a parallel reduction in steady-state mRNA levels of pro-atherogenic molecules [60, 99]. The magnitude of the inhibitory effect of ω-3 PUFA on endothelial activation is related to the extent to which ω-3 PUFA incorporate into cellular lipids. Nutritional supplementation can produce large enough elevations in DHA concentration to observe noticeable effects in *in vitro* studies [61, 97]. The exact mechanisms of the inhibitory effects of ω-3 PUFA on endothelial activation are unknown. However, the reduction of steady state levels of adhesion molecule and growth factor mRNA by ω-3 PUFA action persists after adhesion is activated. Furthermore, the effects of ω-3 PUFA occur prior to translation of mRNA into protein and are independent of receptor activation [98, 99].

The multiple double bonds of ω-3 PUFA can have a direct physical effect on NF-κB-induced expression of pro-inflammatory substances. The double bonds may inactivate superoxide anions that are generated early in cytokine-induced intracellular signal transduction, inhibiting hydrogen peroxide formation, which is directly responsible for the activation of NF-κB and induction of adhesion molecules [60].

Ω-3 PUFA display anti-atherogenic effects through direct modulation of nitric oxide (NO) production and release [96, 101]. Nitric oxide is synthesized from L-arginine by NO synthases (NOS), specifically endothelial (eNOS) and inducible (iNOS) synthases. Nitric oxide regulates vascular relaxation and inhibits key atherosclerotic processes such as platelet aggregation, monocyte adhesion, and vascular smooth muscle cell (VSMC) proliferation and migration. The cellular mechanisms by which ω-3 PUFA improve endothelial function remain unclear. Recent reports, however, suggest ω-3 PUFA enhance eNOS and iNOS production of NO. Translocation and activation of eNOS induced by EPA results in endothelium-dependent vasorelaxation [102]. Docosahexaenoic acid increases NO production by potentiating IL-1β-induced iNOS mRNA expression in VSMC through activation of the p44/42 mitogen-activated protein kinase (MAPK) signaling cascade [94, 103].

Effects of ω-3 PUFAs on VSMCs proliferation and migration

Migration and proliferation of VSMC causes intimal hyperplasia, contributing to development of atherogenic lesions. Activated platelets aggregate at sites of endothelial dysfunction and release peptide growth factors, such as platelet-derived growth factor (PDGF), and non-peptide growth factors, like serotonin (5HT) and adenosine diphosphate (ADP). Previous studies indicate PDGF, 5HT, ADP, and TxA_2 can stimulate VSMC to proliferate [100]. Although epidemiological and clinical evidence suggests ω-3 PUFA may reduce both VSMC proliferation and excitability, very few studies demonstrate the cellular mechanisms by which ω-3 PUFA modulate VSMC. Ω-3 PUFA may inhibit VSMC proliferation through multiple signal transduction pathways that modulate growth factors. Alternatively, EPA and DHA block 5HT-induced SMC proliferation due to

increases in mRNA levels of the 5-HT_2 receptor [100]. Ω-3 PUFA also inhibit receptor binding of PDGF, reducing mRNA expression of early genes involved in the development of atherosclerosis [3].

Anti-aggregatory effects of ω-3 PUFAs

Narrowing of the blood vessels due to an atherosclerotic plaque can provide a setting in which a thrombus can more readily block blood flow and cause a myocardial infarction. As described above, ω-3 fatty acids compete for the same elongation and desaturation enzymes as the ω-6 fatty acids. While eicosanoids derived from both parent fatty acids are pro-aggregatory, those derived from AA (2- and 4-series) are 100-fold greater in activity than those derived from EPA (3- and 5-series). Consequently, the EPA metabolites are generally considered as anti-aggregatory. An improved balance between AA and EPA could reduce the likelihood of clot formation.

A variety of models have been used to observe the effects of enriching the diet with ω-3 fatty acids. Experiments in which either saturated fat or PUFA (either ω-3 or ω-6) was added to the diet found striking results. In response to ADP, platelet aggregation was significantly increased in plasma obtained from rabbits consuming the ω-6 rich diet compared to the ω-3 fed groups consuming fish oil (EPA and DHA) or flaxseed oil (ALA) [104]. This increased aggregation was even greater than that observed in the coconut oil group (saturated fat). The same trends applied when platelet activation was initiated by collagen, but not thrombin [104]. The inhibitory effects of the platelet aggregation were directly related to the fatty acid composition of the platelet lipids. It was also hypothesized that a PUFA effect on membrane viscosity could affect the activity

of the proteins in the platelet membranes that are involved in aggregation as receptors or enzymes.

Although beneficial in preventing the potential blocking of a blood vessel by a thrombus, extreme inhibition of clotting mechanisms could have side effects. Increases in bleeding times have been reported in humans with increased intake of fish oils [105, 106] but not flaxseed oil [107, 108]. There is some anecdotal evidence of persons taking aspirin and ω-3 supplements experiencing hematuria and spontaneous nosebleeds likely resulting from severe effects on platelet aggregation [109].

Anti-arrhythmic effects of ω-3 PUFAs

Arrhythmias commonly precede a myocardial infarction and may ultimately cause death. Ω-3 PUFAs have demonstrated anti-arrhythmic effects in several animal models. Experiments performed on isolated hearts found that PUFAs antagonized the depressed ventricular arrhythmia threshold in hypoxia [110] and animals consuming ω-3 fatty acids exhibited significant reductions, or even elimination, of arrhythmias compared to control groups [111, 112]. Direct intravenous injection of a concentrated fish oil emulsion proved effective at preventing fatal ventricular fibrillation (VF) in dogs subjected to exercise stress tests following a coronary artery ligation [113]. Follow-up studies demonstrated that injections of purified EPA and DHA, as well as the parent ω-3, ALA, were all equally protective against VF [114].

The most common way to consume ω-3 fatty acids has been in the form of marine oils like fish. However, dietary compliance for fish supplementation is often an issue due to concerns about environmental toxins, palatability and eructation [115]. Sadly, no

matter how good a product is for you, if the general population will not ingest it, it will never provide the expected benefits. Finding an alternative source of ω-3 PUFAs, therefore, could be an important issue for cardiovascular health. There is an increasing interest in nutritional interventions that may prevent the development of atherosclerosis and protect against the vascular function abnormalities induced by cholesterol consumption. Flaxseed is one such dietary intervention.

FLAXSEED

Nutritional composition

Flaxseed, a small brown seed produced from the blue flowers of the flax crop *Linum usitatissimum* grown in the Canadian prairies, is the richest plant source of the ω-3 PUFA α-linolenic acid (ALA; C18:3n-3) (Figure 11) [115]. Flaxseed is also known as linseed. ALA demonstrates significant beneficial effects against heart disease [116-119]. ALA can also be converted in the body to the cardioprotective ω-3 PUFAs found in fish oils, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [120]. Flaxseed exists in 4 main forms, whole seed, ground, partially defatted flaxseed meal or as flaxseed oil. The nutritional composition of the four forms of flaxseed is listed in Table 3. Whole and ground flaxseed contains 41% fat, of which 73% of this is PUFA. ALA accounts for approximately 57% of total fatty acids in flax. Flaxseed is also a good source of two other nutrients which contribute to the cardioprotective effects of flaxseed; dietary fibre and phytoestrogens called lignans. The high soluble and insoluble fibre content of flaxseed forms a typical viscous gum that once it is digested may lower circulating total cholesterol (TC) and low-density lipoprotein (LDL) cholesterol levels by enhancing transit time and increasing bile acid excretion. The seed coat of flaxseed contains the

highest known source of lignan phytoestrogens. The main lignan in flaxseed is secoisolariciresinol diglucoside (SDG). Plant lignans are phenolic compounds that once ingested are converted by gut bacteria to the bioactive mammalian lignans enterolactone and enterodiol. SDG is a potent antioxidant [121, 122]. Flaxseed oil does not contain any dietary fibre or lignans, whereas partially defatted flaxseed meal is rich in fibre and lignans and poor in ALA content.

Figure 11. Flax and flaxseed
© 2009 Flax Council Canada. Images used with permission from the Flax Council of Canada on August 10, 2009 [123]

Table 3. Nutritional composition of four common forms of flaxseed

TABLE 3

Nutritional composition of four common forms of flaxseed

(per 100g)

	Fat (g)	ALA (g)	Protein (g)	CHO (g)	Dietary Fibre (g)	Lignan (mg)	Calories (kcal/g)
Whole flaxseed	41.0	22.8	20.0	28.9	27.8	82-2600	450
Ground flaxseed	40.8	23.1	20.0	29.2	27.7	82-2600	450
Flaxseed oil	100.0	57.0	0	0	0	0	884
Partially defatted flaxseed meal	11.1	6.0	38.9	38.9	33.3	2500	389

The nutritional composition of whole flaxseed, ground flaxseed, and flaxseed oil is adapted from [124]. The lignan content of flaxseed was obtained from [125]. The nutritional composition of partially defatted flaxseed meal, including the lignan content was provided by Omega Nutrition, Canada. Abbreviations: ALA, alpha-linolenic acid; CHO, carbohydrates (includes sugars, starches, and total dietary fibre).

Interest in flaxseed as a functional food capable of providing protective effects against CHD is rising. In recent years, several animal and clinical trials have been conducted to help elucidate the mechanism by which flaxseed is cardioprotective. Whole ground flaxseed or the derivatized components of flaxseed have exhibited cardioprotective effects both clinically [126-133] and in several animal models [121, 134-141]. Flaxseed has demonstrated many anti-atherogenic properties [121, 122, 134-138, 140, 141]. However, the exact mechanism(s) by which dietary flaxseed inhibits atherosclerotic development are only beginning to be understood.

Animal data

Effects on circulating lipids, atherosclerosis and oxidation

Dietary flaxseed can significantly prevent atherosclerotic development under various conditions and in multiple animal models [121, 134-138]. In general, studies using whole ground flaxseed, low ALA partially defatted flaxseed meal, or the lignan SDG as the dietary treatment report an overall reduction in TC levels, and a

corresponding decrease in atherosclerosis [121, 134]. The only study directly investigating the role of flaxseed oil on plasma lipids and atherosclerosis reported no effect [142]. Since the hypolipidemic effect of flaxseed is not always correlated to a decrease in atherogenesis, it is at best likely to be only one of the contributing factors to the anti-atherogenic potential of flaxseed. The powerful anti-oxidative effects of the lignans within flaxseed probably contribute to its anti-atherogenic potential [121, 122, 138]. It is well known that antioxidants can reduce atherogenesis in animals [143-145].

Effects on vascular function

The benefits of consuming flaxseed may extend beyond its anti-atherogenic potential. The endothelial lining of arteries normally acts as a barrier and modulator of vascular contraction and relaxation. The endothelium can become damaged and exhibit dysfunctional relaxation kinetics under conditions of prolonged stress induced by elevated blood pressure or cholesterol levels. Flaxseed has been demonstrated to protect against the loss of endothelial-dependent vascular relaxation induced by hypertensive conditions [139]. Vessels from hypertensive animals fed flaxseed exhibited normal vascular relaxation in response to acetylcholine and bradykinin, two endothelial-dependent vasorelaxants, as compared to vessels from hypertensive animals fed a control diet. Flaxseed had no effect on endothelial-independent routes of modulating vascular response [139].

Effects on ischemia/reperfusion-induced arrhythmias

Arrhythmias commonly precede a myocardial infarction and may ultimately cause death. Dietary flaxseed supplementation completely suppressed the incidence of ventricular fibrillation (VF) normally observed during ischemia, and significantly reduced the incidence of VF during reperfusion in hearts obtained from cholesterol-fed rabbits [146]. Flaxseed appears to exert its protective effect by shortening the QT interval and action potential duration of heart beats [146]. The ALA content of the diet was primarily responsible for the anti-arrhythmic effects, presumably through its inhibitory action on Na^{+}-Ca^{2+} exchange [147].

Clinical data

Hypolipidemic effects

Tables 4, 5 and 6 summarize the clinical findings from several dietary intervention studies using flaxseed or one of its extracted components. The majority of the studies report that flaxseed modestly reduces TC and LDL cholesterol levels in normolipidemic [128, 132, 133] and hypercholesterolemic patients [126, 129, 131] and has less impact on HDL and TG levels. Ground flaxseed also reduces the levels of other atherogenic lipoproteins including $Lp_{(a)}$, apolipoproteins A-1 and B [127, 130]. However, the results are not always consistent. Dodin and colleagues' recent trial in healthy menopausal women conflict with these findings, as they report that consuming either 40g/d of ground flaxseed or the wheat germ placebo for 12 months both result in elevated levels of apolipoproteins A-1 and B, $Lp_{[a]}$, and decreased LDL peak particle size [148]. In addition, several intervention trials report no effect of ground or whole flaxseed on plasma lipids [148-152]. The variable effects of whole ground flaxseed on plasma TC and

LDL cholesterol levels reported do not always appear to be related to the dose of flaxseed provided (15-50g/d), or to general patient characteristics, such as age, sex, or circulating lipid status. As little as 20g of ground flaxseed per day has been reported to have potent cholesterol (↓17%) and triglyceride (↓36%) lowering effects in mildly hypercholesterolemic adults [126] whereas higher doses of flaxseed (40g/day) has been reported to have no effect on plasma lipids in mildly hypercholesterolemic patients [148, 151]. Dietary intervention studies using flaxseed oil have not reported any beneficial effect on TC, LDL, HDL, and TG levels, despite a rise in plasma ω-3 PUFAs [153-155]. The only two studies reporting an effect on plasma lipids demonstrate that flaxseed oil may cause more harm than good by increasing circulating TC and decreasing HDL levels [154, 156]. Jenkins et al. reported that 50g/d of partially defatted flaxseed meal, poor in ALA, but rich in dietary fibre and lignans, reduces TC and LDL cholesterol within 3 weeks of feeding [129]. The direct effects of extracted flax lignans on plasma lipids have only recently been investigated. Whereas one study investigating the effects of flax SDG lignans in healthy postmenopausal women report no effect on plasma lipids [157], Zhang et al. recently report that SDG lignan, at doses of 300 and 600mg/d in hypercholesterolemic adults, reduces TC by up to 22% and LDL cholesterol by up to 24%, with no effect observed on HDL or TG levels [158]. Based on the investigations to date, the lignan and/or fibre content of flaxseed appear to contribute to the hypolipidemic effect of flaxseed. It is doubtful that the ALA in flaxseed oil has any hypolipidemic action in humans.

Table 4. Clinical trials reporting cardiovascular effects of whole ground flaxseed or partially defatted flaxseed meal

TABLE 4

Clinical trials reporting cardiovascular effects of whole ground flaxseed or partially defatted flaxseed meal

Reference	Study Design	Patient Population (sample size)	Flaxseed intervention	Treatment Duration	Control group (Comparison group)	% change in ω-3 PUFA	% change in TC	% change in LDL	% change in HDL	% change in TG	Other health effects	Other markers tested, but NS
Bloedon et al. (2008)[130]	RCT parallel	Hypercholesterolemic adults (n=62)	40g/d of ground flaxseed-containing baked products	10 weeks	wheat bran products	↑ ALA‡	No change	↓ 13% (5wk)† ↓ 7% (10wk) NS	↓ 16% (men 5wk)* ↓ 9% (men 10wk)*	No change	↓ Lp[a] 14%* ↓ HOMA-IR 23.7%*	inflammation or oxidative stress markers
Patade et al. (2008) [131]	RCT parallel	Hypercholesterolemic postmenopausal women (n=55)	30 g/d flaxseed	3 months	oat bran fibre	Not measured	↓7%	↓ 10%	No change	No change		Other clinical and hematological parameters
Dodin et al. (2008)[148]	RCT parallel	Healthy menopausal women (n = 191)	40 g/d flaxseed, 20g in bread, 20g as ground grains	12 months	Wheat germ	↑ ALA‡ ↑ DPA‡ ↑ ω-3s‡	No change	No change	No change	No change	↑ apo A ↑ apo B ↑ Lp(a) ↓ LDL particle size*	fibrinogen, CRP, insulin, glucose
Faintuch et al. (2007)[149]	RCT crossover	Morbidly obese adults (n=41)	30 g/d flaxseed flour	2 weeks	fat-free commercial manioc flour	Not measured	No change	No change	No change	No change	↓ WBC 9.6%* ↓ CRP 22.6%* ↓ SAA 17.8%* ↓ Fibronectin 10.9%*	glucose, albumin, insulin, leptin, C3 & C4
Mandasescu et al.(2005) [126]	RCT parallel	Mildly hyperlipidemic adults (n=40)	20 g/d ground flaxseed	60 days	hypolipidic diet (+/- statin)	Not measured	↓ 17%*	↓3.9%*	No change	↓ 36%*	↓ TC/HDL-C ratio 33.5%*	BMI
Dodin et al. (2005) [159]	RCT parallel	Healthy postmenopausal women (n=179)	40 g/d ground flaxseed, ground seed or in bread	12 months	Wheat germ	Not measured	↓vs placebo*	No change	↓vs placebo*	No change	↓ SBP 3.8%† ↓ DBP 5.3%‡ ↓ weight 0.8kg* ↓BMI 0.3kg/m²*	bone mineral density
Stuglin et al. (2005) [150]	Uncontrolled trial	Healthy men (n=15)	3 muffins/d with 32.7 g total flaxseed	4 weeks	None	Not measured	No change	No change	No change	↑ 29%*	↓ creatinine 7.8%‡	HR, markers of hepatic and renal function
Lucas et al. (2002) [127]	RCT parallel	Postmenopausal women (n=58)	40 g/d ground flax-seed, also 1,000 mg/d calcium and 400 IU/d vitamin D	3 months	Wheat	Not measured	↓ 6%#	↓ 4.7% NS	↓ 4.8% NS	↓ 13% NS	↓ apo A-1 6%* ↓ apo B 7.5%‡	markers of bone formation and resorption, estrogenic effects
Lemay et al. (2002) [151]	RCT crossover	Hypercholesterolemic menopausal women (n=25)	40 g/d ground flaxseed, seed or baked in bread	2 months	(HRT therapy: estrogen or progesterone)	Not measured	No change	No change	No change	No change	↓ glucose*	fibrinogen and PAI-1

TABLE 4 (continued)

Reference	Study Design	Patient Population (sample size)	Flaxseed intervention	Treatment Duration	Control group (Comparison group)	% change in ω-3 PUFA	% change in TC	% change in LDL	% change in HDL	% change in TG	Other health effects	Other markers tested, but NS
Clark et al. (2001) [152]	RCT crossover	Adults with lupus nephritis (n=15)	30 g/d ground flaxseed	1 year	Period with and without dietary treatment	↑ ALA 70%‡	No change	No change	No change	No change	↓ creatinine 3.1%*	albumin
Jenkins et al. (1999) [129]	RCT crossover	Hyperlipidemic adults (n=29)	50 g/d partially defatted flaxseed in muffins (20 g fiber/d)	3 weeks	Wheat bran muffins	Not measured	↓4.6%‡	↓7.6%‡	No change	↑10%*	↓apo B 5.4%‡ ↓apo A-I 5.8%† ↑oxidation (↓thiol 11%†)	Lp ratios, HDL, ex vivo androgen or progestin activity
Arjmandi et al. (1998)[160]	RCT crossover	Hypercholesterolemic postmenopausal women (n=38)	38 g/d whole flaxseed in baked products	6 weeks	Sunflower seed in baked products	Not measured	↓6.9%#	↓14.7%‡	No change	No change	↓ Lp[a] 7.4%*	
Cunnane et al. (1995) [132]	RCT crossover	Healthy young adults (n=10)	50 g/d flaxseed in 2 muffins	4 weeks	Wheat flour muffins	↑ ALA* ↑ EPA* ↑ DPA*	↓6%*	↓ 9%*	No change	No change	↑ urinary lignan excretion	Indexes of lipid peroxidation
Clark et al. (1995) [128]	Uncontrolled trial	Adults with lupus nephritis (n=9)	15, 30, and 45g/d ground flaxseed for 4 weeks at each dose	12 weeks	none	↑ ALA(30g) ↑ EPA(30g)	↓ 11%# (30g) ↓ 9%# (45g)	↓ 12% (30g) #	No change	↓ 4% (45g) NS	↓creatinine ↑ C3 ↓ blood viscosity ↓ PA	BP
Cunnane et al. (1993) [133]	Uncontrolled trial	Healthy young women (n=9)	50 g/d ground flaxseed as supplement or baked in bread	4 weeks	None (supplement versus baked in bread)	↑ ALA#	↓ 9%#	↓ 18%#	No change	No change	↓ postprandial blood glucose 28 %#	

*P < 0.05; †P < 0.005; ‡P < 0.001

Abbreviations: RCT, Randomized controlled trial; g/d, grams per day; ω-3 PUFA, serum omega-3 polyunsaturated fatty acid levels; TC, total cholesterol; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; TG, triglycerides; NS, not significant; ALA, alpha-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; DHA, docosapentaenoic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; TNF-α, tumor necrosis factor-α; IL, interleukin; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; C3, complement 3; C4, complement 4; PA, platelet aggregation; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular cell adhesion molecule-1; sE-selectin, soluble E-selectin; TxB_5, thromboxane; PGE_5, prostaglandin, EPA:AA, fatty acid ratio (marker for thromboxane production and platelet aggregation).

Table 5. Clinical trials reporting cardiovascular effects of flaxseed oil

TABLE 5

Clinical trials reporting cardiovascular effects of flaxseed oil

Reference	Study Design	Patient Population (sample size)	Flaxseed intervention	Treatment Duration	Control group (Comparison group)	% change in ω-3 PUFA	% change in TC	% change in LDL	% change in HDL	% change in TG	Other health effects	Other markers tested, but NS
Kaul et al. (2008)[155]	RCT parallel	Healthy adults (n=86)	2g/d flaxseed oil, in two 1g capsules	12 weeks	placebo, (fish oil, hempseed oil)	↑ ALA	No change	No change	No change	No change		Oxidative modification of LDL, platelet aggregation, inflammatory markers
Paschos et al. (2007)[153]	RCT parallel	Dyslipidaemic men (n=87)	15mL/d flaxseed oil (8 g ALA/day)	12 weeks	15 mL/d safflower oil (11 g/d LA)	Not measured	Not measured	Not measured	Not measured	Not measured	↓ SBP‡ ↓ DBP‡ ↓ MAP‡	
Nelson et al. (2007)[161]	RCT parallel	Young, healthy, overweight adults	flaxseed oil capsules (increasing ALA to 5% of total energy intake)	8 weeks	Isocaloric regular diet	↑ ALA* ↑ EPA* ↑DHA*	Not measured	Not measured	Not measured	Not measured		BMI, WC, IL-6, TNF-α, CRP, SAA
Harper et al. (2006)[156]	RCT parallel	Healthy adults (n=56)	5.2 g/d flaxseed oil (3 g/d of ALA)	26 weeks	olive oil	Not measured	↑ 8.3%*	No change	No change	No change		Lp particle size
Thies et al. (2001)[162]	RCT parallel	Healthy adults (n=48)	9 oil capsules/d with 2g of ALA from flaxseed oil	12 weeks	Palm/sunflower oil (80:20) (Fish oil, GLA, AA, DHA)	Not measured	Not measured	Not measured	Not measured	Not measured	↓ sVCAM-1 16% ↓ sE-selectin 23%	inflammatory cell numbers, TNF-α, IL-1β, IL-6, sICAM-1
Nestel et al. (1997)[154]	RCT parallel	Obese adults with markers for insulin resistance (n=15)	20 g/d from flax oil margarine products	4 weeks	Sunola sunflower oil	↑ ALA‡ ↑ DPA*	No change	No change	↓8.3%*	No change	↑ systemic arterial compliance 46%‡ ↓ MAP 12.5%* ↑ LDL oxidizability‡ ↓ insulin sensitivity*	glucose tolerance tests
Caughey et al. (1996)[163]	RCT parallel	Healthy adults (n=28)	ALA rich diet 13.7 g/d from flaxseed oil products	4 weeks	sunflower oil products	↑ ALA* ↑ EPA* ↑DHA*	Not measured	Not measured	Not measured	Not measured	↓TNF-α 27%* ↓ IL-lβ 31%* ↓T_xB_5 30%* ↓ PGE_5 33%*	
Allman et al. (1995)[164]	RCT parallel	Healthy young males (n=11)	40 g/d flaxseed oil	23 days	Sunflower oil	↑ platelet EPA*	Not measured	Not measured	Not measured	Not measured	↑ EPA:AA* ↓ platelet aggregation*	

*P < 0.05; †P < 0.005; ‡P < 0.001

Abbreviations: RCT, Randomized controlled trial; g/d, grams per day; ω-3 PUFA, serum omega-3 polyunsaturated fatty acid levels; TC, total cholesterol; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; TG, triglycerides; NS, not significant; ALA, alpha-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; DHA, docosapentaenoic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; TNF-α, tumor necrosis factor-α; IL, interleukin; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular cell adhesion molecule-1; sE-selectin, soluble E-selectin; TxB_5, thromboxane; PGE_5, prostaglandin, EPA:AA, fatty acid ratio (marker for thromboxane production and platelet aggregation).

Table 6. Clinical trials reporting cardiovascular effects of SDG lignan from flaxseed

TABLE 6

Clinical trials reporting cardiovascular effects of SDG lignan from flaxseed

Reference	Study Design	Patient Population (sample size)	Flaxseed intervention	Treatment Duration	Control group (Comparison group)	change in plasma lignan	% change in TC	% change in LDL	% change in HDL	% change in TG	Other health effects	Other markers tested, but NS
Zhang et al. (2008) [158]	RCT parallel	Hypercholesterolaemic adults (n=55)	300 or 600 mg/d SDG	8 weeks	Placebo (no SDG)	↑ SDG* ↑ ED* ↑ EL*	↓ 22%†	↓24%†	No change	No change	↓ glucose 25%*	
Hallund et al. (2008) [165]	RCT crossover	Healthy postmenopausal women (n=22)	500mg/d SDG in muffin	6 weeks	Placebo (no SDG)	Not measured	Not measured	Not measured	Not measured	Not measured	↓ CRP 15%*	IL-6, TNF-α, sICAM-1, sVCAM-1, and MCP-1
Hallund et al. (2006) [157]	RCT crossover	Healthy postmenopausal women (n = 22)	500mg/d SDG in muffin	6 weeks	Placebo (no SDG)	↑ ED‡ ↑ EL‡	No change	No change	No change	No change		lipoprotein oxidation lag time, antioxidant capacity

$^{*}P < 0.05$; $^{\dagger}P < 0.005$; $^{\ddagger}P < 0.001$

Abbreviations: SDG, secoisolariciresinol diglucoside; ED, enterodiol; EL, enterolactone; RCT, Randomized controlled trial; g/d, grams per day; ω-3 PUFA, serum omega-3 polyunsaturated fatty acid levels; TC, total cholesterol; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; TG, triglycerides; NS, not significant; CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; IL, interleukin; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1.

Anti-inflammatory effects

Atherosclerosis is a complex disease involving factors other than hyperlipidemia. Infectious disease and inflammation, for example, represent an important causative mechanism in atherosclerosis [166]. Ω-3 fatty acids exert an anti-inflammatory action [167, 168]. Faintuch et al. reported that obese adults consuming 30g of flaxseed flour per day had significantly lower circulating levels of C-Reactive Protein (CRP) (↓23%), serum amyloid A (SAA) (↓18%), and fibronectin (↓11%) following only 2 weeks of dietary treatment [149]. Flaxseed appears to also exert anti-inflammatory actions in a healthy population. Three studies report impressive anti-inflammatory effects of either flaxseed oil or SDG lignan in healthy adults, including significant reductions in inflammatory markers, such as TNF-α, IL-lβ, TxB_5, PGE_5, soluble VCAM-1, soluble E-selectin, and serum CRP levels [162, 163, 165]. However, the majority of clinical dietary intervention trials investigating the effects of ground flaxseed, flaxseed oil, or SDG lignan, on markers of inflammation have reported no effect on serum levels of IL-6, TNF-α, sICAM-1, sVCAM-1, MCP-1, CRP, and SAA [130, 148, 155, 161, 165].

Anti-aggregatory effects

The effects of flaxseed supplementation on platelet aggregation have now been investigated in several clinical intervention trials and the results remain inconsistent. Only two trials report that dietary flaxseed provides protection against platelet aggregation. Clark et al. report that 15 to 45 grams of flaxseed ingested daily increases the amount of platelet activating factor (PAF) required to cause platelet aggregation in

patients with lupus nephritis [128]. Allman et al. report that 40g/d of flaxseed oil increases the ratio of EPA to arachidonic acid in platelets, considered a marker for thromboxane production and platelet aggregation potential, and protects against collagen-induced aggregation response in healthy young males [164]. However, larger and more recent studies do not find that flaxseed provides any protection against platelet aggregation [107, 148, 151, 155].

Hypotensive effects

The effects of flaxseed on arterial blood pressure have rarely been reported. However, a large 12 month study in healthy postmenopausal women reported a significant reduction in both systolic and diastolic blood pressure after consuming 40g of ground flaxseed (providing ~9g ALA) per day [159]. Two groups also report that consuming flaxseed oil (8-20g/d ALA) significantly reduces systolic, diastolic and mean arterial pressure within a shorter time period [153, 154].

Antioxidant effects

Because flaxseed possesses great antioxidant potential [121, 122, 138], it would be expected that it would generate impressive antioxidant effects in the body. Conversely two trials report that flaxseed or flaxseed oil causes an increase in markers of oxidative stress including a decrease in serum protein thiol groups [129] and LDL oxidation [154]. The effects of flaxseed on physiological markers of oxidative stress clearly needs further examination in clinical trials.

In summary, consuming moderate doses of ground flaxseed (1-4 tbsp) per day can modestly reduce circulating TC and LDL cholesterol levels, as well as lower various markers associated with atherosclerotic cardiovascular disease in humans. Evidence to date suggests that the dietary fibre and/or lignan content of flaxseed provide the hypocholesterolemic action. The ω-3 ALA found in the flax oil fraction likely contributes to the anti-atherogenic effects of flaxseed via anti-inflammatory mechanisms. Dietary flaxseed may also protect against ischemic heart disease by improving vascular relaxation responses and by inhibiting the incidence of ventricular fibrillation. The data supports the contention that DHA and EPA are not the only cardioprotective PUFAs and ALA can be considered as well. The body of research now effectively argues for the initiation of careful, randomized controlled trials of dietary flaxseed in a patient population with symptoms of atherosclerotic heart disease.

trans FATTY ACIDS (TFAs)

There are dietary fatty acids that may induce significant deleterious effects upon our cardiovascular health. TFAs are unsaturated fatty acids that have undergone a hydrogenation process to reduce the number of double bonds in the fat. This process changes the original *cis* configuration of the double bonds into a *trans* configuration, thereby resulting in a more linear fatty acid chain. TFAs are, therefore, similar in conformation and behaviour to saturated fatty acids. TFAs are found naturally in some meat and dairy products. They are also formed during the partial hydrogenation of liquid oils, forming semi-solid fats like margarine and shortening (Figure 12). The majority of

trans fats in a typical Canadian diet come from margarines, commercially fried foods, and bakery products.

Figure 12. Partially hydrogenated vegetable shortening

© 1996-2006 Lori Alden. The Cook's Thesaurus. Image used with Permission from Lori Alden on August 10, 2009 [169]

TFAs, especially those from partially hydrogenated sources, have been demonstrated to have harmful effects on CVD, circulating lipids, inflammation, endothelial function, and oxidative stress [18, 24, 170, 171]. As a result of the multiple adverse effects reported due to manufactured TFA intake, numerous agencies and governing bodies recommend limiting TFAs in the diet and reducing TFAs in the food supply [27, 172]. The *trans* fat content of foods must now be listed on nutrition information labels (Figure 13) [26, 27]. *Trans* fats have also been banned for use in restaurants in several American cities (New York, Philadelphia, Stamford, CT, Montgomery County, MD), and in the state of California [173]. However, these conclusions have been largely derived from investigations lacking definitive mechanistic evidence in support of a role for TFAs in atherosclerotic disease. An in depth study to identify the direct mechanism(s) responsible for the detrimental effects of TFAs on

cardiovascular health is warranted. It also remains unclear how specific TFA isomers from different sources vary in their biological activity and mechanism of action.

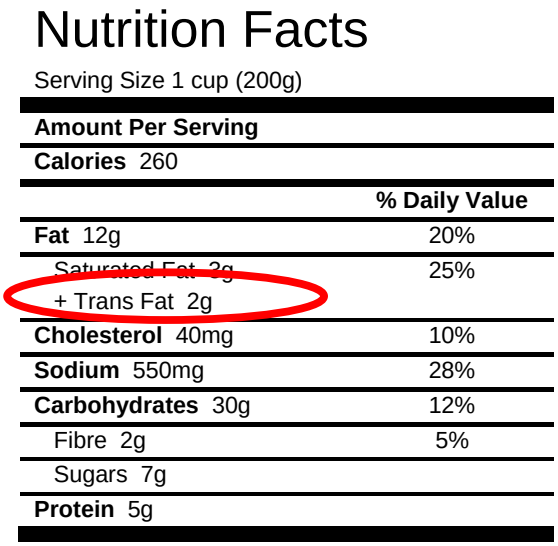


Figure 13. Reporting of *trans* fat content on nutrition information labels

Commercially hydrogenated versus naturally occurring TFAs

Evidence suggests that the source and configuration of TFAs could have a strong impact on the role of TFAs in cardiovascular health, as TFA isomers elicit differential biological effects, both favorable and adverse [172].

Commercially produced TFAs: Elaidic acid (EA)

Elaidic acid (C18:1 *trans*-9) is the main TFA isomer of oleic acid formed during the commercial hydrogenation of vegetable oils, which contain 10-40% TFAs. Industrially produced TFAs are often abbreviated as iTFA. Elaidic acid is, therefore, elevated in products containing partially hydrogenated margarines or vegetable shortening, such as fried foods, cookies, donuts and crackers. Of noteworthy concern is

that elaidic acid has been found in atherosclerotic lesions and adipose tissue of obese and cardiac patients [174-176].

Naturally occurring TFAs: Conjugated linoleic acid (CLA) and Vaccenic acid (VA)

TFAs also occur naturally in low levels in ruminant meat and dairy products. Vaccenic acid (C18:1 *trans*-11), also a *trans* isomer of oleic acid, is naturally produced during bacterial fermentation in ruminant animals (dairy cows and sheep), which results in the biohydrogenation of dietary unsaturated fatty acids. Vaccenic acid is the main ruminant TFA and is present in milk, yoghurt, cheese, butter and meats at low concentrations (~3-8% by weight) [172]. Vaccenic acid can be bioconverted to rumenic acid, an isomer of the cardioprotective TFA conjugated linoleic acid (CLA), in mice and humans [177, 178]. CLA comprises of a group of *trans* isomers of linoleic acid. The two predominant isomers are *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA. CLA has been demonstrated to have anti-obesity, anti-atherogenic, anti-oxidant, and anti-carcinogenic effects, however the data remains controversial [179].

TFAs & cardiovascular disease

Several epidemiological and interventional investigations have consistently shown a significant positive association between the intake of TFA and the risk for coronary heart disease (CHD) [12-23]. Three of these studies further suggest that this direct association was primarily accounted for by industrially produced hydrogenated TFAs (iTFAs) [19, 20, 23]. Recent research suggests that there is a stronger association between CHD risk and TFA intake than with the consumption of SFAs [180]. The Seven Countries Study, the Nurses' Health Study (NHS), and the Zutphen Elderly Study all

report a strong association between TFA intake and CHD risk and long term CHD mortality rates [19, 181, 182]. Women consuming the highest level of TFAs in the NHS had an 80% higher risk for infarction or CHD death [19]. A summary analysis of prospective studies on TFA intake and CHD risk, published by Oomen and colleagues in 2001, estimates a relative risk ratio of 1.25. They showed that there is a 25% increase in CHD risk for every 2% increase in energy from TFAs [183]. As previously mentioned, TFA intake is estimated to be approximately 2.6% of energy in North America [26]. Thus, if we were to remove this 2% of energy from TFAs from the diet, the risk would go down by 1.0, which would be associated with a 20% decrease in the incidence of CHD [183].

TFA intake has been associated with atherogenic risk factors, such as elevated cholesterol and triglyceride levels and plasma markers of inflammation and endothelial dysfunction [12-18, 184]. The relationship of TFAs with atherosclerosis has been determined largely through indirect evidence. For example, several studies have found TFAs present in atherosclerotic lesions and adipose tissue of obese, diabetic, and cardiac patients [174-176]. The TFA content in adipose tissue is now considered an important biomarker measure for the long-term intake of TFAs [183]. Many observational studies report that patients consuming TFAs become dyslipidemic and have elevated plasma markers of inflammation and endothelial dysfunction [13-18, 24].

Effects on circulating lipids

Concern regarding the impact of consuming iTFAs on circulating lipid levels dates back to the 1950s. The majority of the studies reported that TFAs from partially

hydrogenated sources elevated cholesterol levels as compared to natural oils, but that the rise in total cholesterol was in general lower than that caused by consuming SFAs [185]. Concern grew over time as studies distinguished among lipoprotein cholesterol classes. In the 1990s, it was observed that both elaidic acid and SFAs raised total and LDL cholesterol levels, but only iTFAs lowered HDL cholesterol levels [186]. Subsequent research has confirmed the hypercholesterolemic effect of hydrogenated TFAs and its contribution to the atherogenic effects of TFAs [171, 185, 187-191]. Lichtenstein and colleagues demonstrated that this rise in LDL-C and drop in HDL-C levels occurs in a dose dependent manner with TFA intake [191]. iTFAs have also been reported to raise triglyceride and Lp(a) levels and decrease LDL particle size as compared to SFAs [171, 192, 193]. TFAs are believed to decrease HDL levels via an inhibition of lecithin:cholesterol acyltransferase (LCAT) activity and by increasing the activity of cholesteryl ester transfer protein [194, 195]. Only a few studies have reported that moderate levels of TFAs have neutral effects on lipid profiles [188, 189, 196].

Effects on atherosclerosis – reports from experimental animal studies

It is important to identify a mechanism to explain the deleterious effects of TFAs on cardiovascular disease. Many of the studies cited above have suggested that TFAs may contribute to cardiovascular disease through a stimulation of atherosclerosis. There are few published reports that have directly investigated the effects of *trans* fats on atherosclerotic development. Surprisingly, only a few studies have reported that *trans* fats stimulate atherogenesis. Rudel and colleagues report that $LDLr^{-/-}$/ApoB100 overexpressing mice fed a 4% *trans* fat blend in the absence of dietary cholesterol following 16 weeks had elevated LDL-cholesterol, VLDL, triglycerides, and

atherosclerotic development [197]. However, the atherosclerosis was measured as an increase in aortic cholesterol ester concentration in the vessel wall and did not present a direct measurement of atherogenic development. An increase in atherosclerosis was also reported in two older studies in swine and rabbits fed high doses of *trans* fat [198, 199]. More recent studies have shown no effect. In three independent studies, Kritchevsky and colleagues refuted an earlier study from their own lab [199] and reported no stimulatory effect of a high TFA diet on atherosclerosis in rabbits, hamsters, and vervet monkeys [200-202]. In fact, the study conducted in hamsters consuming a diet with 0.12% cholesterol and 8.8% *trans*-C18:1 found that the aortic fatty streak area was lowest in animals consuming the *trans* fat diet [202]. Cassagno and colleagues also found that partially hydrogenated rapeseed oil, providing 3% of energy as elaidic acid had no effect on circulating cholesterol levels or atherosclerosis, however an increase in triglycerides, apoptosis, and oxidative stress was reported [170].

It is important to resolve this controversy and explain the opposing findings if we are to fully understand the contribution of *trans* fats to CHD. It is possible that TFAs may influence cardiovascular health in a manner independent of atherogenesis or, alternatively, it is possible that some aspects of the studies conducted by Kritchevsky and others were sub-optimal to detect an effect of the TFA diets. The low density lipoprotein receptor deficient ($LDLr^{-/-}$) mouse may be a better animal model in which TFAs can be studied because it is an excellent representation of human atherosclerotic disease [54, 203]. It is also possible that co-administration of TFAs with dietary cholesterol may mask the atherogenic effects of TFAs.

Cellular effects of trans fatty acids

There is evidence that TFAs may contribute to CVD via cellular mechanisms that could stimulate atherosclerosis. TFAs have been observed to increase levels of tumor necrosis factor (TNF) activity, TNF receptors, and circulating inflammatory markers (IL-6, CRP) in both healthy individuals and patients with established heart disease [16, 18, 204]. TFAs have also been reported to have detrimental effects on endothelial function. TFA intake is associated with increased levels of soluble adhesion molecules (sICAM-1, sVCAM-1), and E-selectin, all markers of endothelial dysfunction [18]. TFAs decreased brachial artery flow-mediated dilation (FMD) by 29% in healthy individuals [18, 205]. FMD is a direct measure of vascular endothelial function and a marker for CVD risk.

The specific mechanism of action for the detrimental effects of TFAs on CVD remains unknown. The changes in circulating lipids, inflammation, and endothelial function are likely due to the incorporation of TFAs into cellular and nuclear membranes, thus resulting in changes in signaling pathways. TFAs have been demonstrated *in vitro* to be incorporated into the phospholipid composition of cell membranes, resulting in altered cell membrane permeability, eicosanoid production and gene expression, and lipoprotein catabolism [184, 206-210]. TFAs are readily incorporated into all lipid classes in tissues, however the pro-inflammatory effects of TFAs may be mainly mediated by responses in adipose tissue. TFA intake has been demonstrated to alter adipocyte gene expression of peroxisome proliferator-activated receptor (PPAR-γ), resistin, and lipoprotein lipase [208]. TFAs may also regulate genes involved in lipid and non-lipid related cardiovascular risk factors by functioning as ligands for nuclear receptors, such as PPARs, liver X receptor (LXR), and sterol regulatory element binding protein-1

(SREBP-1) [211]. The extent to which the effects of TFAs on circulating lipids, systemic inflammation and endothelial function described above, mediate increased risk of cardiovascular outcomes is not known [211]. However, elevated blood lipids, inflammation and endothelial dysfunction are all independent risk factors for CVD, suggesting that these lipid-mediated and cellular effects may account for the harmful effects on cardiovascular health with which TFAs are associated.

Isomer-specific effects: Intake of ruminant trans fatty acids and risk of coronary heart disease

Epidemiological studies have suggested that TFA intake from industrially produced hydrogenated vegetable oils (iTFA), as found in shortening and margarine, but not those from ruminant fats (rTFA), present in meat and butter, are associated with an increased risk of CVD [19, 20, 183, 212]. The few studies that have investigated the association between intake of rTFA and CHD risk have indicated that low to moderate consumption of rTFA is innocuous or even protective against CHD [19, 20, 213-215]. Willett and colleagues report that the relative risk of CHD for the highest versus the lowest quintile was 0.59 and Pietinen and colleagues report a relative risk of 0.83 [19, 214]. For rTFA, the quintiles of intake range from 0.5 to 2.5g/day, whereas typically the quintiles for iTFA range from 0.1 and 5.1g/day [216]. Motard-Belanger and colleagues recently reported that while moderate amounts of rTFA have neutral effects on plasma lipids and other cardiovascular disease risk factors, higher intakes of iTFA and rTFA both raise cholesterol levels, however only iTFAs raised LDL cholesterol and reduced HDL levels in healthy women [213]. The effects of rTFAs on HDL levels remain unclear. Chardigny and colleagues report that the HDL-cholesterol lowering effect of TFAs seems

to be specific to industrial sources [217]. Conversely, Tholstrup et al. report that a vaccenic acid rich butter reduced both LDL and HDL cholesterol levels in healthy adults, as compared to a regular butter rich in SFAs [218]. The vaccenic acid rich butter also had no appreciable effect on plasma CRP, oxidative stress, insulin, or hemostatic variables [217, 218]. However, the results may be confounded as the VA-rich butter contained higher MUFA and lower SFA levels than the regular butter. Clearly more research is needed to determine the effects of individual isomers on clinical endpoints [172].

No substantial hypotheses have been suggested in the literature regarding the intake of rTFA and its effects on CHD risk factors [216]. There is no clear biological explanation for the variable effects of TFA isomers on lipid and lipoprotein profiles observed across studies. The two oleic acid isomers vary only in the position of the monounsaturated *trans* double bond; elaidic acid at carbon 9 and vaccenic acid at carbon 11. There is a need for more experimental studies investigating the differential effects of TFA isomers on CVD risk factors. One study reports that hamsters do not show differential effects between EA and VA on circulating lipids to substantiate the epidemiological observations that EA is more detrimental to cardiovascular health than VA [219]. The response may lie in the gut. Adipose tissue TFA levels correlate more strongly with iTFA from vegetable sources than with rTFA from animal sources [220, 221]. This stronger correlation may be attributable to differences in the metabolism of the *trans* isomers [221]. EA, but not VA, has been shown to increase TG secretion and incorporation into chylomicrons in *in vitro* experiments in intestinal cells [222]. Further studies in this regard are needed.

CHAPTER II: HYPOTHESES

1. We hypothesize that dietary flaxseed will limit atherosclerotic development and protect against cholesterol-induced vascular contractile dysfunction by lowering plasma cholesterol levels and indices of inflammation.

2. We hypothesize that adding iTFAs in the diet will promote hyperlipidemia, inflammation and atherogenesis in mice when administered in the absence or presence of a dietary cholesterol.

3. We hypothesize that adding rTFAs in the diet will not promote hyperlipidemia, inflammation and atherogenesis in mice.

4. We hypothesize that adding flaxseed in combination with iTFAs in the atherogenic diet will protect against the harmful effects of iTFAs.

CHAPTER III: OBJECTIVES

1. To determine if dietary flaxseed will induce anti-atherogenic effects in two models of atherosclerosis, the cholesterol-fed New Zealand White (NZW) rabbit and the low-density lipoprotein receptor deficient ($LDLr^{-/-}$) mouse.
2. To identify the mechanisms by which dietary flaxseed exerts its antiatherogenic potential.
3. To determine the effects of flaxseed supplementation on vascular contractile function.
4. To determine the effects of flaxseed supplementation on the expression of markers related to inflammation, cell proliferation and obesity-associated co-morbidity.
5. To determine if TFAs will exert detrimental effects on the development of atherosclerosis.
6. To identify the mechanism(s) responsible for atherogenic effects of TFAs.
7. To determine if additional risk factors for cardiovascular disease augment the detrimental effects of TFAs.
8. To determine if TFAs from commercial and natural sources exert the same atherogenic effects.
9. To determine if dietary supplementation with flaxseed can counteract the detrimental cardiovascular effects of TFAs.

CHAPTER IV: MATERIALS AND METHODS

Materials and methods common to all studies

Food intake

Four grams of the experimental diets was provided per day for each mouse in the $LDLr^{-/-}$ mice studies. Since the mice were usually housed five to a cage, 20g of the diets was provided to the mice daily. The amount of diet provided was reduced according to the number of mice in the cage. The following day, before new food was added to the cage, any remaining chow was collected. The amount remaining was weighed and daily food intake was calculated by subtracting this value from the amount of diet provided the previous day. Average food intake was determined by calculating the mean +/- the standard error over the course of the study.

Weight gain

Weight gain for all experimental animal models was determined by calculating terminal fasted body weight (g) – initial fasted body weight (g) / initial fasted body weight (g) x 100. Average weight gain for an experimental group was determined by calculating the mean +/- standard error for all of the animals included in the group.

Nutritional composition of the experimental diets

The nutritional composition of the experimental diets was analyzed by Norwest Laboratories in Lethbridge, AB, or by Industrial Laboratories of Canada in Mississauga, ON, for proximate analysis of crude protein, carbohydrate, fat, fiber, ash and digestible energy.

Blood & tissue collection

Plasma and/or serum was collected at the end of the experimental feeding period and stored at -80^{o}C until analyzed for fatty acid, triglyceride and cholesterol content. Serum was obtained by centrifuging the blood samples at 6,800 x g for 10 minutes at 4^{o}C. Plasma was obtained by collecting the blood samples in syringes containing EDTA, followed by centrifugation at 14,000 x g for 10 minutes at 4^{o}C.

Circulating cholesterol and triglyceride levels

Serum cholesterol and triglyceride levels were quantified using commercial enzymatic kits according to the manufacturer's protocols (Thermo Electron Corporation, Walthan, MA, USA). Serum cholesterol and triglyceride levels are reported as mg/dL. To convert the values reported into International Units (mmol/L), divide the cholesterol values by 38.67 and divide the triglyceride values by 88.97.

Fatty acid profile of the experimental diets

Lipids were extracted from the diets and aortic tissue and derivatized using the method of Folch *et al.* [223]. One gram of diet or aortic tissue was minced and homogenized by Polytron on ice in 20 ml of chloroform-methanol (2:1). The homogenate was combined with 4.2 ml 0.73% NaCl in a separatory funnel, mixed vigorously and allowed to separate overnight. The chloroform layer was then dried with Na_2SO_4, filtered and evaporated by rotary evaporation. The lipids were then resuspended and diluted to 1.0 mg/ml in chloroform. Nitrogen gas was used to evaporate 100 µl of this extract in a test tube to which 1.0 ml 7% boron trifluoride-methanol was added. The tube was sealed with a Teflon cap and heated to 90^{o}C for one hour [224]. The tube and contents were cooled to room temperature and the methylation was terminated by the addition of 1.0 ml

H_2O. The fatty acid methyl esters were then extracted into hexane, washed with water, dried with Na_2SO_4, filtered, concentrated under nitrogen gas and finally resuspended in hexane.

Extraction and derivitization of fatty acids from plasma

Total fatty acids were extracted from the plasma samples and derivatized using the method of Lepage and Roy [225]. Briefly, 100 µl of plasma was added to 2 ml of methanol-benzene (4:1) in a test tube. While vortexing, 200 µl of acetyl chloride was added to the tube. The tubes were sealed and heated to 90^{o}C for one hour. Five ml 6% K_2CO_3 was then added to neutralize the solution and the upper benzene layer was removed for analysis.

Gas chromatographic separation of fatty acid methyl esters

A Varian CP-3800 gas chromatograph (Mississauga, ON, Canada) equipped with a flame ionization detector and Varian CP-Sil 88 capillary column (50 m´0.25 mm´0.20 mm) was used to analyze 0.5 µL of each extract, which were injected with a CP-8400 autosampler at a split ratio of 1:100. Flow rate of the helium carrier gas was 1 ml/min. The initial oven temperature was held at 80^{o}C for 1 min, raised to 140^{o}C at 30^{o}C/min, and then raised to 225^{o}C at 5^{o}C/min and held for 10 min. The total run time for each sample was 30 min. Components were identified by comparison with authentic standards (Nu-Chek Prep, Elysian, MN, USA).

Fatty acid analysis methods specific to the trans fat studies

Lipids were extracted from plasma (100 µL) and a 1 g sample of ground diet using chloroform:methanol (2 :1, v/v) [223]. Conversion of the fatty acids to their methyl

esters was accomplished using a modification of the method by Park and Goins [226]. Briefly, the extracted lipids, (stored in 100 μL of dichloromethane containing the internal standard, C11 :0), were first esterified using 1 ml of 0.5N methanolic NaOH at 90°C for 10 minutes under an atmosphere of nitrogen. After a brief cooling period, 1 mL of 14% BF_3-methanol was added and the contents heated as described above. The reaction was terminated upon the addition of 1 ml distilled water and the FAMEs extracted into 300 μL hexane. Completion of the derivitization reaction was verified using TLC (thin-layer chromatography) using conditions detailed by Cruz-Hernandez and colleagues [227]. FAMEs were then analyzed using gas chromatography coupled with flame-ionization detection (GC-FID). The separation methodology is based upon the 150°C GC temperature program detailed by Kramer and colleagues [228]. FAMEs were analyzed against an authentic standard, GLC 469A with the addition of eicosapentaenoic acid (EPA), (Nu-Chek, Prep.).

Assessment of atherosclerotic lesion formation

Aortae were grossly dissected and cleaned of adventitial tissue, followed by a wash in cold phosphate buffered saline (PBS) solution containing (mM): KCl 2.7, KH_2PO_4 1.5, NaCl 136.9, Na_2HPO_4 4.3 adjusted to pH 7.4, before evaluating the tissue for atherosclerotic lesions by *en face* analysis. For *en face* analysis, the aorta from the ascending arch to the iliac bifurcation was opened longitudinally, pinned flat, digitally photographed, and the luminal images were analyzed using the Silicon Graphics Imaging (SGI) software. The lesion area index was calculated as the ratio of areas with lesions versus total luminal surface area x 100 (mean ± standard error). The aortic tissues were subsequently flash frozen and stored at -80°C until further use.

For Oil Red O analysis of lipid deposits in aortic sinus tissue, the heart samples were embedded in tissue freezing medium (O.C.T. compound), frozen at -80^{o}C and sectioned using a cryostat and thaw-mounted onto positive glass slides. The entire aortic root (400-µm) was sectioned into consecutive 6- to 8-µm thick sections. The distal end of the aortic sinus was recognized by the disappearance of the three aortic valve cusps as previously described [229, 230]. Every sixth section was stained with Oil Red O and hematoxylin, digitally photographed under 40x magnification and evaluated for atherosclerotic lesions using a morphometric imaging system. The measurements were expressed in arbitrary units (pixels). The lesion area index was calculated as the percentage of aortic lumen area covered by atherosclerotic lesions (mean ± standard error).

Statistical analyses

Statistical analysis of data was performed using SigmaStat software (version 2.03, SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard error (SEM). Statistical comparisons were made using one-way analysis of variance (ANOVA), followed by the Fisher's least significant difference *post hoc* test for multiple parametric comparisons using the Sigma Stat software. Differences between means were considered significant when $P < 0.05$.

A. Effects of dietary flaxseed on atherosclerotic development and endothelial function in hypercholesterolemic rabbits

Animals & dietary interventions

Ninety-six male albino New Zealand White (NZW) rabbits (Southern Rose Rabbitry Farm, St. Claude, Canada), weighing 2.5-3 kg upon arrival, were individually housed in metal cages in a room with controlled temperature, humidity, and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed [231]. Animals were randomly assigned to 4 groups of 8 animals per feeding duration based on dietary treatment. Animals were fed for 6, 8, or 16 weeks. The four diets included a regular control diet (**RG**) of regular rabbit chow (CO-OP Complete Rabbit Ration, Federal Co-operatives Limited, Saskatoon, Canada), a 10% ground flaxseed supplemented chow (**FX**), a 0.5% cholesterol supplemented chow (**CH**), or a diet supplemented with 0.5% cholesterol and 10% ground flaxseed (**CF**). The Promega flaxseed, provided from Polar Foods Inc. in Fisher Branch, Canada, contained 71% alpha-linolenic acid (ALA). All diets were dry mixed and repelleted to incorporate the added components. Rabbits were fed 125 grams of the appropriate dietary treatment per day. Water was given *ad libitum*.

Blood sampling

Blood samples were taken at baseline (0 weeks) and following 6, 8 and 16 weeks of dietary intervention from the marginal ear vein prior to daily feeding.

Preparation of tissues

Following 6, 8 or 16 weeks of dietary treatment, rabbits were anesthetized with isofluorane (5%, in oxygen, 2L/min) and heparinized. The aorta and carotid arteries were excised and immediately placed in cold Krebs Henseleit 1.9 mM calcium solution (115 mM NaCl, 25mM $NaHCO_3$, 1.38 mM KH_2PO_4, 2.5 mM KCl, 2.46 mM $MgSO_4$, 1.9 mM $CaCl_2$, 11.2 mM dextrose, pH 7.4). The aorta was carefully dissected from the distal end of the aortic arch to the base of the diaphragm. The aorta and carotids were cleaned of adventitial tissue and prepared for vascular function testing, gas chromatography (GC), sectioning or en face analysis.

Assessment of atherosclerotic lesion formation

Atherosclerotic lesions along the distal aorta and carotid artery were evaluated *en face* and by cross-sectional analysis. For *en face* analysis, the aorta and carotid arteries were prepared and analyzed as described above.

Aortic tissue, fixed in 4% buffered paraformaldehyde and rinsed with 30% sucrose solution buffered in 1x PBS, was embedded in tissue freezing medium (O.C.T. compound), frozen at -20^{o}C, cut into 10 μm thick sections using a cryostat, and thaw-mounted onto positive glass slides. Sections were stained with Oil Red O and counterstained with hematoxylin as previously described [232]. Following en face atherosclerosis analysis, the aortae were stored at –80^{o}C, thawed, and homogenized in preparation for chloroform:methanol lipid extraction as described above.

Experimental protocol for assessing vascular response

Aortic tissue, dissected into 3 mm width rings from the distal end of the aortic arch, were fastened in an organ bath with surgical wire, perfused with the Krebs Henseleit solution, aerated with 95% O_2 and 5% CO_2, and equilibrated at 37^{o}C and pH 7.4. Vascular function was measured with a force transducer as mechanograms of tension (tension (g)/tissue wet weight (g)). The aortic rings were brought to a basal tension of 5.5-6.5 grams of tension and then contracted three times with 47 mM KCl with wash-out periods using Krebs solution between each contraction. Tissues were allowed to return to baseline tension during wash-outs. A dose response curve to norepinephrine (NE) was constructed with concentrations of 10^{-9} M to 10^{-4} M. After the final dose of NE, the tissues were washed-out with 37^{o}C Krebs Henseleit solution and allowed to return to baseline tension. To test the ability of the tissue to relax after pre-contraction with NE, a second dose of 10^{-6} M NE was administered to the bath and the tissues were allowed to reach a steady state of contraction. Acetylcholine (ACh) was then administered without wash-out at concentrations of 10^{-8} M to 10^{-5} M to develop a relaxation response curve to ACh. Following wash-out and a third dose of 10^{-6} M NE, sodium nitroprusside (SNP) was administered in selected experiments at concentrations of 10^{-8} M to 10^{-5} M to generate a relaxation response curve.

B. Effects of flaxseed on atherosclerotic development in the LDLr$^{-/-}$ mouse

Animals and Dietary Interventions

One hundred five (105) female C57BL/6J LDL receptor deficient mice (Jackson Laboratory, Bar Harbour, USA), 5 to 7 weeks old, were randomly assigned, following a 1

week acclimatization period, to 7 dietary treatment groups of 15 animals. The 7 diets included a regular RMH 3000 rodent chow (TestDiet, Richmond, IN, USA) diet (**RG**), a 10% ground flaxseed (Promega Flax, Polar Foods Inc., Fisher Branch, Manitoba, Canada) supplemented chow diet (**FX**), or an atherogenic chow diet supplemented with 2% cholesterol, alone (**CH**), or supplemented with 10% ground flaxseed (**CF**), 5% ground flaxseed (**CF5**), 1% ground flaxseed (**CF1**), or 5% coconut oil, a rich source of saturated fatty acids (**CS**). The Promega flaxseed, provided from Polar Foods Inc. in Fisher Branch, Canada, contained 71% alpha-linolenic acid (ALA). Mice were given 4 grams of one of the seven diets daily. Water was provided *ad libitum*. The mice were housed in plastic cages (maximum 5 animals per cage) in a room with controlled temperature, humidity, and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed [231].

Western Blot Analysis

The expression levels of proliferating cell nuclear antigen (PCNA), the macrophage marker M3/84 (mac-3), interleukin-6 (IL-6), vascular cell adhesion molecule-1 (VCAM-1), and peroxisome proliferative activated receptor gamma (PPAR-γ) were measured by Western immunoblotting techniques. Frozen aortae were homogenized using a mortar and pestle and liquid nitrogen. The homogenates were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and 1mM EGTA, pH 7.5, with 1 mM PMSF, 1 mM benzamidine, and a protease inhibitor cocktail) and centrifuged at 14,000 x g for 15 minutes at 4^{o}C to remove cellular debris. Aliquots of lysates were analyzed by SDS-

PAGE electrophoresis and proteins were transferred onto nitrocellulose membranes using either a wet or semi-dry transfer protocol. The membranes were then blocked and probed with the following primary antibodies: anti-PCNA (1:2,000 dilution; 13-3900, Zymed Laboratories), anti-mac-3 M3/84 (1:200 dilution; Sc-19991, Santa Cruz Biotechnology), anti-IL-6 (1:500 dilution; MAB406, R&D Systems), anti-VCAM-1 (1:500 dilution; sc-1504, Santa Cruz Biotechnology), and PPAR-γ (1:500 dilution; sc-7196, Santa Cruz Biotechnology). The membranes were incubated with an appropriate horseradish peroxidase (HRP) conjugated secondary antibody, the signal was developed using West Pico chemiluminescence substrate (Pierce) or Femto substrate (Pierce) and measured quantified by densitometry analysis using Quantity One software on a Bio-Rad GS-670 FluorSMax Imaging Densitometer. Equal protein loading and transfer were verified by Coomassie Blue and Ponceau S staining. Protein levels were normalized against total actin (Sigma) expression and are represented as a percent of the control (RG) group (arbitrary unit).

Immunohistochemistry

Aortic arch cross-sections were immunostained with antibodies against mac-3 M3/84 (1:50 dilution), IL-6 (1:50 dilution), and PCNA (1:50 dilution). After washing, sections were then incubated with anti-rat (Sigma) and anti-mouse (Chemicon) HRP-conjugated secondary antibodies at 1:200 dilutions. Immunocomplexes containing mac-3, IL-6, and PCNA antibodies were detected using diaminobenzidine tetrahydrochloride dihydrate substrate (DAB; Sigma). A brown-to-black precipitate was indicative of the presence of mac-3, IL-6 or PCNA. Negative controls were performed in the absence of both primary and secondary antibodies as well as the DAB substrate. Adventitial tissue

surrounding the exterior of aortic sections was also detected by DAB staining. Sections were mounted in Permount and digitally photographed using a Nikon microscope under 20x magnification.

C. Effects of *trans* fatty acids on atherosclerotic development in the LDLr$^{-/-}$ mouse

Animals and Dietary Interventions

Thirty-five (35) female C57BL/6J LDL receptor deficient mice (Jackson Laboratory, Bar Harbour, USA), 5 to 7 weeks old, were randomly assigned, following a 1 week acclimatization period, to 7 dietary treatment groups of 5 animals. The seven experimental diets consisted of a base of a delipidated RMH3000 diet from TestDiet (Richmond, IN, USA) with the fat content replaced by one of two fat sources at two concentrations: 4% or 8% regular fat (porcine/soy) and 4% or 8% manufactured partially hydrogenated vegetable shortening (*trans* fat). The 4% partially hydrogenated vegetable shortening provided 1.4% TFA, mainly in the form of elaidic TFA, to the diets. The 8% partially hydrogenated vegetable shortening provided 2.8% TFA, mainly in the form of elaidic TFA, to the diets. The seven experimental diets are as follows: 1) low (4%) regular fat chow (**LR**), 2) low (4%) *trans* fat chow (**LT**), 3) high (8%) regular fat chow (**HR**), 4) high (8%) *trans* fat chow (**HT**), 5) 2% cholesterol and low (4%) regular fat chow (**C+LR**), 6) 2% cholesterol and low (4%) *trans* fat chow (**C+LT**), and 7) 2% cholesterol and high (8%) *trans* fat chow (**C+HT**). Mice were given 4 grams of one of the seven diets daily. Water was provided *ad libitum*. The mice were housed in plastic cages (maximum 5 animals per cage) in a room with controlled temperature, humidity,

and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed [231].

D. Effects of *trans* fatty acids from commercial and natural sources on atherosclerotic development in the LDLr-/- mouse

Animals & dietary interventions

Forty (40) female C57BL/6J LDL receptor deficient mice (Jackson Laboratory, Bar Harbour, USA), 5 to 7 weeks old, were randomly assigned, following a 1 week acclimatization period, to 8 dietary treatment groups of 5 animals. The eight experimental diets consisted of a base of a delipidated RMH3000 diet from TestDiet (Richmond, IN, USA) with the fat content replaced by one of four fat sources: 4% regular fat (porcine/soy) (RG), 4% manufactured partially hydrogenated vegetable shortening providing 1.5% TFA, mainly in the form of elaidic TFA (ES), 15% regular butter providing 0.3% naturally present TFA (RB), and 15% butter providing 1.5% naturally occurring TFA, mainly in the form of vaccenic TFA (VB). The eight diets contained one of the four fat sources with or without added dietary cholesterol: 1) regular fat (**RG**), 2) elaidic shortening (**ES**), 3) regular butter (**RB**), 4) vaccenic butter (**VB**), or atherogenic diets supplemented with 2% cholesterol with 5) regular fat (**CH**), 6) elaidic shortening (**CH+ES**), 7) regular butter (**CH+RB**), or 8) vaccenic butter (**CH+VB**). Mice were provided with 4 grams of one of the eight diets daily (20 g/d in a cage with 5 mice). Water was provided *ad libitum*. The mice were housed in plastic cages (maximum 5 animals per cage) in a room with controlled temperature, humidity, and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed [231].

Butter Production

The low TFA control butter was obtained from the Canadian Dairy Commission (Québec). The TFA enriched milk was produced by modification of the cows' diet. Cows (n=28) were fed a mixed diet composed of concentrates and grass silage with 4% safflower oil. Milk samples were obtained after 4 weeks and the *trans*-C18:1 content of milk fat was determined by gas chromatography with flame ionization detection (GC-FID). Cows with the greatest concentration of *trans*-C18:1 (n=7) were kept on this diet for the purpose of milk collection and the manufacturing of high-TFA butter. Raw milk was first separated into skim milk and cream using a cream separator. Cream was then standardized to 39% fat, immediately pasteurized at 78°C for 16 sec using a heat exchanger, then churned at 4°C to obtain butter.

E. Effects of combining dietary flaxseed and *trans* fatty acids on atherosclerotic development in the LDLr-/- mouse

Animals and Dietary Interventions

One hundred forty (140) female C57BL/6J LDL receptor deficient mice (Jackson Laboratory, Bar Harbour, USA), 5 to 7 weeks old, were randomly assigned, following a 1 week acclimatization period, to 14 dietary treatment groups of 10 animals. The fourteen experimental diets consisted of a base of a delipidated RMH3000 diet from TestDiet (Richmond, IN, USA) with the fat content replaced by one of two fat sources at two concentrations: 4% or 8% regular fat (porcine/soy) and 4% or 8% manufactured partially hydrogenated vegetable shortening (*trans* fat). The 4% partially hydrogenated vegetable shortening provided 1.4% TFA, mainly in the form of elaidic TFA, to the diets. The 8% partially hydrogenated vegetable shortening provided 2.8% TFA, mainly in the form of

elaidic TFA, to the diets. The diets were supplemented with either 2% dietary cholesterol (C), whole ground flaxseed (F), or one of the components of flaxseed; alpha-linolenic acid (ALA), dietary fibre (partially defatted flaxseed meal; P), or the lignan secoisolarecinol diglucoside (L). The fourteen experimental diets are as follows: 1) low (4%) regular fat chow (**LR**), 2) low (4%) *trans* fat chow (**LT**), 3) low (4%) *trans* fat + 10% flaxseed chow (**TF**), 4) low (4%) *trans* fat + 4.4% ALA chow (**TALA**), 5) high (8%) regular fat chow (**HR**), 6) high (8%) *trans* fat chow (**HT**), 7) high (8%) *trans* fat + 10% flaxseed chow (**HTF**), 8) high (8%) *trans* fat + 4.4% ALA chow (**HTALA**), 9) 2% cholesterol + low (4%) regular fat chow (**CR**), 10) 2% cholesterol + low (4%) *trans* fat chow (**CT**), 11) 2% cholesterol + low (4%) *trans* fat + 10% flaxseed chow (**CTF**), 12) 2% cholesterol + low (4%) *trans* fat + 4.4% ALA chow (**CTALA**), 13) 2% cholesterol + low (4%) *trans* fat + 7% partially defatted flaxseed meal chow (**CTP**), and 14) 2% cholesterol + low (4%) *trans* fat + 0.44% lignan chow (**CTL**). Mice were given 4 grams of one of the fourteen diets daily. Water was provided *ad libitum*. The mice were housed in plastic cages (maximum 5 animals per cage) in a room with controlled temperature, humidity, and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed [231].

CHAPTER V: RESULTS

A. Effects of dietary flaxseed on atherosclerotic development and endothelial function in hypercholesterolemic rabbits

Experimental diets & animal weights

Animals in all four treatment groups consumed 125g of chow daily. Animal body weights did not differ significantly amongst the four groups prior to feeding (0 weeks) or at the end of the feeding trials (6, 8 or 16 weeks) (data not shown), which suggests that the energy content of the experimental diets did not differ significantly. The data measuring the nutritional composition of the experimental diets supports this contention. The addition of 10% flaxseed notably elevated the total fat and ALA content of the FX and CF diets (Tables 7 and 8). The ratio of ω-6 to ω-3 polyunsaturated fatty acids (PUFA) was ninefold less in the flaxseed-supplemented diets compared with the RG and CH diets (Table 8).

Table 7. Nutritional composition of the experimental diets

TABLE 7

NUTRITIONAL COMPOSITION OF THE EXPERIMENTAL DIETS

Group	Diet description	Protein	Fat	CHO	Fibre	Ash
				g/100g dry diet		
RG	Regular chow	21.3	5.4	51.4	13.5	8.4
FX	10% flaxseed	20.5	8.1	51.7	11.7	8.1
CH	0.5% cholesterol	20.4	5.2	52.5	13.6	8.1
CF	0.5% cholesterol +10% flaxseed	20.5	8.9	50.6	12.4	7.7

Abbreviations: Protein = Crude Protein; Fat = Crude Fat; CHO = Carbohydrates; Fibre = Crude Fibre.

Table 8. Fatty acid composition of the experimental diets

TABLE 8				
FATTY ACID COMPOSITION OF THE EXPERIMENTAL DIETS				
	RG	**FX**	**CH**	**CF**
Fatty acid		*g/100g fatty acids*		
C14:0	0.8	0.6	0.7	0.5
C16:0	18.3	14.2	17.6	13.4
C18:0	6.2	5.9	5.7	5.2
C16:1 (ω-9)	1.1	0.8	1.0	0.6
C18:1 (ω-9)	30.0	25.4	28.6	23.4
C18:1 (ω-7)	4.8	4.1	4.6	3.7
C22:1 (ω-9)	0.1	0.4	0.3	0.1
C18:2 (ω-6)	31.4	17.5	34.3	19.6
C18:3(ω-3)	5.6	29.9	5.9	32.2
(ω-6)/(ω-3)	5.6	0.6	5.8	0.6

Fatty acids not shown represent less than 0.5 g / 100 g fatty acids in the diet.
Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet; ω, omega.

Circulating cholesterol and triglyceride levels

Initial plasma cholesterol and triglyceride levels were not significantly different among the four groups (Fig. 17). After 6, 8, and 16 wk of dietary treatment, animals fed a proatherogenic diet (CH and CF groups) had up to a 15-fold increase in plasma cholesterol levels compared with the RG and FX groups (Figure 14A). There was no significant difference in plasma cholesterol levels between the CH and CF groups at any point in the trials. The addition of 10% flaxseed to the atherogenic diet had no effect on plasma cholesterol levels. Plasma triglyceride levels were also elevated in the CH group at all time points (Figure 14B). The addition of 10% flaxseed to the cholesterol diet significantly attenuated this rise. A general decrease in plasma triglyceride levels was noted in the cholesterol-fed groups with an increasing length of feeding trial.

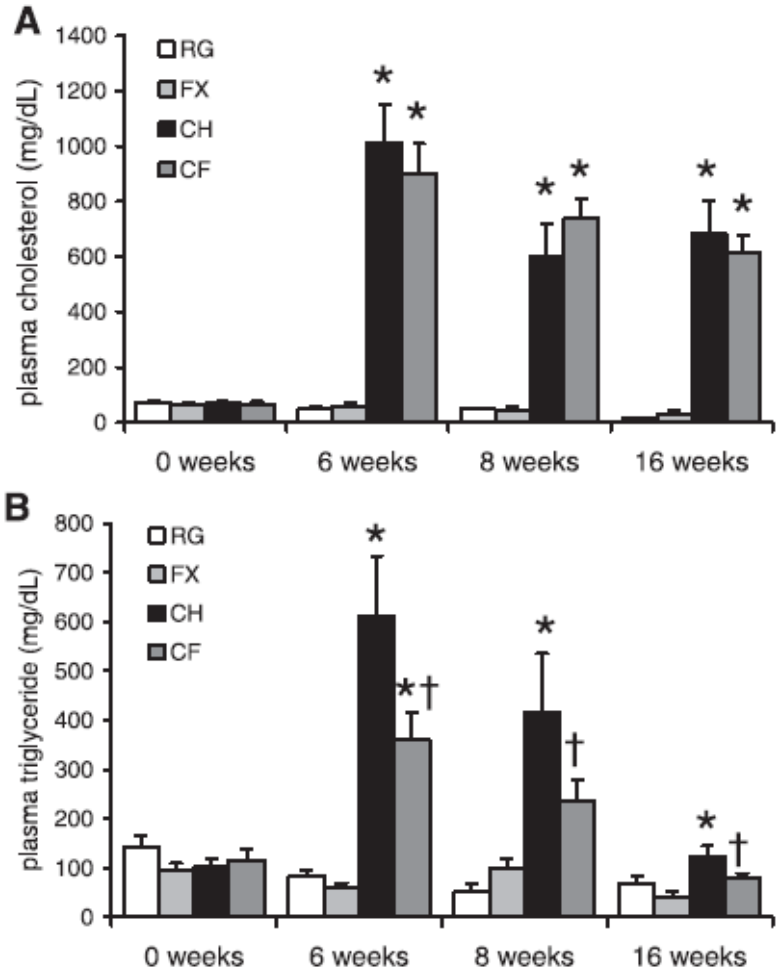


Figure 14. (a) Plasma cholesterol and (b) plasma triglyceride levels in rabbits before (0 weeks) and following 6, 8, or 16 weeks of dietary interventions

Values are means ± SE; n = 4–8. *P < 0.05 vs. RG and FX groups. †P < 0.05 vs. CH group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

Plasma total fatty acid (TFA) levels were measured in all of the groups (Table 9). The total fatty acid content was elevated in the plasma of the cholesterol fed groups (CH and CF) following all end points. Notable differences in plasma fatty acid content are as follows: myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2 ω-6), and arachadonic acid (C20:4

ω-6) levels were all significantly higher in the cholesterol fed groups (CH and CF) versus the RG and FX groups following 6, 8 and 16 weeks, however levels of these fatty acids were not as elevated in the CF group as compared to the CH group at 16 weeks. The addition of 10% flaxseed on its own had no significant effect on plasma alpha-linolenic acid (ALA, C18:3 ω-3) levels, however a small rise was noted in plasma ALA levels in animals fed cholesterol for 16 weeks. The combination of cholesterol and dietary flaxseed resulted in a 60 fold increase in plasma ALA levels as compared to the RG group. The long chain omega-3 fatty acid, eicosapentaenoic acid (EPA; C20:5 ω-3) was only detected in the cholesterol fed groups following 16 weeks, with greater levels observed in the CF group. Docosahexaenoic acid (DHA; C22:6 ω-3) levels in the plasma were only detected in trace amounts. The ratios of ω-6/ω-3 PUFAs were significantly lower in the flaxseed supplemented groups (as much as 91-fold) as compared to the RG and CH groups, with the difference diminishing with the length of feeding trial.

Table 9. Circulating fatty acid levels of rabbits fed experimental diets for 6, 8 and 16 weeks

	6 wk				8 wk				16 wk			
	RG	FX	CH	CF	RG	FX	CH	CF	RG	FX	CH	CF
14:0	t	t	t	t	t	t	0.02±0.00*§	t	t	t	0.09±0.02*	0.04±0.01*†
16:0	0.11±0.01	0.09±0.01	0.71±0.08*	0.76±0.07*	0.15±0.02	0.09±0.02	0.82±0.07*	0.80±0.05*	0.41±0.04	0.26±0.01	4.14±0.24*	3.13±0.40*†
18:0	0.12±0.01	0.11±0.01	0.26±0.03*	0.29±0.05*	0.14±0.01	0.11±0.02	0.28±0.02*	0.34±0.03*	0.17±0.01	0.12±0.02	1.40±0.09*	1.11±0.13*†
16:1	t	t	0.18±0.02*	0.14±0.02*	0.02±0.01	t	0.20±0.02*	0.14±0.01*†	0.03±0.01	t†	0.91±0.11*	0.50±0.06*†
18:1	0.12±0.01	0.09±0.01	1.22±0.12*	1.36±0.15*	0.17±0.02	0.10±0.05	1.43±0.10*	1.43±0.12*	0.64±0.07	0.35±0.05	8.31±0.46*	5.88±0.38*†
18:2(n-6)	0.11±0.01	0.09±0.01	0.62±0.08*	0.75±0.07*	0.13±0.02	0.09±0.02	1.43±0.09*	0.81±0.07*	0.43±0.03	0.27±0.02	4.26±0.32*	2.12±0.30*†
20:3(n-6)	t	t	t	0.02±0.00*	t	t	t	0.02±0.00*	t	t	t	t
20:4(n-6)	0.02±0.00	0.02±0.01	0.03±0.00*	0.03±0.00*	0.02±0.00	t	0.04±0.00*	0.03±0.00*†	0.02±0.00	t	0.26±0.02*	0.16±0.02*†
18:3(n-3)	t	0.04±0.01	0.12±0.02	0.97±0.12*†	t	0.06±0.02	0.17±0.02	1.08±0.14*†	0.04±0.01	0.18±0.02	0.54±0.04*	2.53±0.18*†
20:5(n-3)	t	t	t	t	t	t	t	t	t	t	0.02±0.00	0.04±0.00*†
22:6(n-3)	t	t	t	t	t	t	t	t	t	t	t	t
n-6/n-3	75.9±20.0	2.4±0.3‡	5.4±0.3*	0.83±0.03*†	40.5±10.4	1.7±0.2‡	4.9±0.3*	0.82±0.04*†	12.9±1.7	1.6±0.2‡	8.0±0.3*	1.3±0.1‡†
TFA, mg/ml	0.50±0.02	0.45±0.04	3.2±0.4*	4.4±0.5*	0.64±0.08	0.47±0.05	3.8±0.3*	4.8±0.4*†	1.7±0.1	1.2±0.1	20.0±1.2*	16.6±1.1*†

Values are means ± SE, in mg/ml plasma. Plasma samples were obtained following 16 weeks of feeding (n = 7–8).). *P < 0.05 vs. RG and FX groups; ‡P < 0.05 vs. RG group; †P < 0.05 vs. CH group. §P < 0.05 vs. RG, FX and CF groups. Abbreviations: RG, regular fed; FX, 10% flaxseed fed; CH, 0.5% cholesterol fed; CF, 0.5% cholesterol plus 10% flaxseed fed; t, trace amounts present (<0.01 mg/100 mg fatty acids); n, omega (ω); TFA, total fatty acids (mg fatty acid in 1 ml of plasma).

The dietary interventions also had an effect on the levels of lipid found in the vascular tissue following 8 and 16 weeks of treatment (Table 10). The total fatty acid (TFA) content was elevated in the aortic tissue of the cholesterol fed groups, with the highest aortic TFA levels in the CF group following 16 weeks. Notable differences in the aortic fatty acid content are as follows: levels of the longer-chain fatty acids (C20:1, C20:2 n-6, C20:3 n-6, C22:1, and C24:1) were elevated in the cholesterol-fed groups. Linoleic acid (C18:2 ω-6) levels were elevated in the cholesterol-fed groups at 8 and 16 weeks and were highest in the CF group at 16 weeks. The long-chain PUFA arachidonic acid (C20:4 ω-6) decreased significantly in the FX, CH, and CF groups vs. the RG group at 16 weeks. Aortic ALA (C18:3 ω-3) levels were elevated with flaxseed supplementation as well as in cholesterol-fed animals at 8 weeks; however, by 16 weeks, ALA levels were elevated only in the flaxseed-fed groups, with the highest levels seen in the CF group. The long-chain omega-3 fatty acid EPA was detected only in the aortic tissue of the cholesterol-fed groups at 8 weeks and, at 16 weeks, was only observed in the CF group. DHA levels were also detected in the FX, CH, and CF groups following 8 weeks but only in the CF group following 16 weeks of feeding. At both time points, the highest values were detected in the CF group. The addition of dietary flaxseed diminished the ω-6/ω-3 PUFA ratio in the aortic tissues of the FX and CF groups compared with the RG and CH groups.

Table 10. Fatty acid levels in aortic tissue from rabbits fed experimental diets for 8 and 16 weeks

	8 wk				16 wk			
	RG	FX	CH	CF	RG	FX	CH	CF
14:0	0.14±0.06	0.18±0.03	0.30±0.05	0.26±0.04	0.09±0.03*	0.09±0.04	0.17±0.03	0.27±0.03*†
16:0	2.45±0.70	2.57±0.40	4.76±0.60*	4.50±0.64*	1.91±0.35	2.00±0.43	3.01±0.57	5.26±0.47*†
18:0	1.14±0.18	1.16±0.08	1.76±0.22*	1.70±0.16*	0.17±0.04	0.19±0.06	0.35±0.06	0.68±0.08*†
20:0	t	t	0.07±0.01*	0.06±0.01*	t	t	0.02±0.01	0.05±0.02*
22:0	0.04±0.01	0.04±0.01	0.07±0.00*	0.06±0.00	t	t	t	0.04±0.01*
24:0	t	t	0.03±0.01*	0.03±0.01*	t	t	t	0.03±0.02
16:1	0.27±0.11	0.26±0.06	0.65±0.07*	0.58±0.10*	0.17±0.03	0.19±0.06	0.35±0.06	0.68±0.08*†
18:1	2.49±0.76	2.55±0.26	8.22±1.08*	6.95±1.01*	2.11±0.41	2.24±0.53	4.92±0.86*	10.46±1.03*†
20:1	t	t	0.24±0.04*	0.22±0.05*	t	t	0.13±0.03*	0.24±0.13*†
22:1	t	t	0.04±0.01*	0.02±0.01	t	t	t	0.05±0.02*
24:1	t	t	0.09±0.02*	0.06±0.02*	t	t	t	0.17±0.03*
18:2(n-6)	1.38±0.42	1.44±0.11	3.83±0.56*	3.64±0.54*	1.24±0.28	1.37±0.30	2.56±0.53‡	4.47±0.43*†
20:2(n-6)	t	t	0.27±0.04*	0.21±0.04*	t	t	0.18±0.05*	0.44±0.06*†
20:3(n-6)	t	t	0.23±0.11*	0.28±0.06*	t	t	0.03±0.02	0.78±0.14*
20:4(n-6)	0.90±0.05	0.75±0.03	0.90±0.04	0.86±0.04	1.11±0.07	0.74±0.04‡	0.82±0.07‡	0.90±0.06‡
18:3(n-3)	0.31±0.12	1.27±0.15‡	1.49±0.52‡	2.23±0.35‡	0.25±0.07	1.24±0.30‡†	0.44±0.12	2.79±0.26*†
20:5(n-3)	t	t	0.05±0.03	0.07±0.01*	t	t	t	0.16±0.02*
22:6(n-3)	t	0.03±0.01‡	0.02±0.01	0.06±0.00*†	t	t	t	0.06±0.02*
n-6/n-3	7.42±0.44	1.67±0.12‡	3.34±0.34*	2.11±0.18‡†	9.44±0.30	1.70±0.28‡†	8.28±0.31	1.97±0.11‡†
TFA	9.53±2.45	10.66±1.05	24.27±3.50*	22.78±3.11*	8.14±1.31	8.98±1.86	16.55±2.59*	29.97±2.71*†

Values are means ± SE, in mg/g tissue. Fatty acids were extracted from aortic tissue following 8 or 16 weeks of feeding (n = 4–5). *P < 0.05 vs. RG and FX groups; †P < 0.05 vs. CH group; ‡P < 0.05 vs. RG group. Abbreviations: RG, regular fed; FX, 10% flaxseed fed; CH, 0.5% cholesterol fed; CF, 0.5% cholesterol plus 10% flaxseed fed; t, trace amounts present (<0.01 mg/g of aortic tissue); ω, omega; TFA, total fatty acids (mg fatty acid in 1 g of aortic tissue).

Aortic atherosclerotic development

Aortae were cut longitudinally and the luminal surface was digitally photographed to measure the atherosclerotic lesion area. Plaque formation was not visible in the aortae of the control and flax-fed animals at any time point. However, extensive atherosclerotic plaques were apparent in animals consuming dietary cholesterol (Figure 15). Both the cholesterol and cholesterol-flax fed groups had significantly greater plaque formation than the control and flax fed groups following all trials. There was a statistically significant inhibition of atherosclerotic plaque formation in the cholesterol-flax group compared with the animals fed cholesterol alone at the 6 and 8 week time points. In contrast, the CF group developed more extensive plaque formation than the CH group following 16 weeks of hypercholesterolemic conditions (Figure 15).

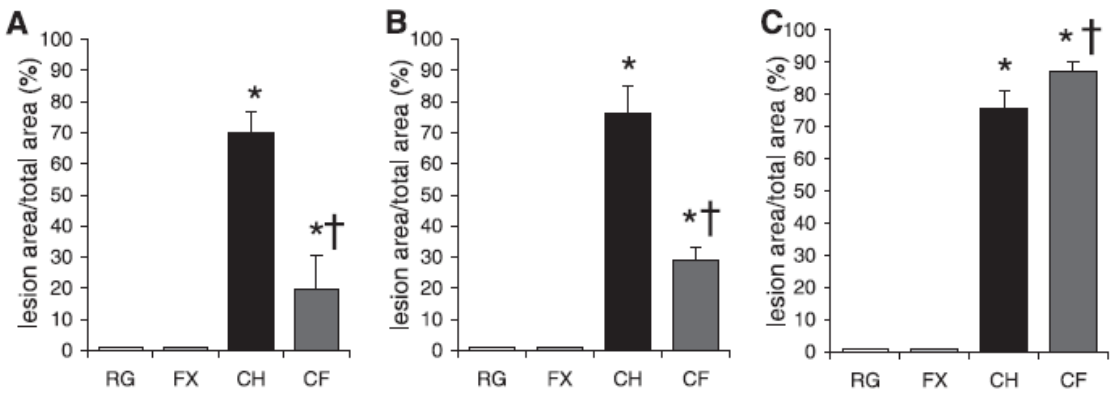


Figure 15. Extent of aortic atherosclerotic lesions in rabbits following 6 (A), 8 (B) and 16 (C) weeks of dietary interventions

The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE; $n = 5–7$. *$P < 0.05$ vs. RG and FX groups. †$P < 0.05$ vs. CH group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

A similar qualitative effect on plaque formation was observed in cross sectional analysis (Figure 16). Plaques were only present in cholesterol-fed animals and were more severe in animals that did not receive flaxseed supplementation. However, at 16 weeks, the protective effect of dietary flaxseed on plaque thickness was not observed.

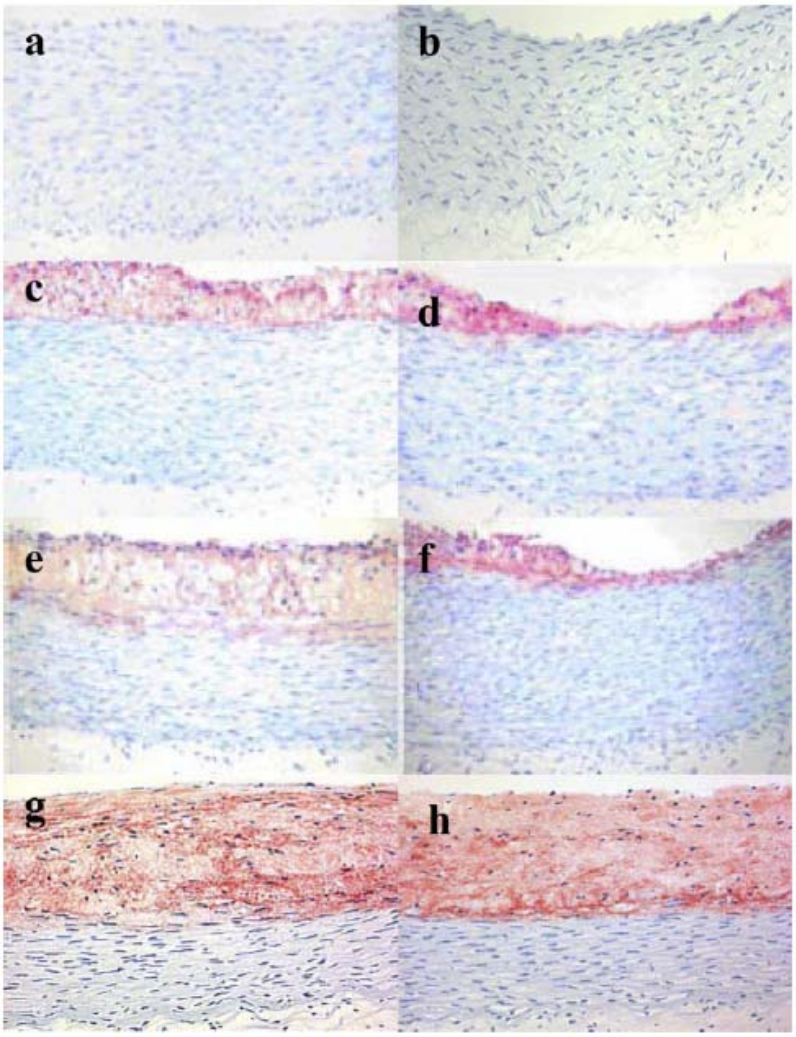


Figure 16. Representative images of aortic cross-sections with Oil Red O-stained lipid deposits

RG group, 6 weeks (a); FX group, 6 weeks (b). Representative pictures from the RG and FX groups at 8 and 16 weeks are not shown as atherosclerotic lesions were not apparent in the RG and FX groups at any time point. CH group: 6 (c), 8 (e) and 16 (g) weeks. CF group: 6 (d), 8 (f) and 16 (h) weeks.

Similar results with respect to atherosclerotic plaque formation were observed in carotid vessels although the extent of the atherosclerosis was not as severe. Atherosclerotic plaque formation in the carotids was only evident in the cholesterol fed groups. Carotids were not collected after the 6 week trial. After 8 weeks of dietary supplementation, atherosclerotic plaque formation in the carotids was inhibited by including flaxseed in the cholesterol diet. This protective effect was lost after 16 weeks of dietary intervention (Figure 17).

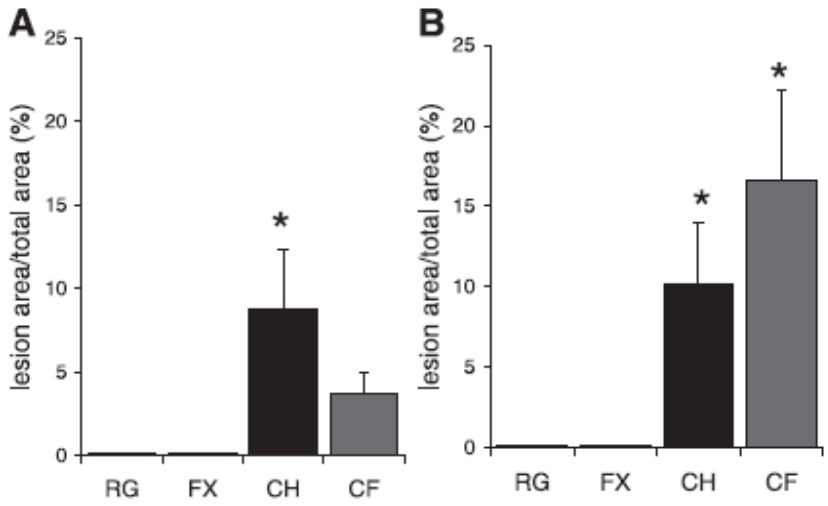


Figure 17. Extent of carotid atherosclerotic lesions following 8 (A) and 16 (B) weeks of dietary treatment

The lesion area was measured as the percentage of luminal area of the carotid arteries covered by atherosclerotic lesions. Values are means ± SE; n = 4–7. *P < 0.05 vs. RG and FX groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

Effects of dietary flaxseed and cholesterol on vascular function

Tissue weights of the 3 mm aortic sections that were used to assess vascular response were not significantly different among the four groups following the 6 and 8 week trials. However, aortic tissue weights were significantly greater, due to large

atherosclerotic lesions, in the CH and CF groups following the 16 week dietary intervention, as compared with the RG and FX groups (CH: 35.4 ± 2.3, CF: 39.0 ± 4.7, RG: 21.5 ± 0.8, and FX: 20.9 ± 1.5 mg). All of the vascular response data was corrected by tissue weight.

The response of aortic rings from animals fed the different dietary regimens was investigated first as a function of the contractile agonists. No differences in KCl-induced vasoconstriction were observed in any groups following 6 and 8 weeks of dietary treatment (Figure 18A and B). However, aortic rings from both of the cholesterol-supplemented groups exhibited an attenuated contractile response to KCl compared with the RG and FX groups after 16 weeks of dietary treatment (Figure 18C).

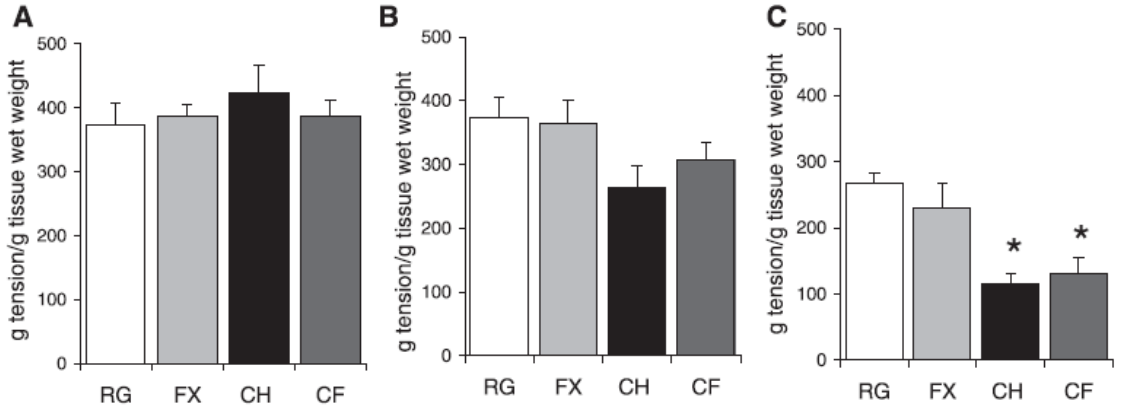


Figure 18. Contractile response to 47 mM KCl of proximal aortic rings isolated after 6 (A), 8 (B) and 16 (C) weeks of dietary treatment

Values are means ± SE; n = 5–8. *P < 0.05 vs. RG and FX groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

A slight depression in the contractile response to NE was observed after 6 weeks of dietary intervention, as aortic preparations from both cholesterol-supplemented groups contracted significantly less in response to 10^{-7} M NE than did the RG and FX groups

(Figure 19A). No difference in NE-induced contraction was observed between the groups following 8 weeks of dietary treatment (Figure 19B). However, the CH and CF groups contracted significantly less in response to a range of NE concentrations (10^{-6} M, 10^{-5} M and 10^{-4} M NE) as compared to the RG and FX groups after 16 weeks of dietary treatment (Figure 19C). There was also a small decline in the overall contractile response to NE observed as a function of the age of all of the animals (Figure 19A-C).

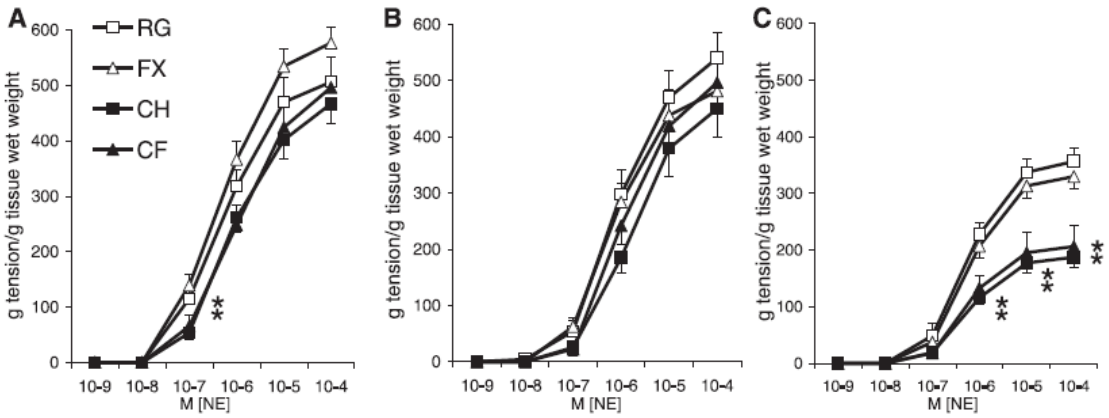


Figure 19. Contractile response to increasing doses of norepinephrine (NE) of proximal aortic rings isolated after 6 (A), 8 (B) and 16 (C) weeks of dietary treatment

Values are means ± SE; n = 6-8. *P < 0.05 vs. RG and FX groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

Aortic relaxation responses were also monitored after precontraction with 10^{-6} M NE as a function of the dietary interventions. Aortic rings from the CH group exhibited significantly less endothelium-dependent relaxation in response to higher doses of ACh (10^{-6} M and 10^{-5} M) than the RG group after 6 weeks of dietary interventions (Figure 20A). Flaxseed added to the diet effectively prevented these cholesterol-induced defects. After 8 weeks of cholesterol feeding, the CH group relaxed less in response to 10^{-6} M

ACh than the RG and FX groups (Figure 20B). Again, this was prevented by including flaxseed in the diet. Following the 16 week trial, the CH group again demonstrated a significant defect in endothelial-dependent relaxation to ACh. The addition of flaxseed partially prevented these cholesterol-induced defects in vascular relaxation; however, the protective effect no longer achieved statistical significance at concentrations of 10^{-5} M ACh (Figure 20C).

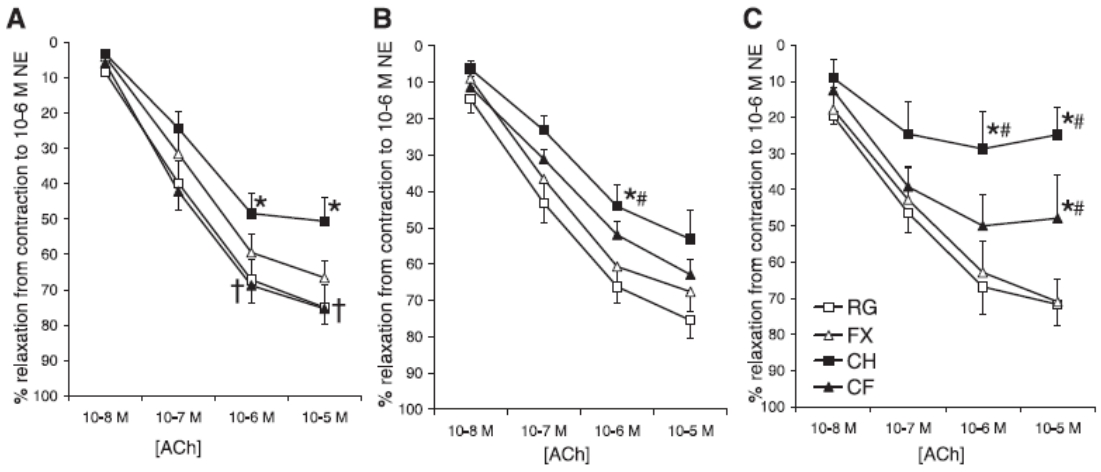


Figure 20. Endothelium-dependent relaxation in response to acetylcholine (ACh) following precontraction with 10-6 M NE of aortic rings isolated after 6 (A), 8 (B) and 16 (C) weeks of dietary treatment

Results are presented as percentage of tension following ACh administration after precontraction with 10^{-6} M NE. Values are means ± SE; n = 6-8. *P < 0.05 vs. RG and FX groups. †P < 0.05 vs. CH group. $^{\#}P$ < 0.05 vs. FX group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

Endothelium-independent vasorelaxation was also investigated using sodium nitroprusside (SNP) following 16 weeks of dietary intervention. There were no significant differences in the extent of endothelium-independent relaxation to SNP amongst the four groups following 16 weeks of dietary intervention (Figure 21A).

Furthermore, the rate of SNP-induced relaxation was also unaltered by the choice of dietary interventions (Figure 21B).

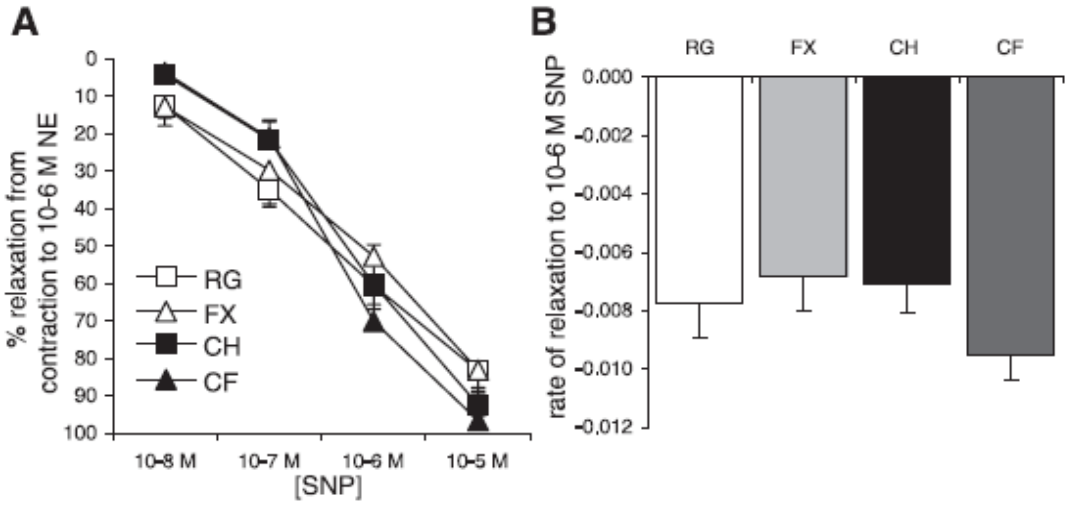


Figure 21. Endothelium-independent relaxation in aortic rings following 16 weeks of dietary treatment

(A) Endothelium-independent relaxation in response to sodium nitroprusside (SNP) following precontraction with 10^{-6} M NE of aortic rings isolated after 16 weeks of dietary treatment. Results are presented as percentage of tension following SNP administration after precontraction with 10^{-6} M NE. Values are means ± SE; n = 7–8. (B) Rate of relaxation in response to 10^{-6} M SNP. Results represent the loss of tension during the first minute after the administration of 10^{-6} M SNP (g tension/sec). Values are means ± SE; n = 7–8. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 0.5% cholesterol-supplemented diet; CF, 0.5% cholesterol- and 10% flaxseed-supplemented diet.

B. Effects of flaxseed on atherosclerotic development in the LDLr$^{-/-}$ mouse

Experimental diets, food intake and weight gain

The nutritional composition of the various diets used in this study was evaluated. As shown in Table 11, the ash, protein, fibre and carbohydrate content of all of the diets was controlled and similar amongst the groups.

Table 11. Nutritional composition of the experimental diets

TABLE 11

NUTRITIONAL COMPOSITION OF THE EXPERIMENTAL DIETS

Group Code	Diet	Ash	Protein	Fibre	CHO	Fat	Calories (kcal/g)	SDG (mg/g)
RG	Regular fat	7.5	25.1	4.5	55.8	7.1	3.61	0
FX	10% Flaxseed	6.8	25.0	4.3	53.5	10.4	3.81	1.37
CH	2% Cholesterol	7.2	24.0	4.4	55.8	8.6	3.70	0
CF	2% Cholesterol + 10% flaxseed	6.5	25.8	4.2	51.0	12.5	3.93	1.37
CF5	2% Cholesterol + 5% flaxseed	6.9	24.1	3.9	54.6	10.5	3.82	0.69
CF1	2% Cholesterol + 1% flaxseed	6.8	24.8	4.1	55.1	9.2	3.75	0.14
CS	2% Cholesterol + 5% coconut oil (SFA)	6.5	23.2	3.8	52.9	13.6	3.98	0

Ash, protein, fibre, CHO, and fat (g/100g diet) are represented as a percent of total nutrients, measured by proximate analysis. Abbreviations: SFA, saturated fatty acid, coconut oil is a good source of SFA; Protein, crude protein; Fibre, crude fibre; CHO, carbohydrates; Fat, crude fat; Calories, metabolic energy(kcal/g); and SDG, the lignan secoisolariciresinol diglucoside. The SDG content of the flaxseed was analyzed by Alister Muir and Kendra Fesyk (BioProducts & Processing, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada). The Promega flaxseed contained 13.73 mg SDG/g. The lignan content of the experimental diets reported was calculated based on the SDG content of the flaxseed multiplied by the amount of flaxseed used in the study.

The metabolic energy levels within the diets were also similar amongst the groups. The lipid content was slightly higher in the flax fed diets. The coconut oil supplemented group was created to provide an internal control for this enhanced lipid load in the diet. Secoisolariciresinol diglucoside (SDG) is the principal lignan found in flaxseed. As expected, the SDG concentration was lower as the content of flaxseed in the chow decreased. The average intake of rodent chow daily was not significantly different between the groups, however, following the 24 week dietary intervention, animals consuming the regular chow (RG) gained significantly less weight as compared to the other experimental groups (Figures 22 and 23).

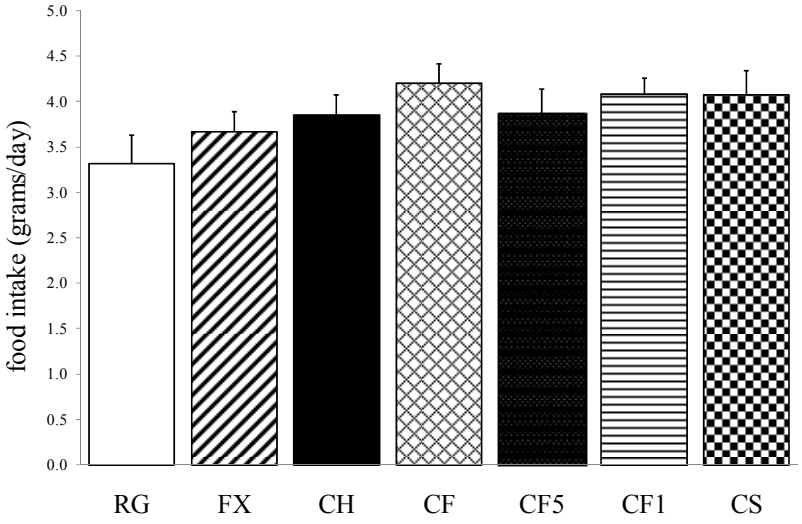


Figure 22. Food intake of LDLr-/- mice fed various dietary treatments for 24 weeks

Values are means ± SE; $n = 3$. No significant difference was found between the groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

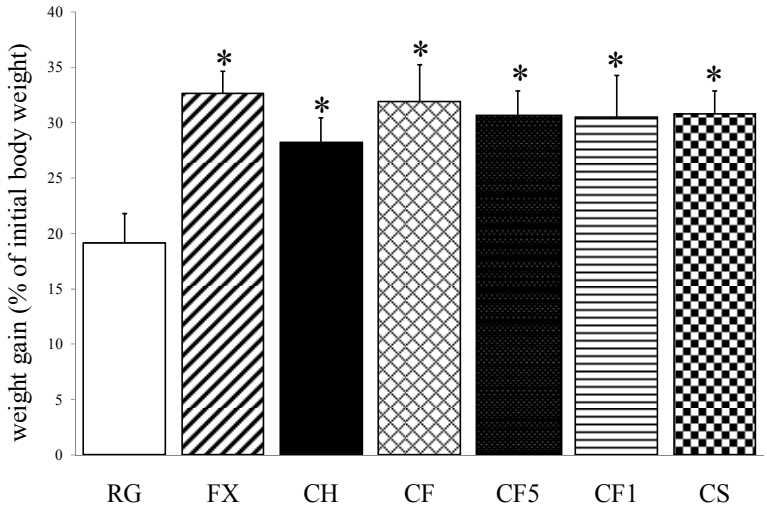


Figure 23. Weight gain in LDLr-/- mice fed various dietary treatments for 24 weeks

Weight gain was measured as weight gain (terminal body weight – initial body weight, g)/initial body weight. Values are means ± SE; $n = 15$. *$P < 0.05$ vs. RG group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

The distribution of SFA, MUFA, and PUFAs is displayed in Figure 24. The PUFA content was highest in the 10% flaxseed groups. The inclusion of flaxseed in the mouse diet resulted in significantly higher levels of ALA (C18:3 ω-3) and reduced levels of LA (C18:2 ω-6) in the chow in comparison to the control, regular mouse chow (Figure 25). As expected, these changes were graded by the amount of flaxseed included in the diet. The cholesterol supplemented diet contained no changes in either fatty acid, unless flaxseed was also included in the mouse chow. The diet supplemented with coconut oil had a significantly lower content of LA but no change in ALA content when compared to the control chow.

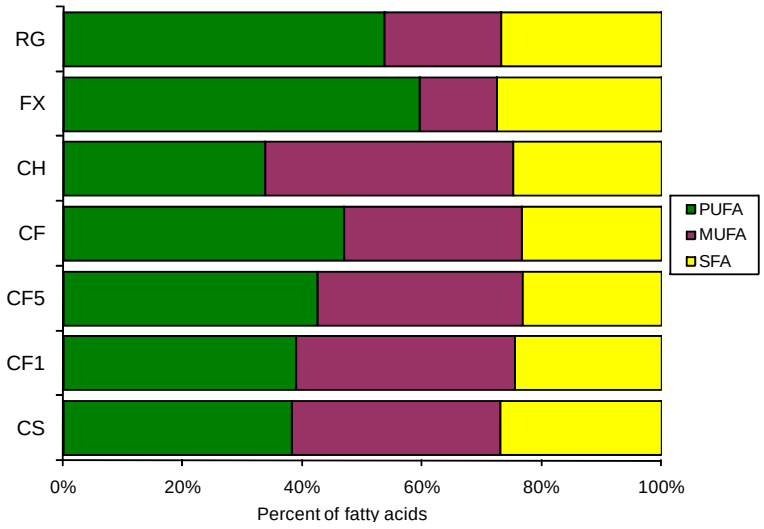


Figure 24. Fatty acid distribution of the experimental diets

Abbreviations: PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

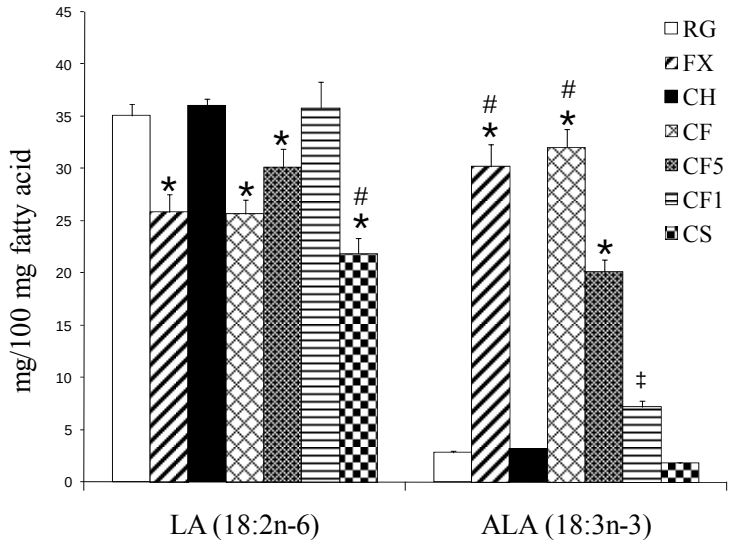


Figure 25. Linoleic (LA) and α-linolenic (ALA) fatty acid content of the experimental diets

Values are means ± SE; $n = 3$. *$P < 0.05$ vs. RG, CH, and CF1 groups. #$P < 0.05$ vs. CF5 group. ‡$P < 0.05$, CF1 vs. all other groups. Abbreviations: n, omega (ω); RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Circulating fatty acid profile

Differences in the plasma fatty acid profile of the animals following the 24 week feeding period were observed (Figure 26). The flaxseed enriched diets induced significant increases in the plasma ALA and EPA (C20:5 ω-3) levels and reduced the arachidonic acid (AA) (C20:4 ω-6) levels as compared to control. Supplementation of the diet with cholesterol resulted in increased LA levels without changes in the other fatty acid species. The addition of flaxseed to the cholesterol enriched diet partially mitigated the cholesterol-induced rise in the plasma LA content, as well as elevated the plasma ALA levels beyond what was observed with flaxseed feeding alone. The extent of the change in these plasma fatty acids was dependent upon the concentration of flaxseed in the chow. All of the dietary treatments had no impact on DHA (C22:6 ω-3) levels in the plasma. The addition of coconut oil in the diet had no effect on plasma fatty acids as compared to

the changes observed in the cholesterol fed group. The ratio of ω-6 to ω-3 PUFAs in the plasma was significantly elevated in the animals that consumed a diet with cholesterol and coconut oil as compared to the control group. The addition of flaxseed to the diet dose-dependently lowered the ω-6/ω-3 ratio in the plasma.

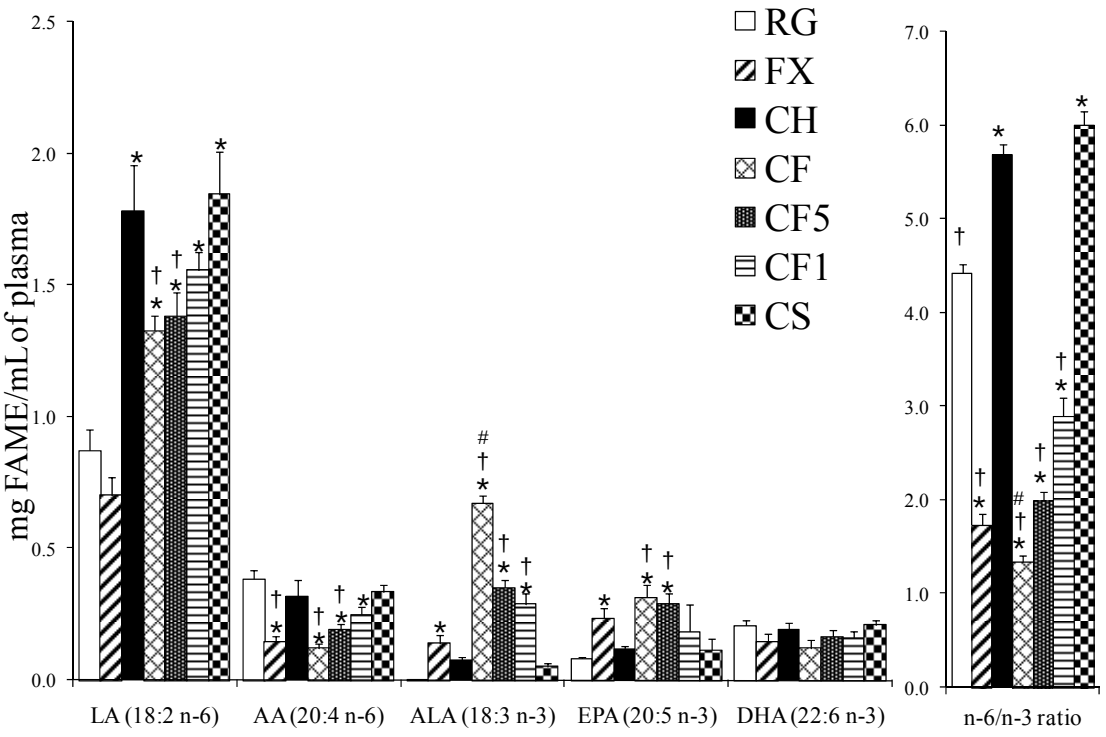


Figure 26. Circulating fatty acid levels and the ratio of ω-6 to ω-3 (n-6/n-3) PUFAs from LDLr-/- mice following 24 weeks of dietary treatment

Values are means ± SE; $n = 3$. *$P < 0.05$ vs. RG group. †$P < 0.05$ vs. CH and CS groups. #$P < 0.05$ vs. CF5 and CF1 groups. Abbreviations: FAME, fatty acid methyl ester; n, omega (ω); LA, linoleic acid; AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Circulating cholesterol, triglyceride and saturated fatty acid levels

As expected, the inclusion of cholesterol in the diet induced a significant increase in plasma cholesterol (Figure 27A). Flaxseed on its own did not alter plasma cholesterol levels in comparison to control levels but when cholesterol was included in the diet, flaxseed mitigated this hypercholesterolemic effect in a concentration dependent manner. Coconut oil did not have a cholesterol-raising effect beyond that seen with cholesterol feeding alone. However, this dietary intervention was the only approach that induced a significant increase in plasma triglyceride levels in comparison to control levels (Figure 27B). Levels of saturated fatty acids (SFA) in the plasma were also elevated in all of the animals consuming cholesterol (Figure 27C). Despite the slightly elevated SFA content in the coconut oil diet in comparison to the other experimental diet (Figure 27C); coconut oil did not extend the rise in plasma SFA levels observed with cholesterol feeding. The addition of flaxseed to the atherogenic diets partially mitigated the effects of cholesterol feeding on plasma SFA levels in a concentration-dependent manner.

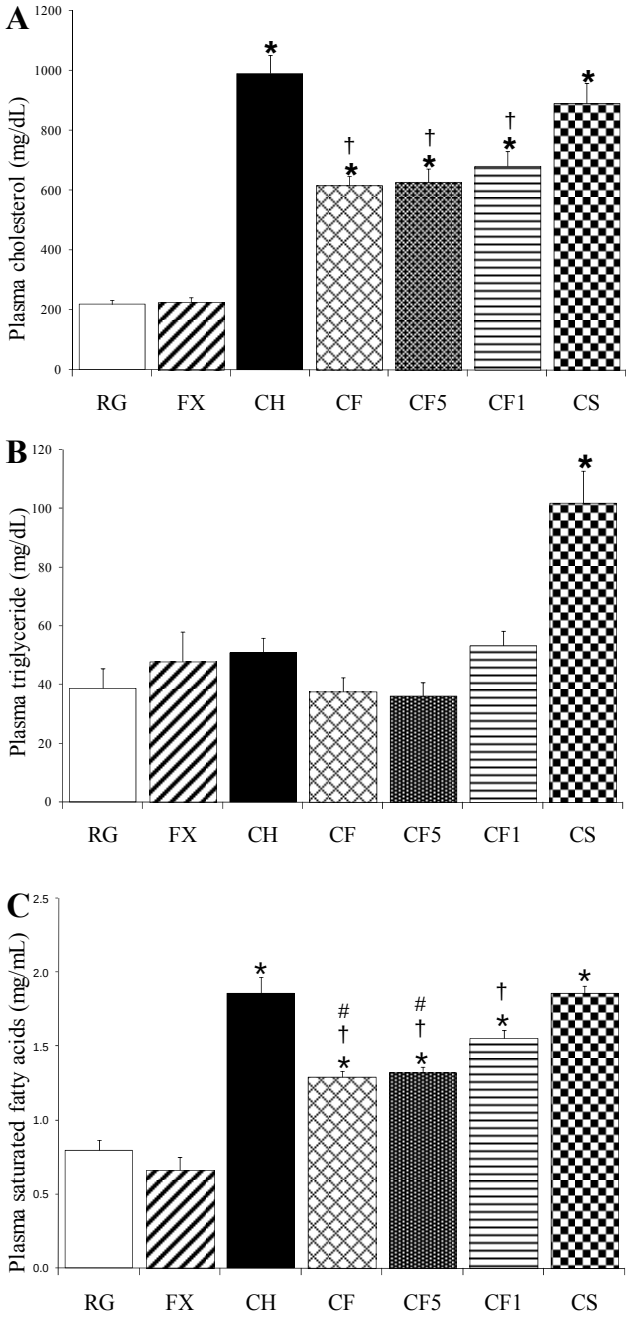


Figure 27. Plasma cholesterol (A), triglyceride (B), and saturated fatty acid (C) levels in LDLr-/- mice following 24 weeks of dietary treatment

Values are means ± SE; n = 13–15. **(A)** plasma cholesterol: *P < 0.05 vs. RG and FX groups. †P < 0.05 vs. CH and CS groups. **(B)** plasma triglycerides: *P < 0.05 CS vs. all other groups. **(C)** saturated fatty acids: *P < 0.05 vs. RG and FX groups. †P < 0.05 vs. CH and CS groups. #P < 0.05 vs. CF1 group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

The inclusion of cholesterol in the diet of the LDLr$^{-/-}$ mice induced a significant atherogenic action in comparison to the control diets. Representative results are shown in Figure 28. Flaxseed included in the diet with cholesterol demonstrated a protective effect (Figure 28D). The results from many animals were pooled and are shown in Figure 29. Mice fed a control diet or one supplemented with flaxseed did not exhibit appreciable atherosclerotic plaque formation. However, a cholesterol-enriched diet induced plaque coverage to about 20% of the luminal surface of the aortic vessel. Flaxseed inhibited this atherogenesis in a dose dependent manner. Coconut oil added to the diet also induced atherosclerotic plaque formation to the same degree as the cholesterol-enriched diet.

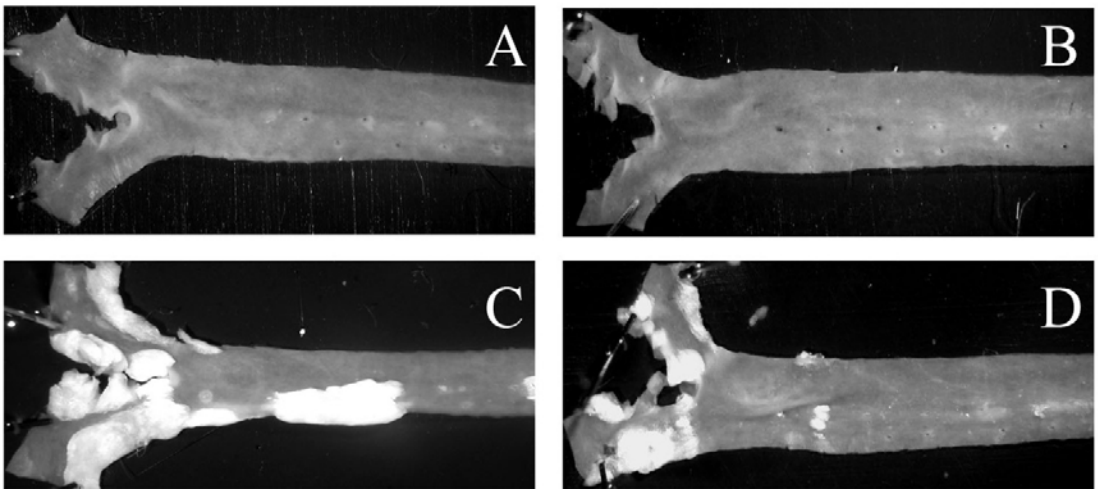


Figure 28. Representative images of aortic atherosclerotic development

Atherosclerotic lesions on the luminal surface of aortae obtained from LDLr$^{-/-}$ mice fed a regular diet (RG) (*A*), a 10% flaxseed supplemented diet (FX) (*B*), a 2% cholesterol supplemented diet (CH) (*C*), or a diet supplemented with 2% cholesterol and 10% flaxseed (CF) (*D*).

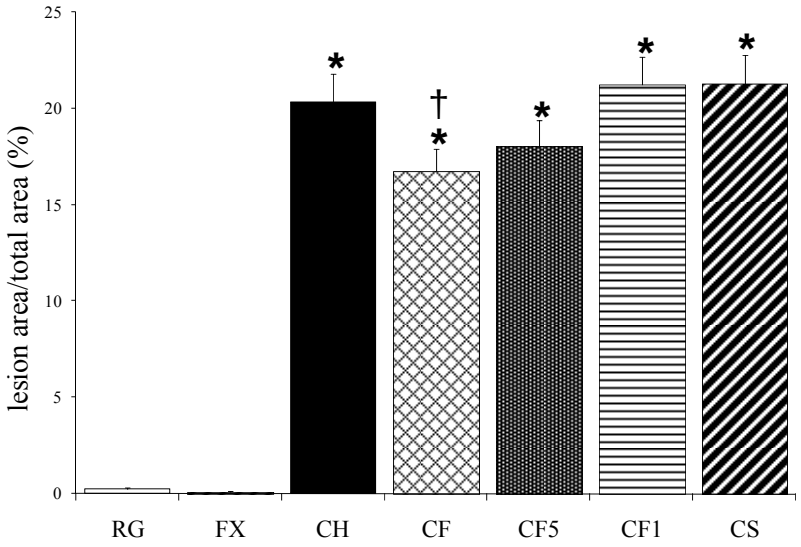


Figure 29. Extent of aortic atherosclerotic lesions following 24 weeks of dietary treatment

The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE; n = 11–15. *P < 0.05 vs. RG and FX groups. †P < 0.05 CF vs. CH, CF1, and CS groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Cross sectional analysis of atherosclerotic development at the aortic sinus revealed lipid deposition with the aid of Oil Red O staining. Representative images are shown in Figure 30. Little lipid deposition was detected in control tissue (Figure 30A) and in animals fed a flaxseed diet (Figure 30B). However, cholesterol supplementation to the diet induced extensive lipid deposits (Figure 30C) that were reduced by the inclusion of flaxseed in the diet (Figure 30D). The extent of atherosclerotic development at the aortic sinus was quantified as a percentage of aortic cross sectional luminal area occupied by Oil Red O stained lipid deposits. The results from many animals were pooled and are shown in Figure 31. Atherosclerotic lesions at the aortic sinus were extensive following

cholesterol feeding. The addition of 5 and 10% flaxseed to an atherogenic diet partially inhibited the development of atherosclerotic lesions at the aortic sinus.

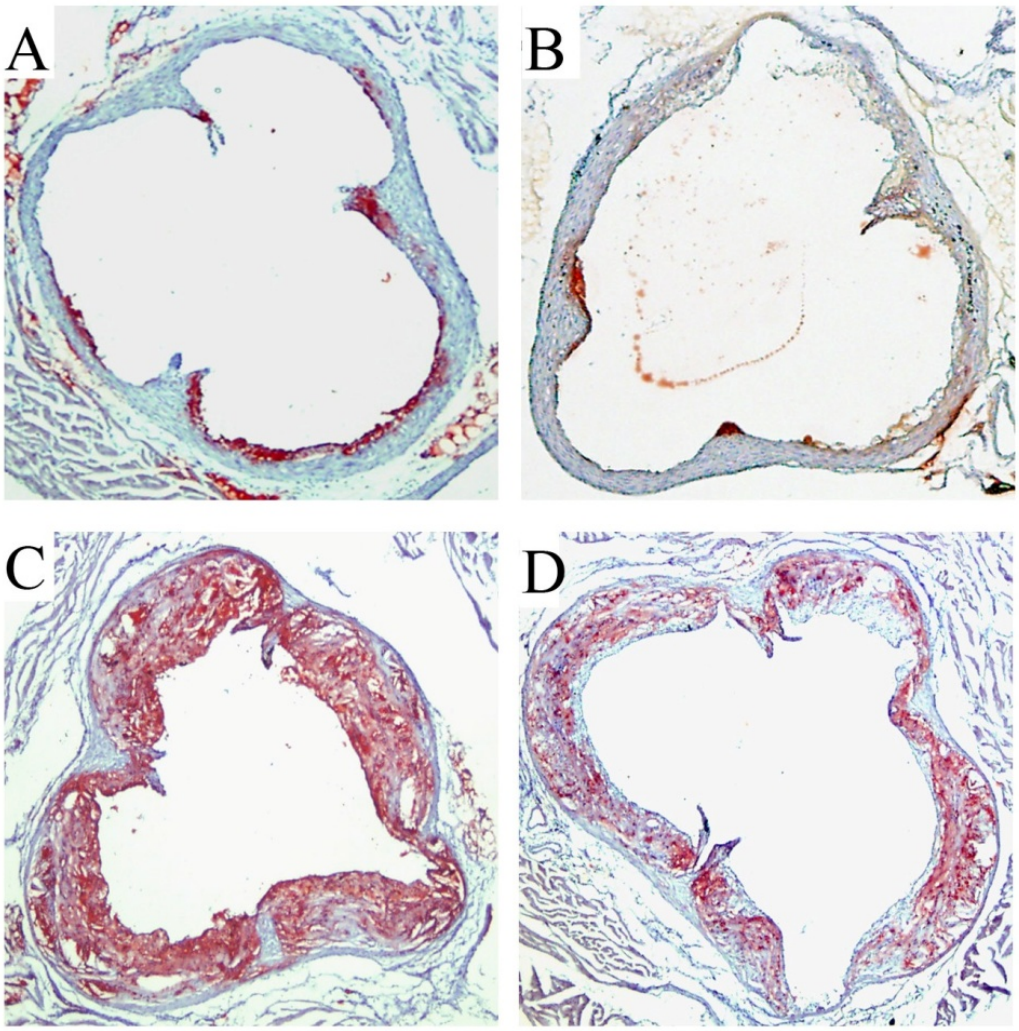


Figure 30. Representative images of aortic cross-sections with Oil Red O-stained lipid deposits

Cross-sections of the aortic sinuses obtained from LDLr$^{-/-}$ mice fed (*A*) RG, regular diet; (*B*) FX, 10% flaxseed supplemented diet; (*C*), CH, 2% cholesterol supplemented diet; or (*D*) CF, 2% cholesterol and 10% flaxseed supplemented diet. The sections were stained with Oil red O for lipid deposition (red) and cross-stained with hematoxylin (blue).

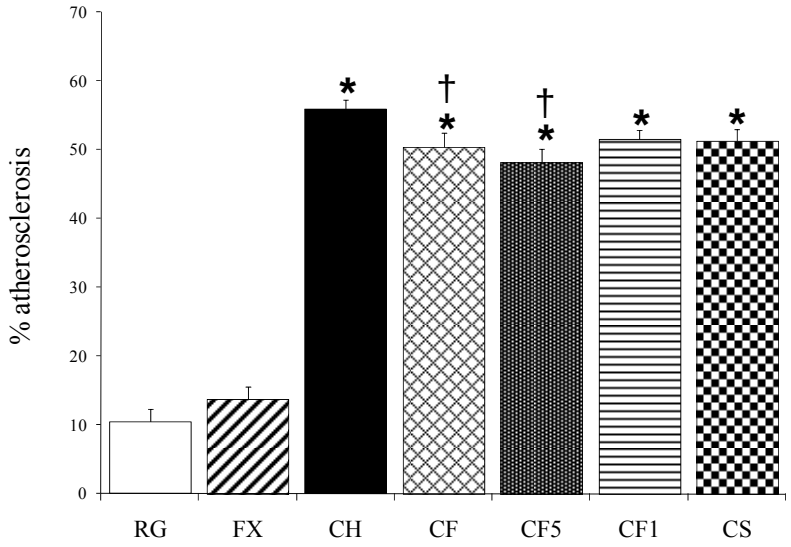


Figure 31. Extent of atherosclerotic lesions at the aortic sinus following 24 weeks of dietary treatment

The lesion area was measured as the percentage of aortic lumen occupied by atherosclerotic lesions. Values are means ± SE; n = 11–15. *P < 0.05 vs. RG and FX groups. †P < 0.05 CF and CF5 vs. CH group. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Anti-proliferative and anti-inflammatory actions of dietary flaxseed

Cell proliferation is associated with atherosclerotic plaque development. The proliferating cell nuclear antigen (PCNA) can be used as an independent marker of cell proliferation within the vessel wall [233-236]. PCNA expression was increased in aortic tissue obtained from mice fed the cholesterol-supplemented diet in comparison to control (Figure 32), as detected by western blots. Flaxseed supplementation on its own did not alter PCNA expression but when included with cholesterol, flaxseed was capable of inhibiting cellular proliferation in a dose dependent manner. Coconut oil in the diet also significantly stimulated cell proliferation. Levels of peroxisome proliferative activated receptor gamma (PPAR-γ) expression in aortic tissue of $LDLr^{-/-}$ mice were not affected by cholesterol, saturated fat, or flaxseed supplementation (Figure 33).

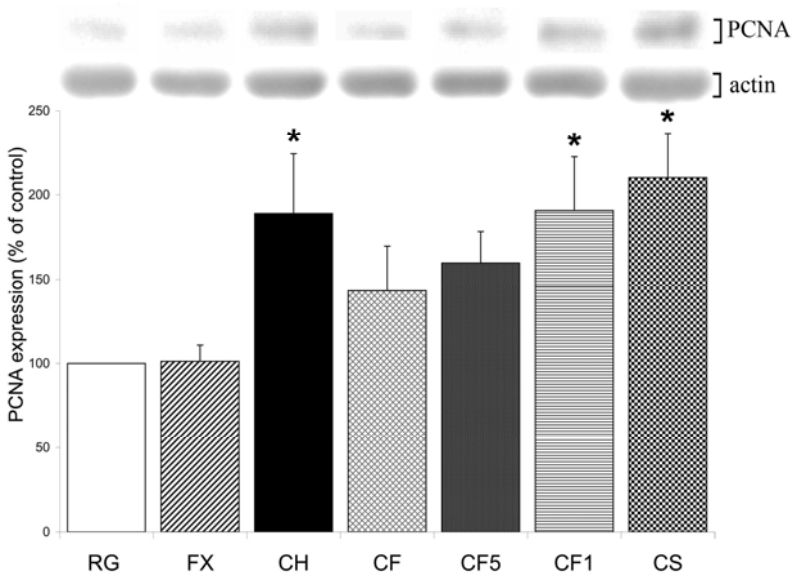


Figure 32. Dietary flaxseed prevents the cholesterol-induced rise in cellular proliferation in atherosclerotic aortic tissues

Expression of proliferating cell nuclear antigen (PCNA) in aortic tissue was detected by Western blotting as a function of the various dietary interventions. Values are means ± SE; $n = 7–8$. *$P < 0.05$ vs. RG and FX groups. A representative image of PCNA expression and total actin as a loading control is displayed at *top*. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

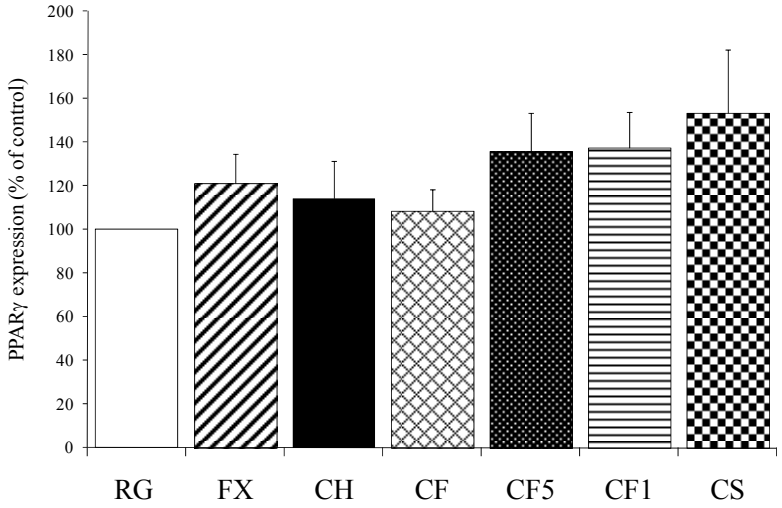


Figure 33. Dietary flaxseed has no effect on PPAR-γ expression

Expression of peroxisome proliferative activated receptor gamma (PPAR-γ) in aortic tissue was detected by Western blotting as a function of the various dietary interventions. Values are means ± SE; *n* = 7–8. No significant differences between the dietary groups. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Because inflammation is now considered to be an important mechanistic process within atherosclerosis, markers of inflammation were also examined as a function of the dietary interventions. The macrophage marker mac-3 has been used as an indicator of inflammatory reactions associated with atherosclerosis [237, 238]. As shown in Figure 34, mac-3 expression was increased significantly in aortic tissue obtained from mice fed cholesterol or coconut oil-enriched diets. Including flaxseed in the cholesterol-supplemented diet significantly inhibited mac-3 expression in a dose dependent manner.

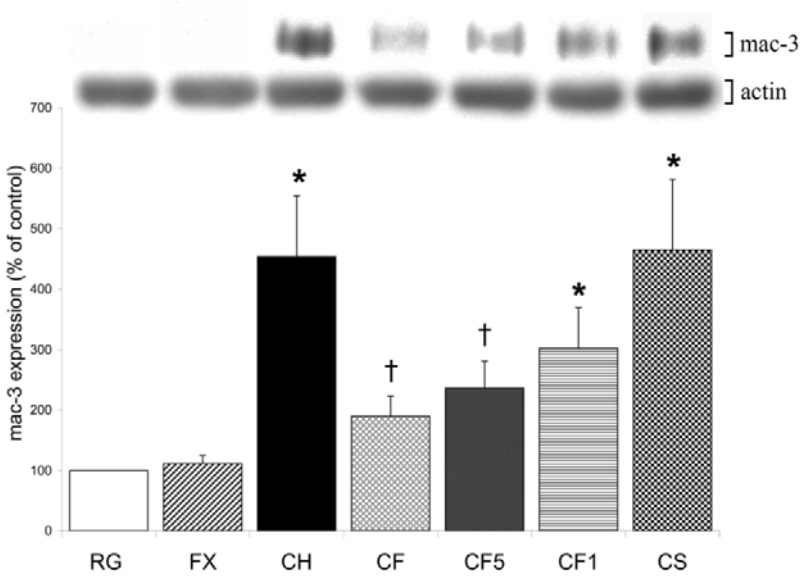


Figure 34. Dietary flaxseed prevents macrophage infiltration into atherosclerotic aortic lesions

Expression of mac-3 in aortic tissue was detected by Western blotting as a function of the various dietary interventions. Values are means ± SE; $n = 7$. *$P < 0.05$ vs. RG and FX groups. †$P < 0.05$ vs. CH and CS groups. A representative image of mac-3 expression and total actin as a loading control is displayed at *top*. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

Inflammation in the aortic tissues was also examined using the pro-inflammatory cytokine interleukin-6 (IL-6). Western blot analysis revealed that IL-6 expression was increased in aortic tissue obtained from mice fed the cholesterol-supplemented diet (CH) and the cholesterol and coconut oil supplemented diet (CS) in comparison to control (Figure 35). Flaxseed supplementation on its own did not alter IL-6 expression but when included with cholesterol, flaxseed in the two highest concentrations (5 and 10%) was capable of mitigating the effects of cholesterol and coconut oil on IL-6 expression.

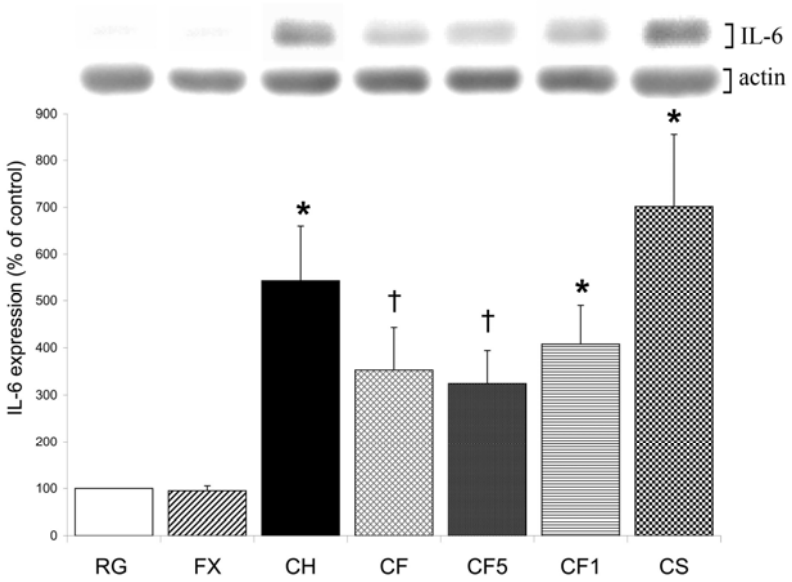


Figure 35. Dietary flaxseed prevents the cholesterol-induced rise in IL-6-mediated inflammation in atherosclerotic aortic tissues

Expression of IL-6 in aortic tissue was detected by Western blotting as a function of the various dietary interventions. Values are means ± SE; n = 9-12. *P < 0.05 vs. RG and FX groups. †P < 0.05 vs. CS group. A representative image of IL-6 expression and total actin as a loading control is displayed at *top*. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

The effects of dietary flaxseed on the inflammatory and atherogenic marker vascular cell adhesion molecule-1 (VCAM-1) are shown in Figure 36. VCAM-1 expression was significantly increased in aortic tissue from mice consuming cholesterol or coconut oil enriched diets. The addition of flaxseed to an atherogenic diet prevented the cholesterol and saturated fat-induced rise in aortic VCAM-1 expression in a dose dependent manner.

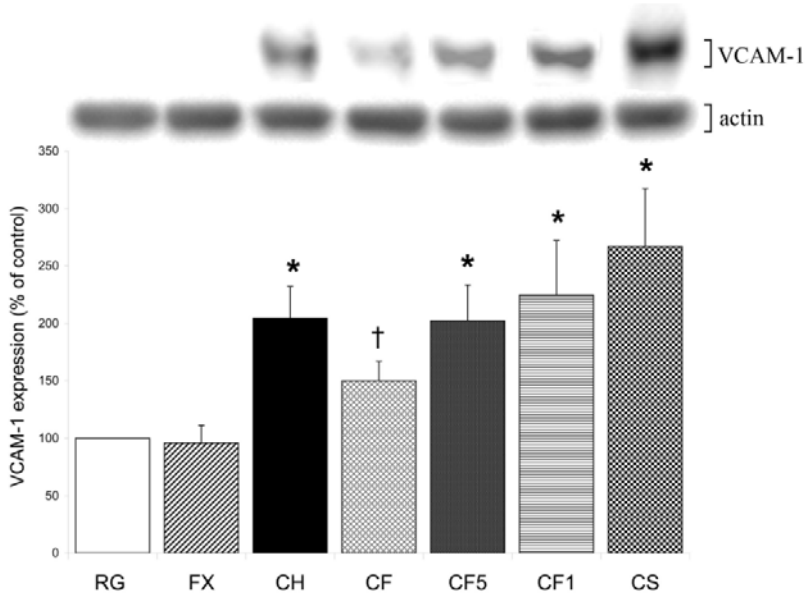


Figure 36. Dietary flaxseed protects against cellular adhesion and inflammation in atherosclerotic aortic tissues

Expression of VCAM-1in aortic tissue was detected by Western blotting as a function of the various dietary interventions. Values are means ± SE; $n = 6$. *$P < 0.05$ vs. RG and FX groups. †$P < 0.05$ vs. CS group. A representative image of VCAM-1 expression and total actin as a loading control is displayed at *top*. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; CF5, 2% cholesterol with 5% flaxseed-supplemented diet; CF1, 2% cholesterol with 1% flaxseed-supplemented diet; CS, 2% cholesterol with 5% coconut oil-supplemented diet.

The expression of markers of proliferation and inflammation was confirmed in cross-sections at the aortic sinus. Representative images from the RG, FX, CH, and CF groups are shown in Figure 37. Little antibody staining is detected in the RG group. Flaxseed on its own has no effect on mac-3, IL-6, and PCNA expression. Abundant immunoreactivity for mac-3 (A) is detected throughout atherosclerotic lesions from $LDLr^{-/-}$ mice fed a cholesterol diet (CH), whereas IL-6 (B) and PCNA (C) expression predominates in the innermost region of atheromas. IL-6 staining is also detected within the media layer of aortic cross-sections from the CH group. The addition of dietary flaxseed to an atherogenic diet reduced the expression of mac-3, IL-6, and PCNA in aortic atherosclerotic lesions in $LDLr^{-/-}$ mice as compared to cholesterol feeding alone.

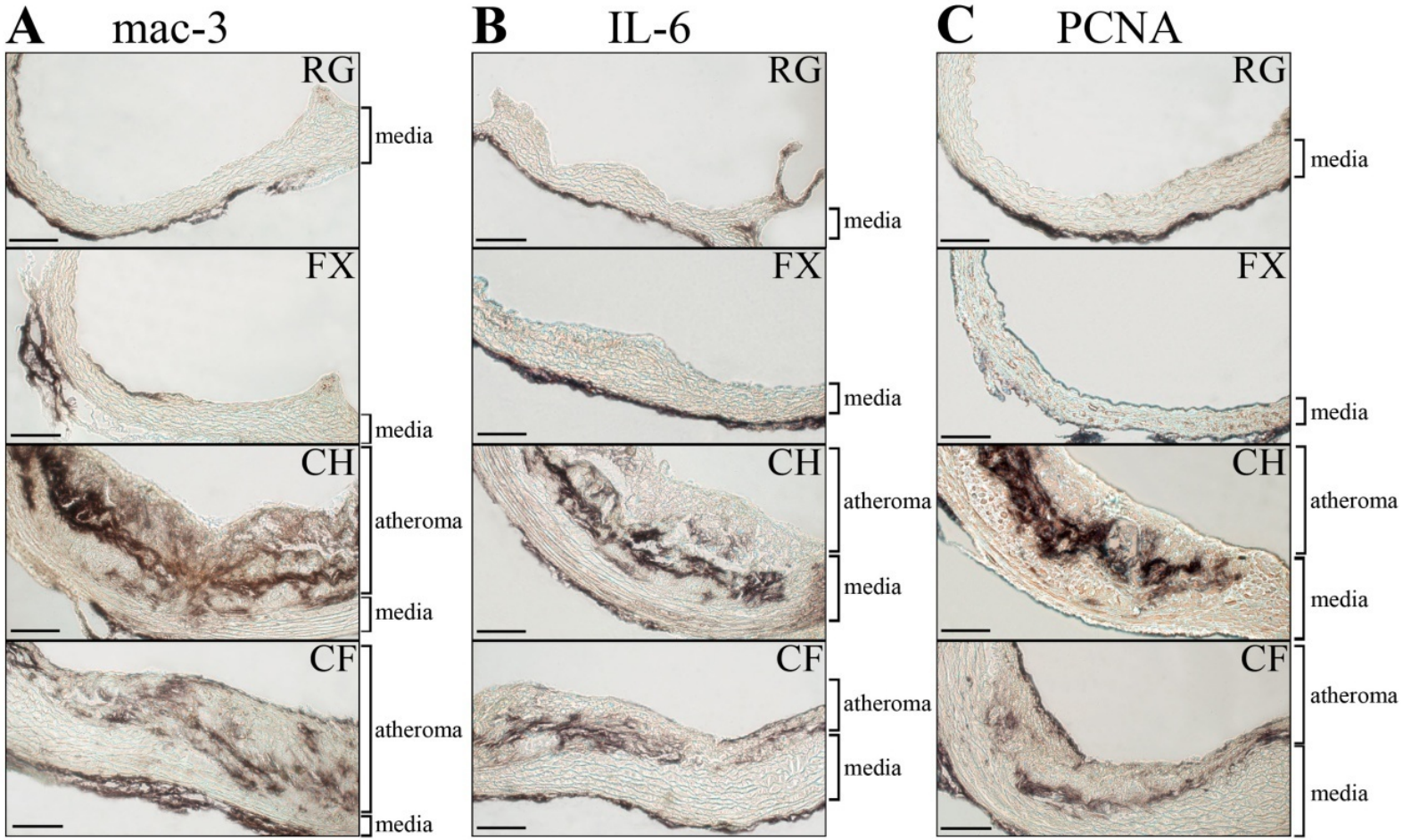


Figure 37. Dietary flaxseed prevents atherosclerotic development via anti-inflammatory and anti-proliferative actions

Representative images of aortic cross sections immunostained with markers of macrophage infiltration, inflammation, and proliferation from LDLr$^{-/-}$ mice fed a RG, FX, CH, or CF diet for 24 wk. Bars in each panel represent 0.1 mm. Immunoreactivity to mac-3 (*A*), IL-6 (*B*), and PCNA (*C*) antibodies is evident with brownish DAB staining. Abbreviations: RG, regular diet; FX, 10% flaxseed-supplemented diet; CH, 2% cholesterol-supplemented diet; CF, 2% cholesterol with 10% flaxseed-supplemented diet; DAB, diaminobenzidine.

C. Effects of *trans* fatty acids on atherosclerotic development in the LDLr$^{-/-}$ mouse

Experimental diets

The nutritional composition of the 7 experimental diets is listed in Table 12. Three diets were created to serve as internal controls for the four diets containing TFAs (LR for LT, HR for HT and C+HT, and C+LR for C+LT and C+HT). The composition of these control diets was identical to the TFA containing diets, apart from the source of dietary fat. The grain content in each of the diets contributed a small amount of dietary fat. As detailed in Table 12, doubling the amount of added fat increased the dietary fat content by approximately 50%, and adding cholesterol to the diet added approximately 25% more dietary fat. The fat content was, therefore, highest in the C+HT group. Both the LT and C+LT diets contained 1.4% TFA, which relates to 3.2% of caloric energy as TFA intake. The HT and C+HT diets contained 2.8% TFA, which provides 6.4% of caloric energy as TFA intake. The ash, protein, fibre and carbohydrate content of all of the diets were controlled and similar amongst the groups. Despite an increase in dietary fat in the higher fat groups, the digestible energy levels provided by the diets were all in a similar range (within 3.5 and 3.7 kcal/gram). Food intake between the groups was similar (Table 12).

Table 12. Nutritional composition of the experimental diets

TABLE 12

NUTRITIONAL COMPOSITION OF THE EXPERIMENTAL DIETS AND AVERAGE FOOD INTAKE

Group Code	Group Name	Dietary fat	Ash	Protein	Fibre	CHO	Fat	Calories (kcal/g)	TFA (% of diet)	TFA (% caloric energy)	Food intake (g/day)
LR	Low Regular fat	4% pork/soy fat	7.0	24.0	4.3	56.1	8.5	3.5	0	0	3.8
LT	Low *trans* fat	4% hydrogenated vegetable shortening	7.0	24.2	4.4	56.2	8.2	3.5	1.4	3.2	3.8
HR	High Regular fat	8% pork/soy fat	6.4	22.2	4.2	54.7	12.5	3.6	0	0	3.7
HT	High *trans* fat	8% hydrogenated vegetable shortening	6.6	21.7	5.2	54.5	12.0	3.6	2.8	6.4	3.8
C+LR	Cholesterol + Low Regular fat	2% cholesterol + 4% pork/soy fat	6.7	23.2	4.3	55.0	10.8	3.6	0	0	3.9
C+LT	Cholesterol + Low *trans* fat	2% cholesterol + 4% hydrogenated vegetable shortening	6.7	22.9	4.8	54.8	10.8	3.6	1.4	3.2	3.8
C+HT	Cholesterol + High *trans* fat	2% cholesterol + 8% hydrogenated vegetable shortening	6.4	22.7	4.2	53.2	13.4	3.7	2.8	6.4	3.8

Ash, protein, fibre, CHO, and fat (g/100g diet) are represented as a percent of total nutrients, measured by proximate analysis (dry matter). TFA content was estimated based on the addition of 4 or 8% partially hydrogenated shortening, containing 35% TFA content, to the diets. Food intake (g/day) represents the average amount of food consumed by $LDLr^{-/-}$ mice fed experimental diets over 14 weeks. There was no significant difference in food intake between the groups (n=5/group). Abbreviations: Protein = Crude Protein; Fibre = Crude Fibre; CHO = Carbohydrates; Fat = Crude Fat; Calories = Digestible Energy (kcal/g); and TFA = *trans* fatty acids

Fatty acid profile of the experimental diets

The experimental diets differed in fatty acid composition (Figure 38). Noteworthy differences include, a general increase in fatty acid levels in experimental diets with higher fat contents (HR, HT and C+HT) and a general, although not always statistically significant, reduction in SFA (C14:0 and C16:0), MUFA (C16:1, C18:1 and C20:1) and PUFA (C18:2 and C18:3) fatty acid levels in groups containing TFAs as compared to their control group (LT vs. LR, HT vs. HR and C+LT vs. C+LR). The regular fat groups (LR, HR and L+LR) had minimal levels of TFAs. The addition of commercially hydrogenated vegetable shortening significantly and dose-dependently raised the TFA fatty acid content of the experimental diets (LT, C+LT, HT and C+HT groups) (Figure 38D).

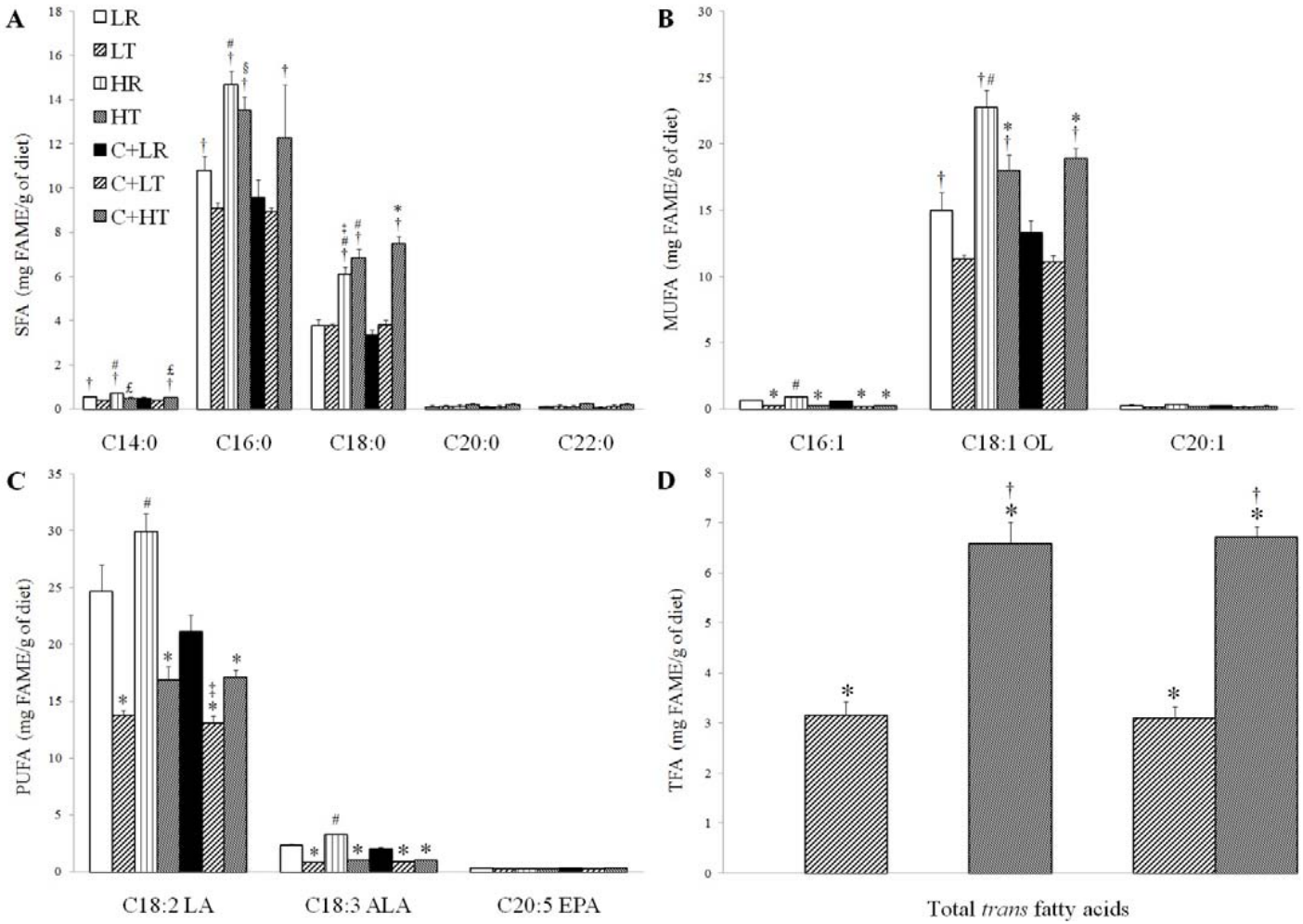


Figure 38. Fatty acid composition of the experimental diets fed to LDLr-/- mice for 14 weeks

Levels of saturated (SFA) (A), monounsaturated (MUFA) (B), polyunsaturated (PUFA) (C), and t*rans* (TFA) (D) fatty acids in the experimental diets (mg/g diet). Values are means ± SE; $n = 3$. $^{*}P < 0.05$ vs. LR, HR, and C+LR groups. $^{\dagger}P < 0.05$ vs. LT and C+LT groups. $^{\#}P < 0.05$ vs. LR and C+LR groups. $^{\ddagger}P < 0.05$ vs. C+HT group. $^{\S}P < 0.05$ vs. C+LR group. $^{\infty}P < 0.05$ vs. LR group. $^{£}P < 0.05$ vs. HR group. Only trace amounts (<0.01 mg/mL) of C13:0, C14:1, C16:1*t*, C17:0, C18:2*tt*, C18:3 GLA, C20:3[11,14,17], C20:3[8,11], C22:1 and C22:6 DHA were detectable (data not shown). Abbreviations: LR, low regular fat diet; LT, low trans fat diet; HR, high regular fat diet; HT, high trans fat diet; C+LR, cholesterol + low regular fat diet; C+LT, cholesterol + low trans fat diet; C+HT, cholesterol + high trans fat diet; OL, oleic acid; LA, linoleic acid; GLA, gamma linolenic acid; AA, arachadonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Circulating fatty acid profile

Differences in the plasma fatty acid profile of the animals following the 14 week feeding period were observed (Figure 39). The cholesterol supplemented regular diet (C+LR) had the highest levels of plasma saturated fatty acids (SFA) (C16:0 and C18:0), monounsaturated fatty acids (MUFA) (C16:1 and C18:1), and polyunsaturated fatty acids (PUFA) (C18:2 linoleic acid (LA), C18:3 alpha-linolenic acid (ALA), C20:4 arachadonic acid (AA), and C20:5 eicosapentaenoic acid (EPA)). Surprisingly, this rise was partially attenuated by the addition of TFAs (C+LT and C+HT) in the diet. Cholesterol supplementation on its own (C+LR group) did not elevate circulating plasma. Cholesterol supplementation on its own (C+LR group) did not elevate circulating plasma TFA levels, however, the addition of TFAs in an atherogenic diet (C+LT and C+HT) had a strong, dose dependent, stimulatory effect on plasma TFA levels.

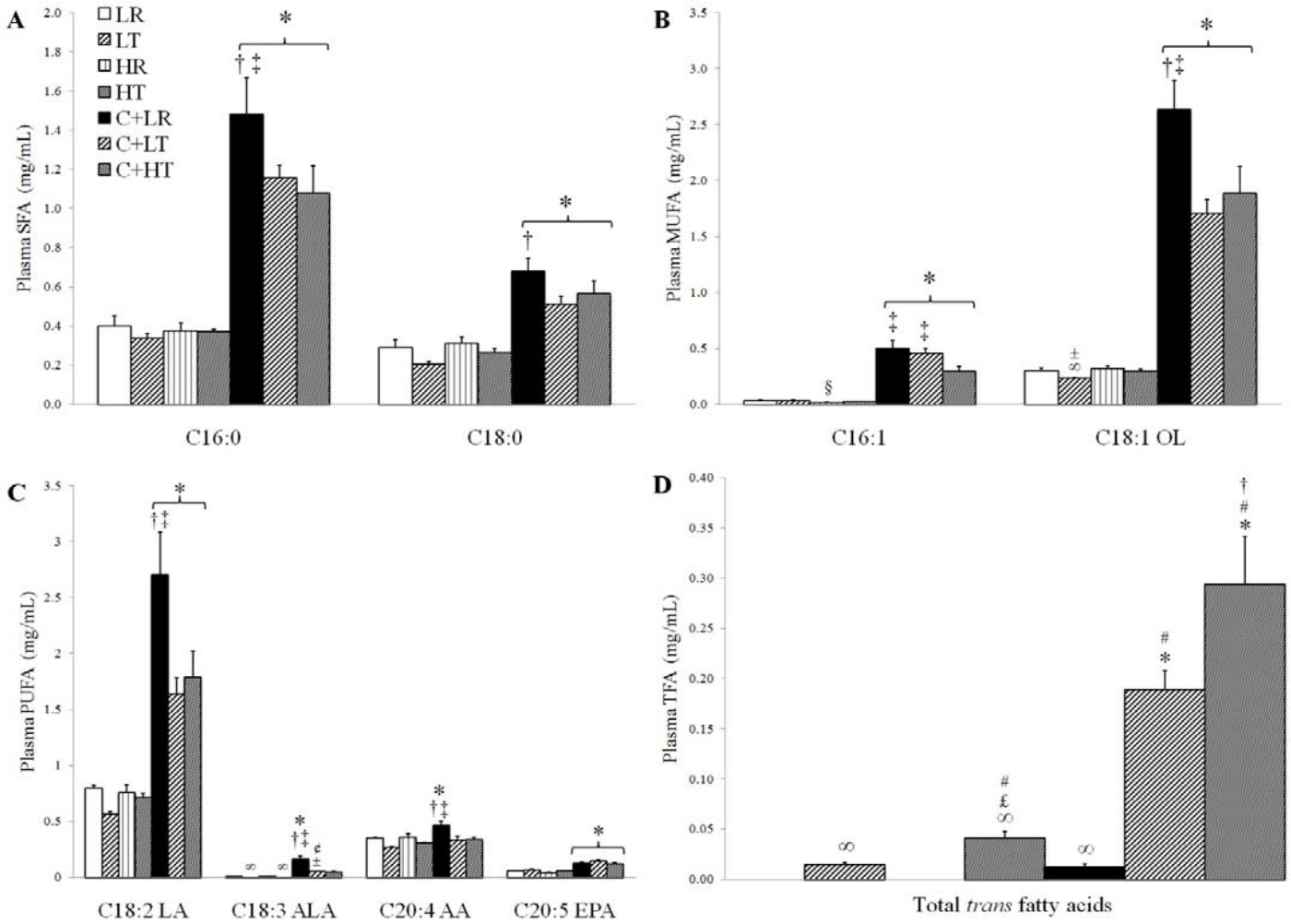


Figure 39. Circulating fatty acid levels in LDLr-/- mice following 14 weeks of dietary treatment

Saturated (A), monounsaturated (B), polyunsaturated (C), and t*rans* (D) fatty acids were quantitated (mg/mL) in plasma from LDLr$^{-/-}$ mice following 14 weeks of dietary interventions. Values are means ± SE; $n = 4$. $^{*}P < 0.05$ vs. LR, LT, HR and HT groups. $^{\#}P < 0.05$ vs. C+LR group. $^{\dagger}P < 0.05$ vs. C+LT group. $^{\ddagger}P < 0.05$ vs. C+HT group. $^{\infty}P < 0.05$ vs. LR and HR groups. $^{\S}P < 0.05$ vs. LR and LT groups. $^{¢}P < 0.05$ vs. LT group. $^{£}P < 0.05$ vs. HR group. $^{\pm}P < 0.05$ vs. HT group. Only trace amounts (<0.01 mg/mL) of C13:0, C14:0, C14:1, C16:1*t*, C17:0, C18:2*tt*, C18:3 GLA, C20:0, C20:1, C20:3^{11,14,17}, C20:3^{8,11}, C22:0, C22:1 and C22:6 DHA were detectable (data not shown). Abbreviations: LR, low regular fat diet; LT, low trans fat diet; HR, high regular fat diet; HT, high trans fat diet; C+LR, cholesterol + low regular fat diet; C+LT, cholesterol + low trans fat diet; C+HT, cholesterol + high trans fat diet; OL, oleic acid; LA, linoleic acid; GLA, gamma linolenic acid; AA, arachadonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Circulating cholesterol and triglyceride levels

Serum cholesterol levels were similar amongst all of the groups at the start of the study (Figure 40). Following 4 weeks of feeding, the addition of dietary cholesterol had a sharp effect on serum cholesterol levels. Substituting the fat source for TFAs partially prevented the dietary cholesterol induced rise in serum cholesterol levels. Serum cholesterol levels rose in the groups consuming TFAs and cholesterol (C+LT and C+HT) over the course of the study (Figure 40). Following 14 weeks of feeding, the substitution of fat for a TFA source in the diet resulted in lower serum cholesterol levels as compared to the LR group. Increasing the level of fat in the diet resulted in a small rise in serum cholesterol levels (HR vs. LR). Including higher levels of TFA in the diet had no effect on serum cholesterol levels (HT vs. HR). As expected, the inclusion of cholesterol in the diet induced a significant increase in serum cholesterol levels. The substitution of regular fat for manufactured TFAs in the atherogenic diets resulted in slightly lower serum cholesterol levels at the conclusion of the study. Increasing the dose of TFAs had no effect on serum cholesterol levels.

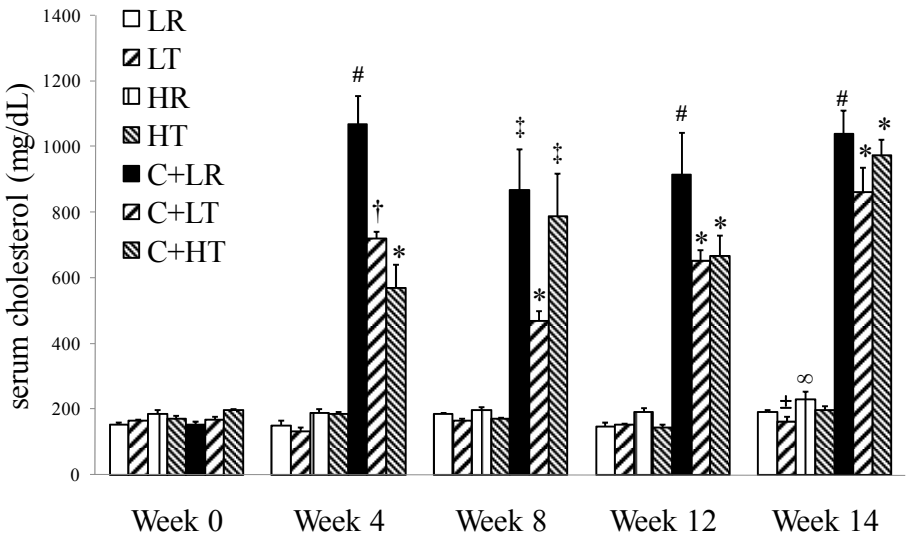


Figure 40. Serum cholesterol levels in LDLr-/- mice following 0, 4, 8, 12, and 14 weeks of dietary treatment

Values are means ± SE, n = 5 at 0, 4, 8, 12 and 14 weeks. $^{\pm}P < 0.05$ versus LR group. $\infty P < 0.05$ versus LR and LT groups. $^{*}P < 0.05$ versus LR, LT, HR, and HT groups. $^{\dagger}P < 0.05$ versus LR, LT, HR, HT, and C+HT groups. $^{\ddagger}P < 0.05$ versus LR, LT, HR, HT, and C+LT groups. $^{\#}P < 0.05$ versus all groups. Abbreviations: LR, low regular fat diet; LT, low *trans* fat diet; HR, high regular fat diet; HT, high *trans* fat diet; C+LR, cholesterol + low regular fat diet; C+LT, cholesterol + low *trans* fat diet; C+HT, cholesterol + high *trans* fat diet.

Substituting the fat source for *trans* fat had no effect on serum triglyceride levels in the absence of dietary cholesterol. Consuming TFAs in the presence of dietary cholesterol had a significant impact on serum triglyceride levels (Figure 41). TG levels were ~1.3 times greater in the C+LT group and ~2.5 times greater in the C+HT group versus the C+LR group.

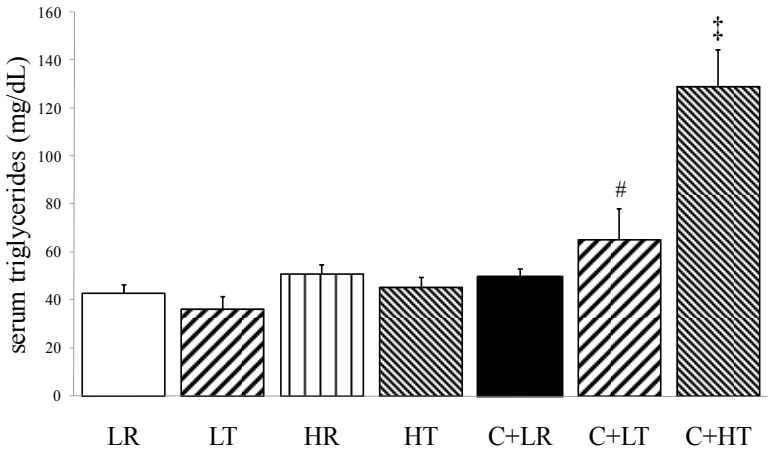


Figure 41. Serum triglyceride levels in LDLr-/- mice following 14 weeks of dietary treatment

Values are means ± SE, n = 4-5. #P < 0.05 versus LT group. ‡P < 0.05 C+HT versus all groups. Abbreviations: LR, low regular fat diet; LT, low *trans* fat diet; HR, high regular fat diet; HT, high *trans* fat diet; C+LR, cholesterol + low regular fat diet; C+LT, cholesterol + low *trans* fat diet; C+HT, cholesterol + high *trans* fat diet.

Aortic atherosclerotic development

The results from many animals on the extent of atherosclerotic lesions developed on the aortic luminal surface following 14 weeks of dietary intervention were pooled and are shown in Figure 42. Mice fed a control diet (LR) did not exhibit appreciable atherosclerotic plaque formation. Including TFAs in the diet from a manufactured hydrogenated vegetable shortening source (LT) stimulated atherosclerotic development on its own, an event not normally observed in the LDLr$^{-/-}$ mouse unless it is fed dietary cholesterol or a strongly atherogenic diet. TFAs initiated atherosclerotic development in a dose dependent manner (LT > LR and HT > HR). The inclusion of cholesterol in the diet of the LDLr$^{-/-}$ mice induced a significant atherogenic action in comparison to the groups without dietary cholesterol. Substituting the fat source for either a low or high concentration of TFA from vegetable shortening in the presence of dietary cholesterol did

not have an additional effect on atherosclerotic development. In the present study, dietary TFAs were directly responsible for the atherogenesis. The higher the circulating [TFA] was, the more extensive were the atherosclerotic lesions (Figure 43).

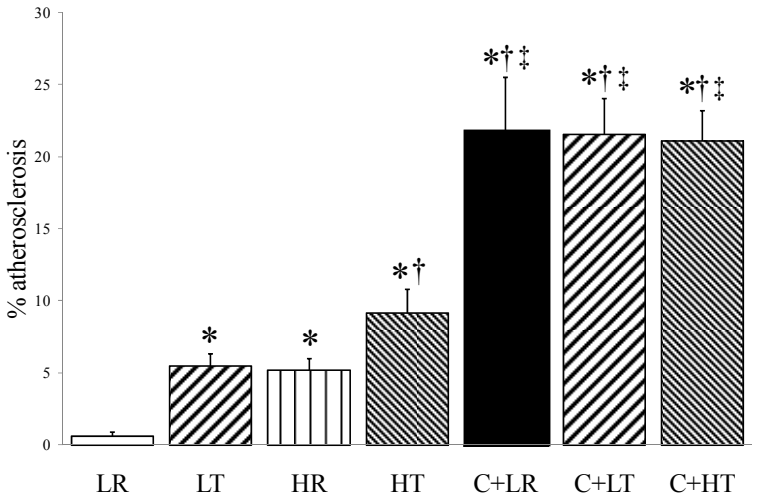


Figure 42. Extent of aortic atherosclerotic lesions in LDLr-/- mice following 14 weeks of dietary treatment

The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE, $n = 5$. *P < 0.05 versus LR group. †P < 0.05 versus LT and HR groups. ‡P < 0.05 C+LR, C+LT, C+HT versus HR group. Abbreviations: LR, low regular fat diet; LT, low *trans* fat diet; HR, high regular fat diet; HT, high *trans* fat diet; C+LR, cholesterol + low regular fat diet; C+LT, cholesterol + low *trans* fat diet; C+HT, cholesterol + high *trans* fat diet.

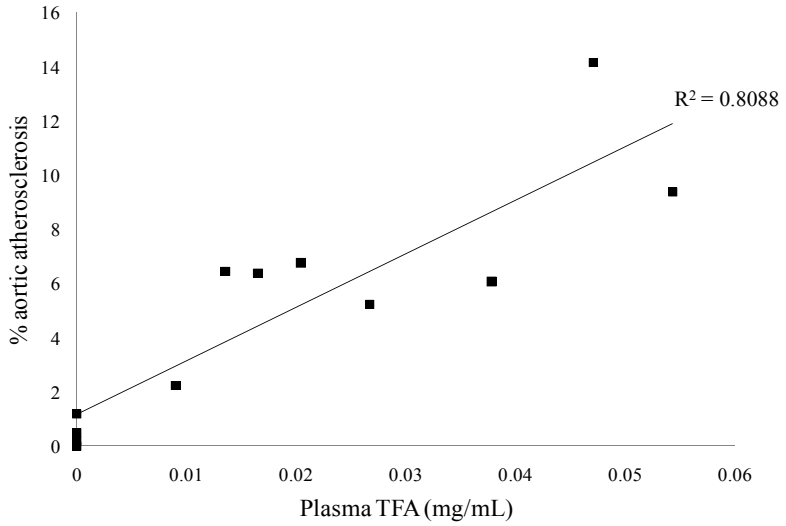


Figure 43. Relationship of atherosclerosis to circulating *trans* fatty acid concentration

Each data point represents pooled values from four mice fed a control diet or one supplemented with low or high *trans* fat. Data are taken from Figures 39D and 42.

D. Effects of *trans* fatty acids from commercial and natural sources on atherosclerotic development in the LDLr$^{-/-}$ mouse

Experimental diets

The nutritional composition of the 8 experimental diets is listed in Table 13. Four diets were created to serve as internal controls for the four diets containing TFAs (RG for ES, RB for VB, CH for CH+ES, and CH+RB for CH+VB). The composition of these control diets was identical to the TFA containing diets, apart from the source of dietary fat. The grain content in each of the diets contributed a small amount of dietary fat. As detailed in Table 13, the fat content in the butter containing groups (RB, VB, CH+RB, and CH+VB) was approximately double that in the regular fat (RG and CH) and elaidic shortening groups (ES and CH+ES). Both the ES and VB fats provided 1.4% TFA to the diet. The ash, protein, fibre and carbohydrate content of all of the diets were controlled and similar amongst the groups. Despite an increase in dietary fat in the butter containing

groups, the digestible energy levels provided by the diets were all in a similar range, and virtually identical between each of the control and TFA matched groups. The fatty acid composition of the two butters is described in Table 14. The concentration of elaidic, vaccenic and total TFAs were all ~4x higher in the VB butter. Vaccenic acid accounts for ~50% of all TFAs in the fortified butter (VB).

Table 13. Nutritional composition of the experimental diets

TABLE 13

NUTRITIONAL COMPOSITION OF THE EXPERIMENTAL DIETS

Group Code	Group Name	Dietary fat	Ash	Protein	Fibre	CHO	Fat	TFA	Calories (kcal/g)
RG	Regular fat	4% pork/soy fat	6.17	21.0	3.8	56.1	7.52	0.0	3.08
ES	Elaidic Shortening	4% hydrogenated vegetable shortening rich in elaidic TFA	6.33	21.8	4.0	56.2	7.37	1.4	3.15
RB	Regular Butter	15% regular butter	5.22	19.2	3.2	50.1	17.73	0.3	3.66
VB	Vaccenic Butter	15% butter rich in vaccenic TFA	5.50	19.5	3.3	49.9	17.56	1.4	3.67
CH	Cholesterol (CH)	2% CH + 4% pork/soy fat	5.97	20.5	3.8	55.0	9.49	0.0	3.19
CH+ES	CH + Elaidic Shortening	2% CH + 4% hydrogenated vegetable shortening rich in elaidic TFA	5.88	20.2	4.3	54.8	9.51	1.4	3.17
CH+RB	CH +Regular Butter	2% CH + 15% regular butter	5.04	18.8	3.0	49.7	18.23	0.3	3.66
CH+VB	CH + Vaccenic Butter	2% cholesterol + 15% butter rich in vaccenic TFA	5.28	19.5	3.4	49.4	17.92	1.4	3.67

Ash, protein, fibre, CHO, and fat (g/100g diet) are represented as a percent of total nutrients, measured by proximate analysis (as fed). TFA content (g/100g diet)was estimated based on the addition of 4% partially hydrogenated shortening (ES) containing 35% TFA content, 15% regular butter (RB) containing 2.4% TFA content, or 15% modified vaccenic acid rich butter (VB) containing 10% TFA content, or to the diets. Abbreviations: Protein = Crude Protein; Fibre = Crude Fibre; CHO = Carbohydrates; Fat = Crude Fat; Calories = Digestible Energy (kcal/g); and TFA = *Trans* fatty acids

Table 14. Fatty acid composition of the regular butter (RB) and the vaccenic acid rich butter (VB)

TABLE 14

FATTY ACID COMPOSITION OF THE REGULAR BUTTER (RB) AND THE VACCENIC ACID RICH BUTTER (VB)
(g/100g)

Fatty Acid	Geometric Isomer	Common Name	**RB** Regular butter	**VB** Vaccenic butter
C4:0			3.80	4.31
C6:0			2.07	1.95
C8:0			1.22	1.01
C10:0			2.67	1.96
C12:0			3.10	2.17
C14:0			9.75	7.78
C15:0			1.18	0.85
C16:0			26.16	18.12
C17:0			2.38	1.59
C18:0			8.02	10.12
C20:0			0.14	0.12
C14:1			0.95	0.84
C16:1			1.59	1.27
C18:1	*cis* 9	Oleic acid	15.04	18.02
C18:1	*cis* 11		0.44	0.44
C18:1	*cis* 12		0.17	0.79
C18:1	*cis* 13		0.06	0.10
C18:1	*cis* 15		0.13	0.09
C18:1	*trans* 5		0.03	0.04
C18:1	*trans* 6-8		0.21	0.55
C18:1	*trans* 9	Elaidic acid	0.17	0.50
C18:1	*trans* 10		0.21	1.06
C18:1	*trans* 11	Vaccenic acid	1.07	4.67
C18:1	*trans* 12		0.31	1.02
C18:1	*trans* 14		0.00	1.17
C18:1	*trans* 15		0.21	0.46
C18:1	*trans* 16		0.23	0.46
C18:2	*cis* 6	Linoleic acid (LA)	1.68	2.34
C18:2	*cis* 9		0.05	0.16
C18:2	*trans* 6		0.01	0.36
C18:2	*trans* 9		0.00	0.10
C18:2	*trans* 11		0.12	0.08
C18:2	9*c*, 11*t*	Conjugated linoleic acid (CLA)	0.50	2.10
C18:3	9*c*, 11*t*, 13*c*	Conjugated alpha-linolenic acid (CLnA)	0.06	0.04
C18:3	*cis* 9	Alpha-linolenic acid (ALA)	0.59	0.30
C22:5			0.10	0.08
C22:6		Docosahexaenoic acid (DHA)	0.12	0.06
Total fatty acids		**g/100 g**	**84.55**	**87.12**
***Trans* fatty acids**		**g/100 g**	**2.44**	**9.95**

Abbreviations: t, *trans*; c, *cis*.

Fatty acid profile of the experimental diets

The experimental diets differed in fatty acid composition (Figure 44). Noteworthy differences include, a general increase in SFA and MUFA levels in the butter groups (RB, VB, CH+RB and CH+VB) and a reduction in linoleic and linolenic PUFAs (C18:2 and C18:3) in the diets supplemented with either vegetable shortening (ES) or butter. The regular fat groups (RG and CH) contained virtually no TFAs. The addition of commercially hydrogenated vegetable shortening (ES) significantly raised the C18:1 *trans* isomer content in the diet. The addition of regular butter in the diet resulted in a moderate increase in all TFA isomers. Substituting the regular butter for a butter rich in vaccenic acid raised the content of C18:1*t*6 and C18:1*t*9 TFA isomers to a similar extent as did the hydrogenated shortening (ES) diets and resulted in a fourfold increase in vaccenic acid (C18:*1t* VA) levels.

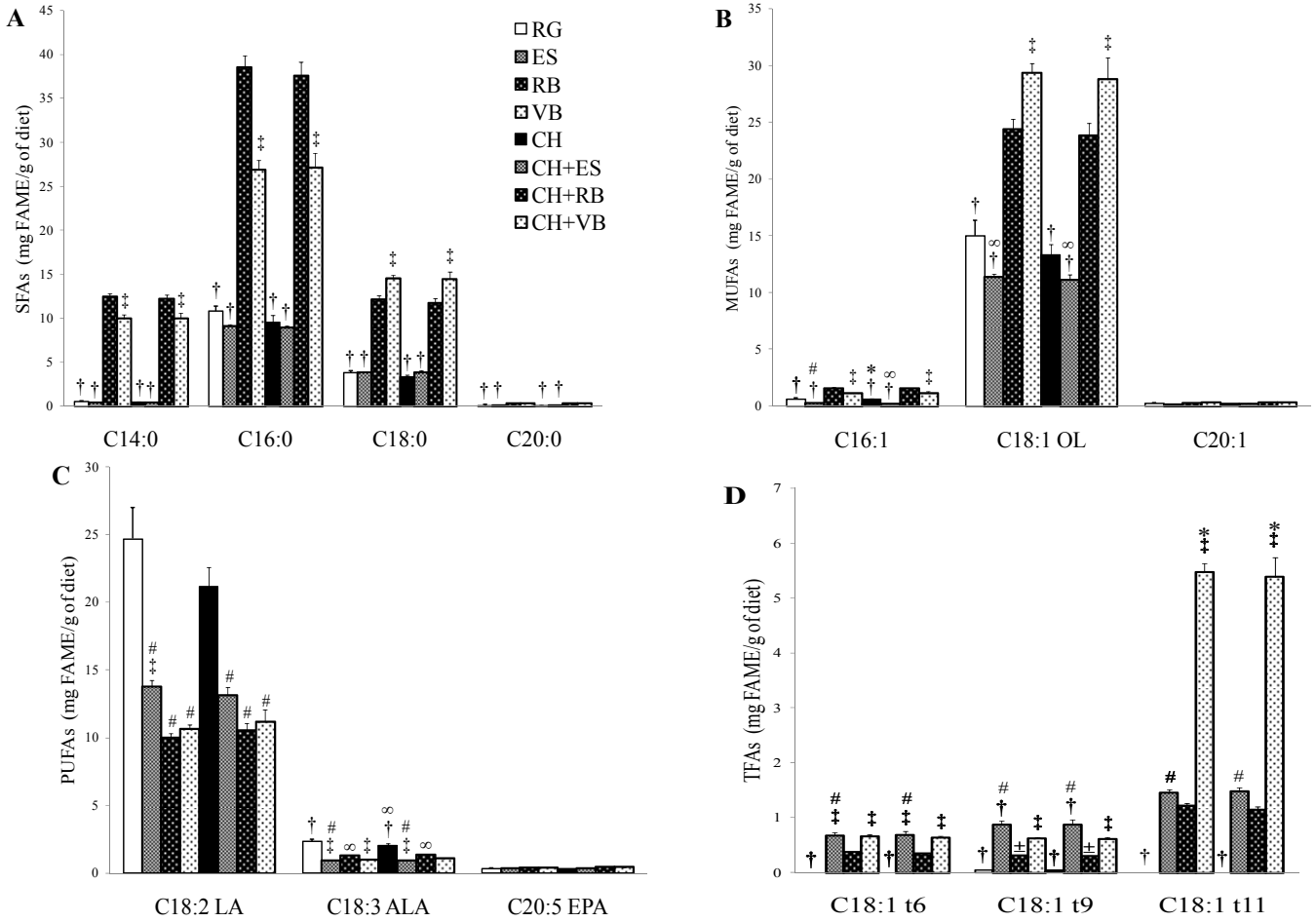


Figure 44. Fatty acid composition of the experimental diets fed to LDLr-/- mice for 14 weeks

Levels of saturated (SFA) (A), monounsaturated (MUFA) (B), polyunsaturated (PUFA) (C), and t*rans* (TFA) (D) fatty acids in the experimental diets (mg/g diet). Values are means ± SE; n = 3. **P* < 0.05 vs. ES and CH+ES groups. $^{\dagger}P$ <0.05 vs. RB, VB, CH+RB and CH+VB groups. $^{\#}P$<0.05 vs. RG and CH groups. $^{\ddagger}P$ <0.05 vs. RB and CH+RB groups. $^{\infty}P$ <0.05 vs. RG group. $^{\pm}P$ <0.05 vs. VB group. Only trace amounts (<0.01 mg/mL) of C13:0, C14:1, C16:1*t*, C17:0, C18:2*tt*, C18:3 GLA, C20:3^{11,14,17}, C20:3^{8,11}, C22:1 and C22:6 DHA were detectable (data not shown). Abbreviations: RG, regular fat diet; ES, elaidic shortening diet; RB, regular butter diet; VB, vaccenic butter; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening diet; CH+RB, cholesterol + regular butter; CH+VB, cholesterol + vaccenic butter diet. OL, oleic acid; LA, linoleic acid; GLA, gamma linolenic acid; AA, arachadonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Food intake and weight gain

Although the animals were provided with the same amount of chow per day (4 grams/mouse/day), food intake varied slightly between the groups. The RG, ES, CH, CH+ES, and CH+RB groups all consumed significantly more chow daily than the RB group. The CH and CH+RB groups also consumed more chow than the mice in the VB and CH+VB groups (Figure 45). Food intake may have been affected by taste or due to the slight difference in caloric intake provided by each of the diets. The amount of food consumed daily did not relate to the amount of weight gain as a percent of initial body weight in the animals. The higher caloric content of the butter diets (0.4 kcal/g) appeared to be a more important factor relating to weight gain over the course of the study. Weight gain was significantly higher in the butter groups (RB, VB, CH+RB, and CH+VB) as compared to the RG, ES, and CH groups. The CH+ES group gained a modest, but significantly lower amount than the VB group (Figure 46).

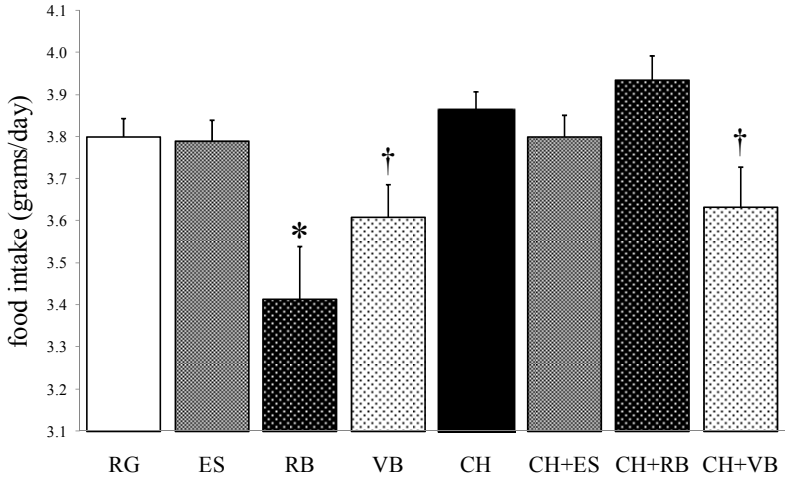


Figure 45. Average food intake in LDLr-/- mice following 14 weeks of dietary treatment

Average daily food intake in LDLr$^{-/-}$ mice during 14 weeks of dietary interventions. Values are means ± SE. *n* = 5. *P < 0.05 versus RG, ES, CH, CH+ES, and CH+RB. † P < 0.05 versus CH and CH+RB. Abbreviations: RG, regular fat diet; ES, elaidic shortening (iTFA) diet; RB, regular butter diet; VB, vaccenic butter (rTFA) diet; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening (iTFA) diet; CH+RB, cholesterol + regular butter diet; CH+VB, cholesterol + vaccenic butter (rTFA) diet; iTFA, industrially hydrogenated *trans* fatty acid; rTFA, ruminant (natural) *trans* fatty acid.

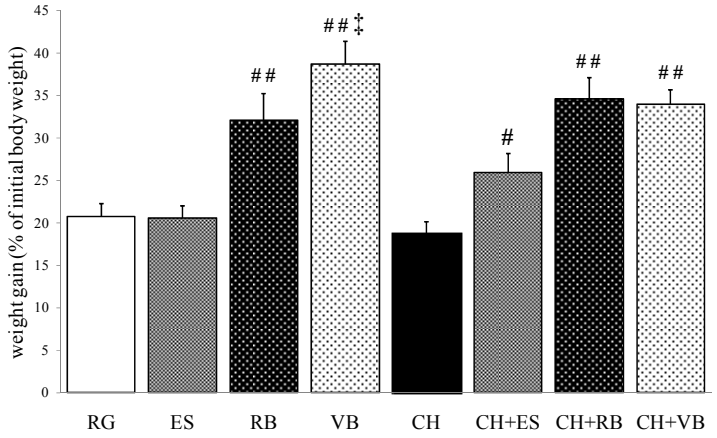


Figure 46. Weight gain in LDLr-/- mice following 14 weeks of dietary treatment

Weight gain in LDLr$^{-/-}$ mice following 14 weeks of dietary interventions, as calculated by terminal fasted body weight (g) - initial fasted body weight (g)/ initial fasted body weight (g) x 100. Values are means ± SE. $n = 5$. $^{\#}P < 0.05$ versus RG, ES, and CH groups. $^{\#\#}P < 0.05$ versus RG, ES, CH, and CH+ES groups. $^{\ddagger}P < 0.05$ versus RB group. Abbreviations: RG, regular fat diet; ES, elaidic shortening diet; RB, regular butter diet; VB, vaccenic butter diet; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening diet; CH+RB, cholesterol + regular butter diet; CH+VB, cholesterol + vaccenic butter diet.

Circulating fatty acid profile

Differences in the plasma fatty acid profile of the animals following the 14 week feeding period were observed (Figure 47). Supplementing the diet with cholesterol resulted in higher circulating levels of SFAs (C16:0 and C18:0), MUFAs (C16:1 and C18:1) and PUFAs (C18:2 LA, C18:3 ALA and C20:5 EPA) than the same diets without cholesterol. Cholesterol alone did not result in a rise in circulating levels of TFA isomers. TFAs are only present in the circulation if consumed. Cholesterol however did roughly triple the levels of circulating TFAs isomers when combined in the diet with a source of TFAs (ES, RB and VB). The CH+ES diet resulted in the highest circulating levels of elaidic acid (C18:1*t*9), whereas the CH+VB diet resulted in the highest levels of vaccenic acid (C18:*1t* VA).

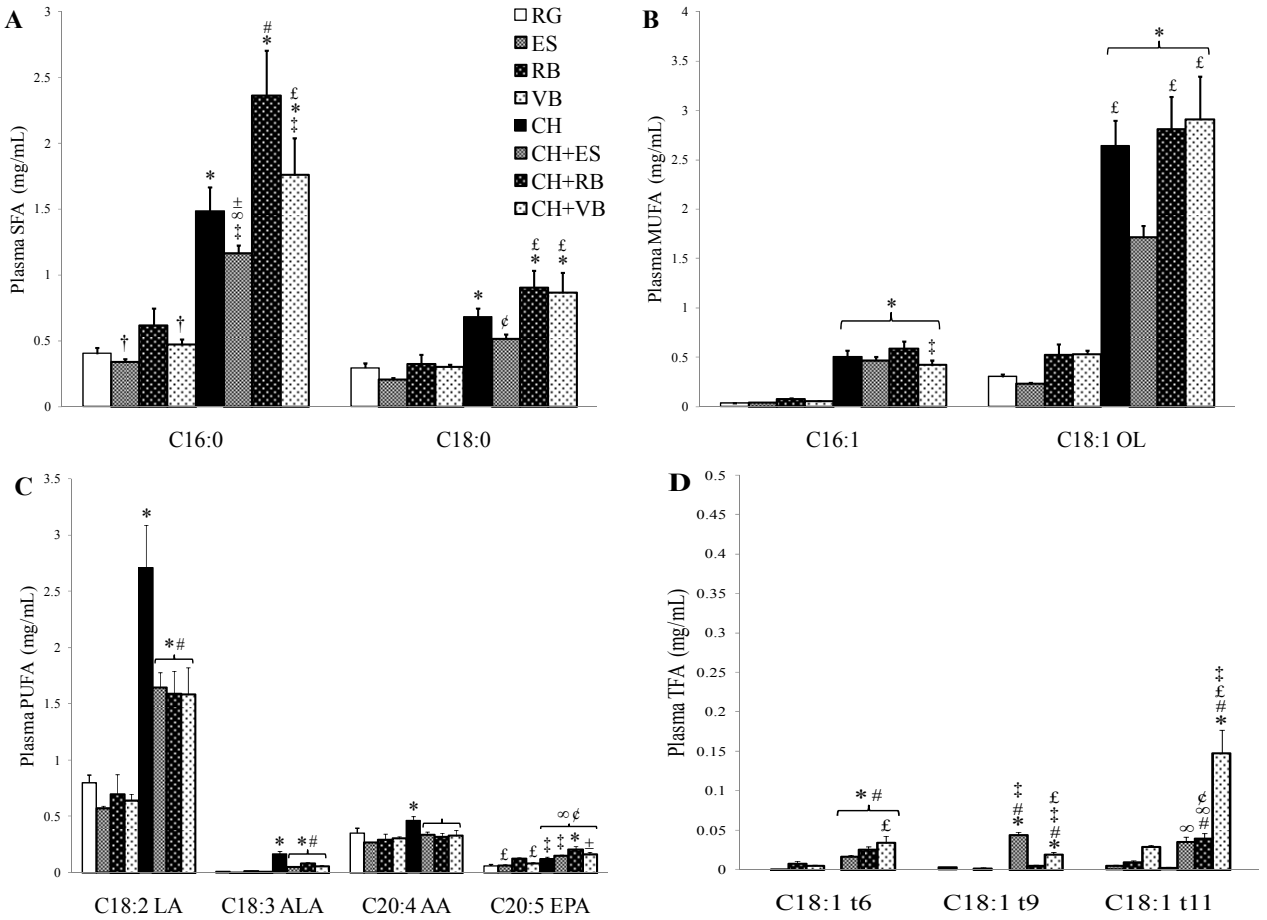


Figure 47. Circulating fatty acid levels in LDLr-/- mice following 14 weeks of dietary treatment

Saturated (SFA) (A), monounsaturated (MUFA) (B), polyunsaturated (PUFA) (C), and t*rans* (TFA) (D) fatty acids were quantitated (mg/mL) in plasma from LDLr$^{-/-}$ mice following 14 weeks of dietary interventions. Values are means ± SE; n = 4-5. $^{*}P < 0.05$ vs. RG, ES, RB and VB groups. $^{\dagger}P < 0.05$ vs. CH, CH+ES, CH+RB and CH+VB groups. $^{\ddagger}P < 0.05$ vs. CH+RB group. $^{£}P < 0.05$ vs. CH+ES group. $^{\#}P < 0.05$ vs. CH group. $^{\pm}P < 0.05$ vs. VB group. $^{\S}P < 0.05$ vs. RG, ES, RB and CH groups. $^{¢}P < 0.05$ vs. ES group. $^{\infty}P < 0.05$ vs. RG group. Only trace amounts (<0.01 mg/mL) of C13:0, C14:0, C14:1, C16:1*t*, C17:0, C18:2*tt*, C18:3 GLA, C20:0, C20:1, C20: 3[11,14,17], C20: 3[8,11], C22:0, C22:1 and C22:6 DHA were detectable (data not shown). Abbreviations: RG, regular fat diet; ES, elaidic shortening diet; RB, regular butter diet; VB, vaccenic butter; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening diet; CH+RB, cholesterol + regular butter; CH+VB, cholesterol + vaccenic butter diet; OL, oleic acid; LA, linoleic acid; GLA, gamma linolenic acid; AA, arachadonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Circulating cholesterol and triglyceride levels

As expected, the inclusion of cholesterol in the diet induced a significant increase in serum cholesterol levels (Figure 48). Serum cholesterol levels were also slightly, but significantly elevated in both the butter groups in the absence of dietary cholesterol (RB and VB groups) as compared to the RG and ES groups. The addition of either manufactured or dairy TFAs did not raise serum cholesterol levels beyond the levels in the cholesterol group alone. Manufactured TFAs did not significantly change serum cholesterol levels as compared to its respective internal control group (ES vs. RG or CH+ES vs. CH). In contrast, dairy TFAs partially mitigated the cholesterol and butter induced rise in cholesterol levels, as compared to the CH+RB group.

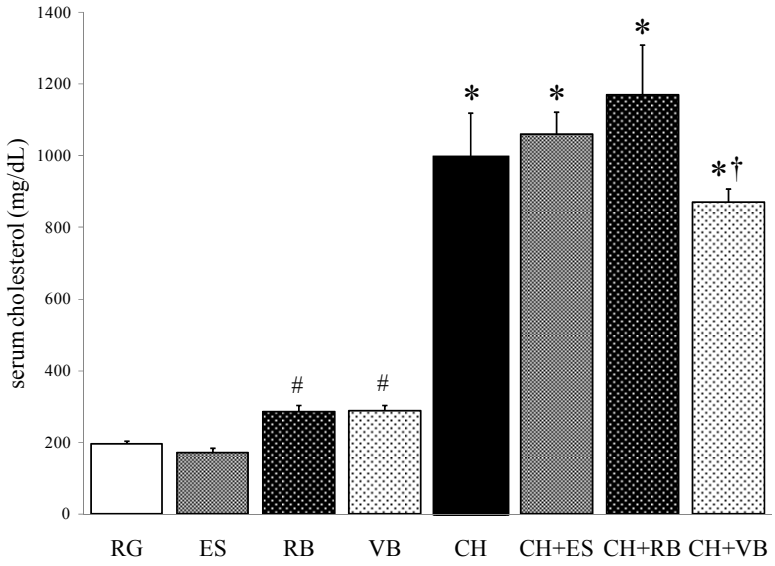


Figure 48. Serum cholesterol levels in LDLr-/- mice following 14 weeks of dietary treatment

Values are means ± SE, *n*=5. $^{\#}P < 0.05$ versus RG and ES groups. *P < 0.05 versus RG, ES, RB and VB groups. $^{\dagger}P < 0.05$ versus CH+RB group. Abbreviations: RG, regular fat diet; ES, elaidic shortening diet; RB, regular butter diet; VB, vaccenic butter; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening diet; CH+RB, cholesterol + regular butter; CH+VB, cholesterol + vaccenic butter diet.

Serum triglyceride levels were slightly elevated in the RB group as compared to the RG and ES groups, but the VB group was only slightly elevated as compared to the ES group (Figure 49). Serum triglyceride levels were highest in the groups consuming a combination of butter and cholesterol. Dairy TFAs (CH+VB) partially mitigated the rise in serum triglyceride levels as compared to the diet with cholesterol and regular butter (CH+RB).

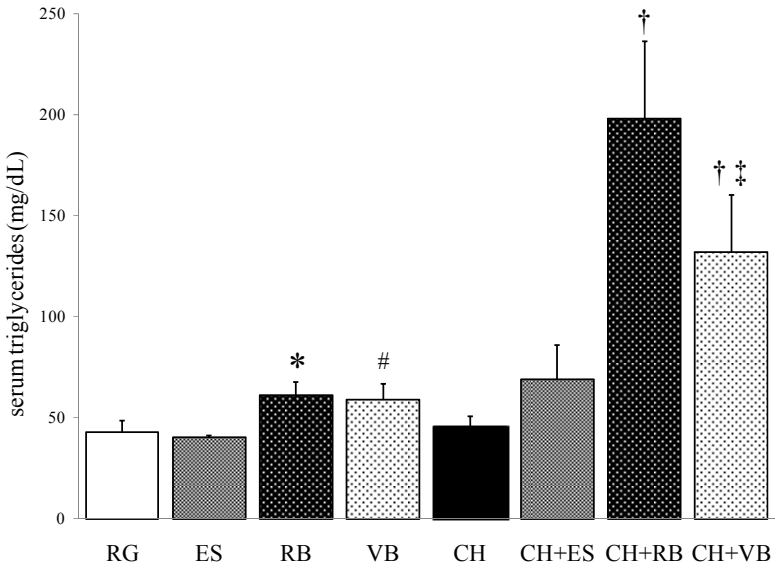


Figure 49. Serum triglyceride levels in LDLr-/- mice following 14 weeks of dietary treatment

Values are means ± SE, $n = 5$. *P < 0.05 versus RG and ES groups. $^{\#}$P < 0.05 versus ES group. †P < 0.05 versus RG, ES, RB, VB, CH and CH+ES groups. ‡P < 0.05 versus CH+RB group. Abbreviations: RG, regular fat diet; ES, elaidic shortening diet; RB, regular butter diet; VB, vaccenic butter; CH, 2% cholesterol diet; CH+ES, cholesterol + elaidic shortening diet; CH+RB, cholesterol + regular butter; CH+VB, cholesterol + vaccenic butter diet.

Aortic atherosclerotic development

The results from many animals on the extent of atherosclerotic lesions developed on the aortic luminal surface following 14 weeks of dietary intervention were pooled and are shown in Figure 50. Mice fed a control diet (RG) did not exhibit appreciable atherosclerotic plaque formation. Including a source of manufactured hydrogenated vegetable shortening (ES) stimulated atherosclerotic development on its own, an event not normally observed in the $LDLr^{-/-}$ mouse unless it is fed dietary cholesterol or a strongly atherogenic diet. The addition of either RB or VB butters to the diet did not significantly stimulate atherosclerotic development beyond that observed in the control group (RG). The inclusion of cholesterol in the diet of the $LDLr^{-/-}$ mice induced a significant atherogenic action in comparison to the control diets. Substituting the fat source for either elaidic shortening or regular butter in the cholesterol-supplemented, atherogenic diet did not have an additional effect on atherosclerotic development. Therefore, consuming TFAs from a manufactured elaidic acid-rich hydrogenated vegetable shortening stimulated atherosclerotic development, but not beyond the effects of dietary cholesterol. Conversely, consuming a vaccenic acid rich butter in the presence of dietary cholesterol protected against atherosclerotic development.

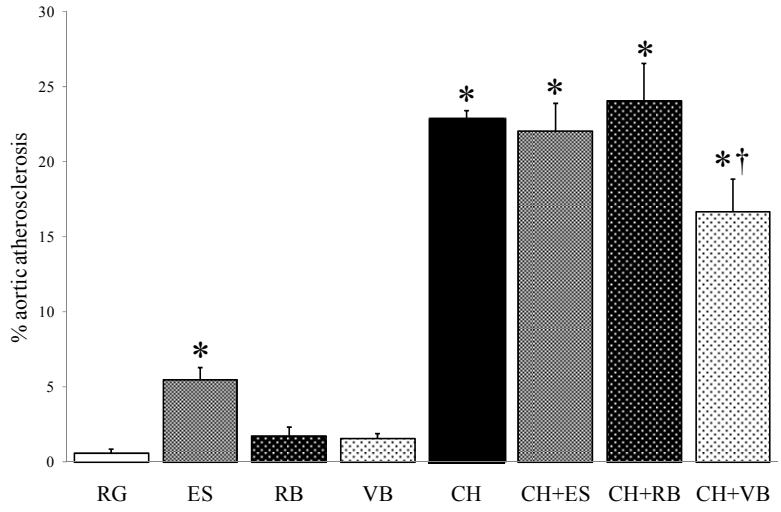


Figure 50. Extent of aortic atherosclerotic lesions in LDLr-/- mice following 14 weeks of dietary treatment

The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE. *P < 0.05 versus RG, RB, and VB groups. †P < 0.05 versus CH, CH+ES, and CH+RB groups. Abbreviations: RG, regular fat diet (*n* = 5); ES, elaidic shortening diet (*n* = 5); RB, regular butter diet (*n* = 5); VB, vaccenic butter (*n* = 5); CH, 2% cholesterol diet (*n* = 5); CH+ES, cholesterol + elaidic shortening diet (*n* = 5); CH+RB, cholesterol + regular butter (*n* = 5); CH+VB, cholesterol + vaccenic butter diet (*n* = 5).

E. Effects of combining dietary flaxseed and *trans* fatty acids on atherosclerotic development in the LDLr$^{-/-}$ mouse

Experimental diets

The nutritional composition of the 14 experimental diets is listed in Table 15. The diets were created to test the antiatherogenic potential of whole ground flaxseed or one of its components, flaxseed oil (ALA), dietary fibre (partially defatted flaxseed meal - PDFM) or lignan, in the presence of cholesterol and TFAs. Three diets containing regular fat (LR, HR and CR) and three diets containing TFAs (LT, HT and CT) were created to serve as internal controls for the eight diets containing flaxseed (TF, HTF and CTF) or one of the components of flaxseed (TALA, HTALA, CTALA, CTP and CTL). The

source of TFAs for all of the diets with a T in the name is industrially produced partially hydrogenated vegetable shortening rich in elaidic *trans* fatty acid (iTFA). The grain content in each of the diets contributed a small amount of dietary fat. As detailed in Table 15, doubling the amount of added fat increased the dietary fat content by approximately 50%, and adding cholesterol to the diet added approximately 25% more dietary fat. Adding 10% whole ground flaxseed or 4.4% flaxseed oil (ALA) increased the fat and caloric content as compared to their respective control groups. The diets containing partially defatted flaxseed meal (CTP) and lignan (CTL) contained a similar fat content to the diets with cholesterol and TFAs. The LT, TF, TALA, CT, CTF, CTALA, CTP and CTL diets contained 1.4% iTFAs, which relates to approximately 3.2% of caloric energy as TFA intake. The HT, HTF and HTALA diets contained 2.8% TFA, which provides approximately 6.4% of caloric energy as TFA intake. The ash, protein, fibre and carbohydrate content of all of the diets were controlled and similar amongst the groups. Despite an increase in dietary fat in the higher fat groups, the digestible energy levels provided by the diets were all in a similar range (within 3.5 and 3.9 kcal/gram).

Table 15. Nutritional composition of the experimental diets

TABLE 15

NUTRITIONAL COMPOSITION OF THE EXPERIMENTAL DIETS

Group Code	Group Name	Dietary fat	Ash	Prot	CHO	Fat	TFA	Calories (kcal/g)
LR	Low Regular fat	4% pork/soy fat	6.17	21.0	56.1	7.52	0.0	3.5
LT	Low *Trans* fat	4% hydrogenated vegetable shortening (HVS)	6.33	21.8	56.2	7.37	1.4	3.5
TF	*Trans* fat + Flax	4% HVS + 10% ground flaxseed	5.70	23.0	50.2	12.10	1.4	3.7
TALA	*Trans* fat + ALA	4% HVS + 4.4% flaxseed oil (ALA)	5.80	22.1	51.2	12.70	1.4	3.7
HR	High Regular fat	8% pork/soy fat	5.71	19.8	54.7	11.21	0.0	3.7
HT	High *Trans* fat	8% HVS	5.94	19.5	54.5	10.78	2.8	3.7
HTF	High *Trans* fat + Flax	8% HVS + 10% ground flaxseed	5.30	23.0	47.2	15.80	2.8	3.9
HTALA	High *Trans* fat + ALA	8% HVS + 4.4% flaxseed oil (ALA)	5.40	22.3	47.4	13.80	2.8	3.9
CR	CH + Regular fat	2% cholesterol + 4% pork/soy fat	5.97	20.5	55.0	9.49	0.0	3.5
CT	CH + *Trans* fat	2% cholesterol + 4% HVS	5.88	20.2	54.8	9.51	1.4	3.7
CTF	CH + *Trans* fat + Flax	2% cholesterol + 4% HVS + 10% ground flaxseed	5.59	20.9	52.0	12.20	1.4	3.7
CTALA	CH + *Trans* fat + ALA	2% cholesterol + 4% HVS + 4.4% flaxseed oil (ALA)	5.60	20.5	50.7	15.00	1.4	3.8
CTP	CH + *Trans* fat + PDFM	2% cholesterol + 4% HVS + 7% partially defatted flaxseed meal	6.17	22.3	53.8	8.81	1.4	3.5
CTL	CH + *Trans* fat + Lignan	2% cholesterol + 4% HVS + 0.44% lignan	5.86	21.2	55.4	8.25	1.4	3.5

Ash, protein, fibre, CHO, and fat (g/100g diet) are represented as a percent of total nutrients, measured by proximate analysis (as fed). TFA content (g/100g diet) was estimated based on the addition of 4% partially hydrogenated shortening (ES) containing 35% TFA content to the diets. Abbreviations: Prot = Crude Protein; Fibre = Crude Fibre; CHO = Carbohydrates; Fat = Crude Fat; Calories = Digestible Energy (kcal/g); and TFA = *Trans* fatty acids

Circulating cholesterol and triglyceride levels

As expected, the inclusion of cholesterol in the diet induced a significant increase in serum cholesterol levels (Figure 51). Serum cholesterol levels were also slightly, but significantly elevated in the HTF group as compared to the LT group. The addition of either one of the three components of flaxseed, ALA, dietary fibre (PDFM) or lignan reduced circulating cholesterol levels as compared to the CR and CT groups. Surprisingly the addition of whole ground flaxseed reduced serum cholesterol levels, but not to a significant extent. The addition of ALA and PDFM, but not lignan, resulted in significantly lower cholesterol levels than the CTF group.

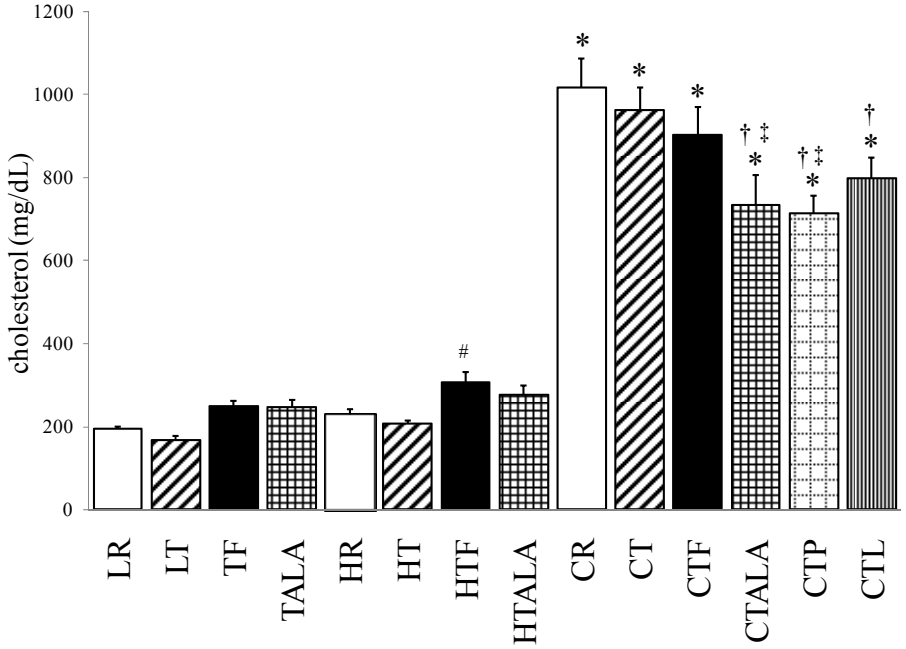


Figure 51. Serum cholesterol levels in LDLr-/- mice following 14 weeks of dietary treatment

Values are means ± SE. *P < 0.05 vs. LR, LT, TF, TALA, HR, HT, HTF and HTALA groups; †P < 0.05 vs. CR and CT groups; ‡P < 0.05 vs. CTF group; #P < 0.05 vs. LT group; Abbreviations: LR, low regular fat (n=9); LT, low *trans* fat (n=10); TF, low *trans* fat + 10% flaxseed (n=9); TALA, low *trans* fat + 4.4% ALA (n=7); HR, high regular fat (n=9); HT, high *trans* fat (n=10); HTF, high *trans* fat + 10% flaxseed (n=9); HTALA, high *trans* fat + 4.4% ALA (n=9); CR, 2% cholesterol + low regular fat (n=9); CT, 2% cholesterol + low *trans* fat (n=10); CTF, 2% cholesterol + low *trans* fat + 10% flaxseed (n=10); CTALA, 2% cholesterol + low *trans* fat + 4.4% ALA (n=9); CTP, 2% cholesterol + low *trans* fat + 7% partially defatted flaxseed meal (n=10); CTL, 2% cholesterol + low *trans* fat + 0.44% lignan (n=9).

Serum triglyceride (TG) levels were significantly elevated in the flaxseed, ALA, and PDFM groups (TALA, HTF, HTALA, CTF, CTALA and CTP), but not in the lignan (CTL) group (Figure 52). Serum TG levels were higher in the CTF group as compared to all other groups. However, serum TG levels were lower in all groups with cholesterol, iTFA and isolated flaxseed compounds (ALA, fibre, lignan) as compared to the whole ground flaxseed group.

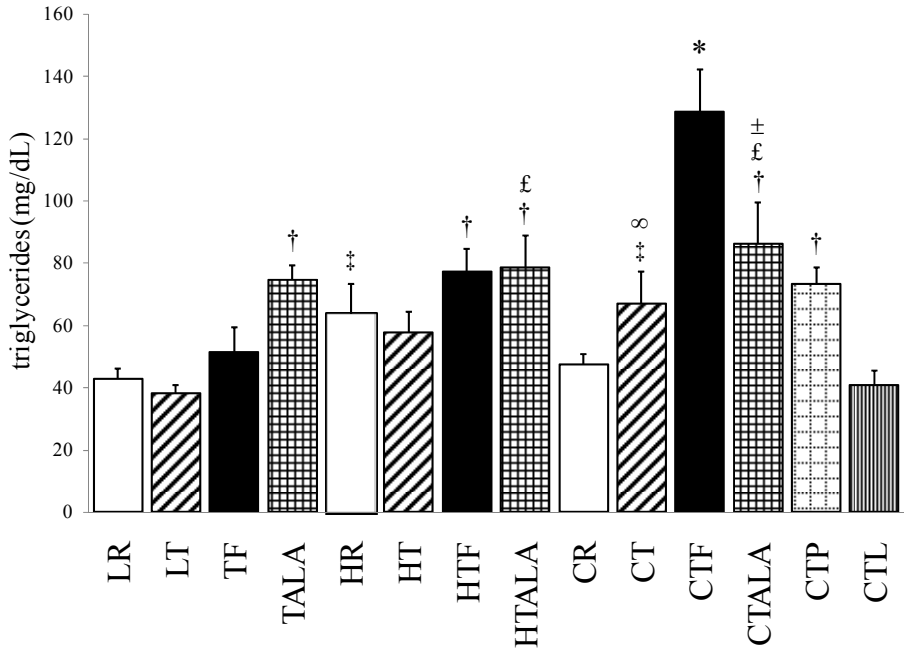


Figure 52. Serum triglyceride levels in LDLr-/- mice following 14 weeks of dietary treatment

Values are means ± SE. *P < 0.05 CTF vs. all other groups; †P < 0.05 vs. LR, LT, CR and CTL groups; ‡P < 0.05 vs. LT and CTL groups; ∞P < 0.05 vs. LR group; £P < 0.05 vs. TF group; ±P < 0.05 vs. HT group; Abbreviations: LR, low regular fat chow (n=10); LT, low *trans* fat chow (n=10); TF, low *trans* fat + 10% flaxseed chow (n=6); TALA, low *trans* fat + 4.4% ALA chow (n=10); HR, high regular fat chow (n=10); HT, high *trans* fat chow (n=10); HTF, high *trans* fat + 10% flaxseed chow (n=10); HTALA, high *trans* fat + 4.4% ALA chow (n=10); CR, 2% cholesterol + low regular fat chow (n=9); CT, 2% cholesterol + low *trans* fat chow (n=10); CTF, 2% cholesterol + low *trans* fat + 10% flaxseed chow (n=10); CTALA, 2% cholesterol + low *trans* fat + 4.4% ALA chow (n=10); CTP, 2% cholesterol + low *trans* fat + 7% partially defatted flaxseed meal chow (n=10); CTL, 2% cholesterol + low *trans* fat + 0.44% lignan chow (n=10).

Aortic atherosclerotic development

The extent of atherosclerotic lesions developed on the aortic luminal surface of $LDLr^{-/-}$ mice following 14 weeks of dietary intervention are shown in Figure 53. Mice fed a control diet (LR) did not exhibit appreciable atherosclerotic plaque formation. Increasing the fat content in the diet resulted in a small but significant increase in atherosclerotic lesions. Substituting the fat source with industrially produced hydrogenated vegetable shortening (iTFA) stimulated atherosclerotic development in a dose dependent manner (LT and HT). Adding whole ground flaxseed or flaxseed oil (ALA) to diets containing low and high doses of iTFAs (TF, TALA, HTF and HTALA) completely prevented atherosclerotic development. Including cholesterol in the diet of the $LDLr^{-/-}$ mice induced a significant atherogenic effect. As previously demonstrated, adding iTFAs in the atherogenic diet did not stimulate atherosclerosis beyond the cholesterol mediated atherogenic action. Adding whole ground flaxseed to a diet with cholesterol and iTFAs (CTF) partially inhibited atherosclerotic development. Conversely, the dietary fibre and lignan content of flaxseed had no appreciable effect on atherosclerosis. However, adding flaxseed oil to the diet significantly inhibited atherosclerotic lesion development. Flaxseed oil reduced the extent of developed atherosclerotic lesions to similar levels as seen in the groups fed iTFAs or higher doses of fat with no added cholesterol (LT, HR and HT).

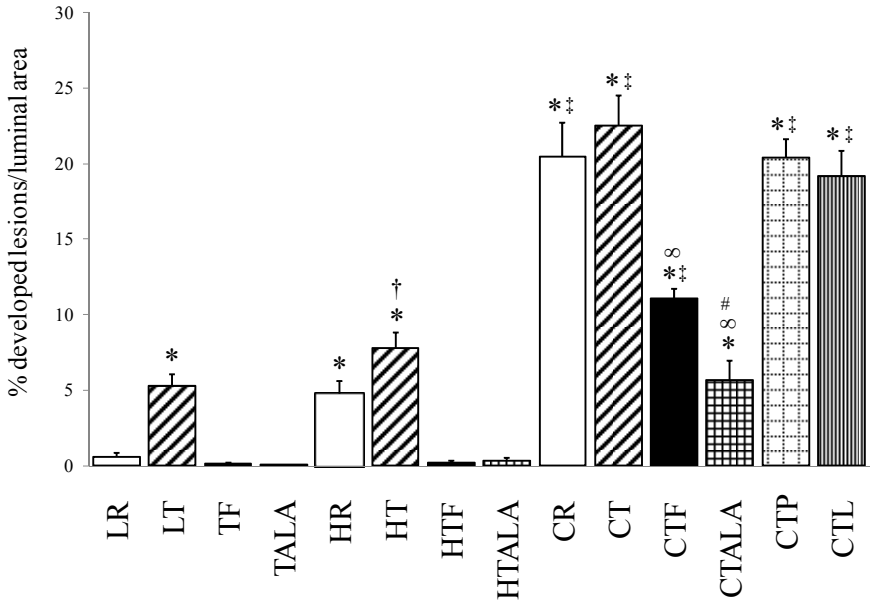


Figure 53. Extent of aortic atherosclerotic lesions in LDLr-/- mice following 14 weeks of dietary treatment

The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SE. *P < 0.05 vs. LR, TF, TALA, HTF and HTALA groups. †P < 0.05 vs. LT and HR groups. ‡P < 0.05 vs. LT, HR and HT groups. ∞P < 0.05 vs. CR, CT, CTP and CTL groups. #P < 0.05 vs. CTF group. Abbreviations: LR, low regular fat chow (n=5); LT, low *trans* fat chow (n=5); TF, low *trans* fat + 10% flaxseed chow (n=8); TALA, low *trans* fat + 4.4% ALA chow (n=7); HR, high regular fat chow (n=5); HT, high *trans* fat chow (n=5); HTF, high *trans* fat + 10% flaxseed chow (n=7); HTALA, high *trans* fat + 4.4% ALA chow (n=7); CR, 2% cholesterol + low regular fat chow (n=10); CT, 2% cholesterol + low *trans* fat chow (n=10); CTF, 2% cholesterol + low *trans* fat + 10% flaxseed chow (n=10); CTALA, 2% cholesterol + low *trans* fat + 4.4% ALA chow (n=7); CTP, 2% cholesterol + low *trans* fat + 7% partially defatted flaxseed meal chow (n=10); CTL, 2% cholesterol + low *trans* fat + 0.44% lignan chow (n=10).

CHAPTER VI: DISCUSSION

A. Effects of dietary flaxseed on atherosclerotic development and endothelial function in hypercholesterolemic rabbits

Long–term flaxseed supplementation resulted in a significant elevation of ALA levels in the plasma and aortic tissue (Tables 9 and 10). This elevation occurred in the absence and presence of additional dietary cholesterol. Although the plasma concentration of the longer-chain omega-3 fatty acid EPA was only detected in low levels with cholesterol feeding following the 16 week trial, EPA and DHA were both detected in the aortic tissue of rabbits consuming flaxseed and cholesterol following 8 weeks of feeding. The highest levels were seen in animals consuming a combination of cholesterol and flaxseed following the longer feeding trials. These results demonstrate that ALA is metabolized to a small extent to longer-chain fatty acids in the rabbit. Flaxseed supplementation also reduced the ω-6 to ω-3 PUFA ratio, primarily as a result of the elevated levels of circulating ω-3 PUFAs. The addition of dietary flaxseed to the atherogenic diet also mitigated the cholesterol induced rise in plasma triglyceride levels. These results are consistent with previous work from our laboratory using this dietary intervention [146].

Our findings that dietary flaxseed can inhibit the development of atherosclerotic lesions on the aortic luminal surface are consistent with previous reports [121, 134-137]. Because dietary flaxseed supplementation did not alter circulating cholesterol levels, either in the presence or in the absence of additional cholesterol in the diet, it is clear that its anti-atherogenic effects were not achieved through a cholesterol-lowering action. These effects are consistent with previous studies [85, 88, 135, 146, 239] but in conflict

with other reports that found that flaxseed supplementation reduces circulating cholesterol levels [121, 134, 136, 138]. Because the antiatherosclerotic effects of fibre are due to a cholesterol-lowering action, the high-fibre content of flaxseed, therefore, is unlikely to be responsible for the anti-atherogenic action in the present study. Alternatively, the lignan SDG in flaxseed has been shown to possess potent hypocholesterolemic and anti-atherogenic properties [121, 135, 240] and is the most likely component within flaxseed to be responsible for these beneficial effects. SDG is also a potent antioxidant [122, 241, 242]. Our results extend these findings to demonstrate that the protection afforded by dietary flaxseed was also observed in an important resistance artery, the carotid. Thus the anti-atherogenic effects of flaxseed on atherosclerosis may have implications for the pathogenesis of stroke as well as heart disease. Consistent with this finding, several clinical studies have shown negative correlations between plasma ALA levels with the incidence of stroke [243-245] and coronary heart disease [116, 118, 246].

Our research demonstrates that dietary flaxseed prevents cholesterol-induced atherosclerotic development in short term studies (up to 8 weeks) [247]. However, there are limits to the antiatherogenic capacity of flaxseed. The cholesterol and flax group developed extensive atherosclerotic lesions after prolonged periods of hypercholesterolemia. The extended duration of hypercholesterolemia appeared to overwhelm the beneficial effects of flaxseed supplementation.

The present study has demonstrated that dietary cholesterol had a deleterious effect on vascular contractile function. These findings are consistent with the impaired vascular response of vessels exposed to a high-cholesterol environment [248-252]. The

present results extend this to an attenuation of agonist-induced vascular contractility as well as an impairment of vascular relaxation. Because we observed defects in both KCl- and NE-induced vascular tension generation, this suggests that there is a general defect in smooth muscle function. Cholesterol-induced changes in ion transport pathways including the regulation of Ca^{2+} homeostasis in vascular smooth muscle cells (VSMCs) may represent the mechanism for this effect [253-258]. This may explain both the contraction and relaxation defects identified in the present study. However, three additional factors may also play a role in the depressed contractile response to KCl and NE. First, tension generation was measured as a function of tissue weight. Tissue weights of the aortic rings from the cholesterol-fed animals were higher than the non-cholesterol-fed animals because the plaque increased the vessel thickness. This increase in tissue weight would tend to artificially decrease tension/tissue weight. Second, the plaque found in the aortic rings would contain nonmuscular cell types such as fibroblasts, foam cells, and macrophages that would contribute to tissue weight but not to total tension generation. Third, atherosclerosis is known to transform the phenotype of existing VSMCs from a contractile to a synthetic phenotype [259-263]. This conversion results in a loss of contractile proteins. Synthetic SMCs are no longer transcriptionally programmed to support contractile activity, resulting in an impaired vascular response in atherosclerotic vessels.

This study is the first to describe the effects of flaxseed supplementation on vascular function in atherosclerotic vessels. The protective action of dietary flaxseed on vascular response was however selective. Flaxseed protected against the changes in vascular relaxation but did not protect against the contractile dysfunction induced by the

elevation in circulating cholesterol. The protective effect of dietary flaxseed on vascular relaxation was only attenuated following prolonged hypercholesterolemic conditions, under which flaxseed no longer prevented atherosclerotic development. Ω-3 fatty acids have been reported previously to improve vascular function by limiting the progression of atherosclerosis and reducing endothelial activation and vascular inflammation [264-267]. The protective effects on relaxation identified in our study clearly involved a selective, endothelium-dependent site of action. This finding is consistent with a study reporting that a high-flaxseed diet can enhance endothelial vasorelaxant function in hypertensive rats, without improving blood pressure [139]. The vascular response to SNP was not different in any of the groups, demonstrating that the endothelial-independent routes of modulating vascular relaxation were unaltered. There is no intrinsic change in relaxation capacity in response to nitric oxide (NO) from SNP, rather, the lesion appears to be due to the generation of NO by ACh. Because we did not observe any beneficial effects on vascular function of the flaxseed-enriched diet on its own compared with a regular diet, this would suggest that flaxseed is not altering the intrinsic characteristics of endothelial cells but instead selectively attenuates the detrimental changes induced by cholesterol. Flaxseed did not achieve this effect by altering the circulating cholesterol levels. In contrast, dietary flaxseed did increase levels of ω-3 PUFAs in the plasma and aortic tissue. The largest changes in PUFA concentrations were in ALA levels in the tissue and plasma compartments, with only minor changes in EPA and DHA levels. Therefore, it is reasonable to hypothesize that ALA induced the protective changes observed. In support of this contention, ALA levels have been shown to lower serum markers of vascular inflammation and endothelial activation [162, 268-271], reduce platelet aggregation

[272], and induce changes in intracellular Ca^{2+} movements [118, 146, 273]. Furthermore, circulating ALA levels have been positively associated with endothelium-dependent vasodilation in normocholesterolemic and hypercholesterolemic subjects [117, 274, 275]. Oxidative stress may be another important mechanistic factor in cholesterol-induced vascular contractile dysfunction [276-279] and in atherosclerosis [240, 280, 281]. For example, statins can improve endothelium-dependent vascular relaxation in hypercholesterolemic patients by decreasing oxidative stress [278]. It is possible, therefore, that the potent antioxidant capacity of flaxseed was responsible for its beneficial action on atherosclerosis and vascular relaxation [121, 241]. The lignan SDG found in flaxseed is thought to be responsible for this antioxidant action [121, 122, 242, 282, 283] and may also have contributed to its protective effect against the cholesterol induced defects in vascular relaxation.

In summary, our data demonstrate for the first time that flaxseed can protect against atherosclerotic plaque deposition in carotid arteries and confirm its anti-atherosclerotic effects in the aorta. We have also shown for the first time that dietary flaxseed can improve endothelium-dependent vascular relaxation in the presence of a high-cholesterol diet. However, dietary flaxseed is not a panacea. If the high-cholesterol diet is prolonged in duration, flaxseed eventually loses its ability to protect against cholesterol-induced lesions. Despite this limitation, our data reinforce the hypothesis that long-term supplementation of the diet with ground flaxseed may be an effective and safe dietary strategy to limit the early and moderate stages of atherogenesis and vascular dysfunction associated with atherosclerosis. The flaxseed dosage used in this investigation is similar to that used previously in human studies [127, 133]. Our results,

therefore, suggest that dietary modification with flaxseed may have an important protective action in humans against vascular disease in both the heart and in stroke.

B. Effects of flaxseed on atherosclerotic development in the LDLr$^{-/-}$ mouse

The dietary interventions used in this study induced significant changes in the plasma fatty acid profile of the LDLr$^{-/-}$ mice. Dietary flaxseed increased plasma ALA levels significantly, as would be expected due to the high ALA content of flaxseed. ALA was metabolized in the mice to the longer chain ω-3 fatty acid EPA, but not to DHA. This differs from the inability of rabbits to metabolize ALA derived from dietary flaxseed to the longer chain ω-3 fatty acids unless dietary cholesterol is provided [146, 247]. The inclusion of cholesterol in the diet with flaxseed resulted in a significant stimulation of ALA levels in the plasma of the LDLr$^{-/-}$ mice. This is similar to the response observed previously and likely represents an enhanced absorption of the fatty acid in the gastrointestinal tract [146, 247]. This stimulatory effect of dietary cholesterol on the entry of ALA into the plasma was not saturated because there was a clear dose dependent rise in ALA with increasing flaxseed concentrations (1-10%) despite the cholesterol level in the mouse chow remaining the same. It is also interesting to note that this stimulatory effect was relatively specific to the medium chain polyunsaturated fatty acid species since AA, EPA and DHA were not increased in the plasma when cholesterol was present in the diet.

The results of the present study demonstrate the anti-atherosclerotic effects of dietary flaxseed in the LDLr$^{-/-}$ mouse. This anti-atherogenic action of flaxseed has been shown previously in the cholesterol-fed rabbit model [121, 134, 135, 138, 247, 284].

However, in view of the limitations of the cholesterol-fed rabbit model of atherosclerosis [285, 286], it was important to confirm these findings in a model that more closely represents atherosclerosis in humans. Our data in the $LDLr^{-/-}$ mice now support the direct evaluation of the anti-atherogenic potential of dietary flaxseed in human trials where the effects of flaxseed on cardiovascular disease are not clear. Flaxseed supplementation (20-50 g/day) has been demonstrated to modestly reduce circulating total and LDL cholesterol levels and have no effect on HDL levels in healthy people [126, 127, 129, 132, 133]. Our results in the $LDLr^{-/-}$ mouse agree with this cholesterol-lowering effect observed in humans, but are in conflict with the results obtained in the cholesterol-fed rabbit where flaxseed did not lower circulating cholesterol levels [247]. This would again argue for the importance of using the data obtained in the $LDLr^{-/-}$ mice as a template for what may occur in the future during any dietary studies with flaxseed in humans. It is important to note however that lipoprotein distribution and metabolism may differ between rodents and humans, since circulating cholesterol in mice is predominantly carried by the HDL lipoprotein.

It is evident from the results of this study that the effects of flaxseed on circulating cholesterol and atherosclerosis are sensitive to the dosage of flaxseed employed. A ten fold reduction in the concentration of flaxseed given to the $LDLr^{-/-}$ mice (10-1%) did not significantly reduce its' capacity to lower circulating cholesterol levels, but it did reduce the ability of flaxseed to inhibit atherosclerotic development. This was similar when the flaxseed dosage was reduced by 50%. It appears that cholesterol levels must be lowered below a certain threshold level to see an anti-atherogenic effect. A 10% flaxseed supplemented diet is similar in energetic load to the 50 g/day dosage used in

human trials [129, 132]. This may suggest that flaxseed dosages near 50g/day may be required to inhibit atherosclerosis significantly in humans. Of course, this still requires direct evaluation in human trials but it does provide a useful starting point.

The mechanism for the anti-atherogenic action demonstrated by dietary flaxseed was investigated in the present study. The cholesterol-lowering effect of flaxseed is likely the main contributing factor to its anti-atherogenic potential, however, since atherosclerosis was only inhibited in animals fed a higher flaxseed dose another mechanism, likely cellular, may be responsible for this anti-atherogenic action. Our data reveals for the first time a significant anti-proliferative and anti-inflammatory action of flaxseed. Atherogenesis is thought to involve an inflammatory reaction [287] and accelerated cell proliferation in the region of the obstructive plaque [233, 288]. IL-6 has been implicated in the pathogenesis of atherosclerosis, including smooth muscle cell and fibroblast proliferation, oxidation of LDL cholesterol, activation of monocytes and macrophages, and amplification of the inflammatory cascade in atherogenesis [289-291]. VCAM-1 has been associated with key steps in atherogenesis, including monocyte recruitment and infiltration into the arterial wall and differentiation into macrophages. Flaxseed effectively inhibited the expression of inflammatory markers like IL-6, mac-3, VCAM-1 and the proliferative marker PCNA. Our data demonstrates for the first time that dietary flaxseed reduces the infiltration of macrophages into the subendothelial space and reduces the inflammatory and proliferative state of atherosclerotic lesions. These effects are likely due to the ω-3 fatty acid content of flaxseed. Dietary supplementation with ALA from flaxseed oil has been demonstrated to reduce circulating levels of several atherogenic and inflammatory markers including, C-reactive protein, serum amyloid A,

IL-6, and sVCAM-1 in dyslipidaemic patients [269, 270]. Ω-3 polyunsaturated fatty acids (ALA, EPA, and DHA) have been demonstrated to exert several direct anti-atherogenic properties, including anti-inflammatory [167, 269] and immunomodulatory effects [61, 292, 293], as well as the ability to inhibit leukocyte adhesion [61, 294], decrease the production of pro-inflammatory eicosanoids and inhibit cellular migration and proliferation [100, 295-297]. The anti-atherogenic effects described in this study may also be associated with the low ω-6 to ω-3 fatty acid ratio in the plasma of the flaxseed fed groups. Previous reports have shown that a low ω-6 to ω-3 fatty acid ratio is associated with low levels of circulating inflammatory markers [298], decreased production of pro-inflammatory eicosanoids [299, 300] and reduced atherosclerotic development [301].

In summary, dietary flaxseed can inhibit the atherogenic effects of a high cholesterol diet in the $LDLr^{-/-}$ mouse. The present investigation demonstrates that this effect is achieved through a capacity to lower circulating cholesterol levels and, at a cellular level, by inhibiting cell proliferation and inflammation. This lends further support to the hypothesis that nutritional interventions have the capacity to alter disease through an anti-inflammatory action [302]. Because this anti-atherogenic effect of flaxseed has now been shown in more than one animal model, and, because the $LDLr^{-/-}$ mouse is a close representation of the clinical condition of coronary heart disease in humans, our study argues strongly for the initiation of careful, randomized controlled trials of dietary flaxseed in a patient population with symptoms of atherosclerotic heart disease.

C. Effects of *trans* fatty acids on atherosclerotic development in the LDLr$^{-/-}$ mouse

TFAs are formed during the partial hydrogenation of liquid oils, forming semi-solid fats like margarine and shortening and are found in fried foods, cookies, donuts and crackers. The hydrogenation process reduces the number of double bonds in unsaturated fatty acids and thereby changes the original *cis* configuration of the double bonds into a *trans* configuration, thereby resulting in a more linear fatty acid. Epidemiological investigations have associated diets that contain higher levels of TFAs with a higher incidence of cardiovascular disease [19-23, 183] but there has never been a proven cause-and-effect relationship between dietary TFAs and atherosclerosis [19, 20, 303]. On the contrary, a high cholesterol/high TFA diet administered to rabbits, hamsters, and vervet monkeys [200-202] did not induce atherogenesis. In view of this current state of information, it is somewhat surprising that some governments have restricted TFA content in food products.

We demonstrate for the first time that TFAs have the capacity on their own to directly stimulate atherosclerotic development in the LDLr$^{-/-}$ mice. This animal model may have been an ideal choice in which to study this dietary intervention. Genetically modified murine models of atherosclerosis have been widely accepted in recent years as ideal to study atherosclerotic development [54, 203] and only develop lesions when fed a lipid supplemented, atherogenic diet. Our hypothesis that the effects of dietary cholesterol may have masked the effects of TFAs on their own in the previous works [200-202] appears to have been correct. In the present study, dietary TFAs were directly responsible for the atherogenesis, as they stimulated atherosclerosis, despite a normal body weight and normal cholesterol and triglyceride levels, similar to the control group. The higher the circulating [TFA] was, the more extensive were the atherosclerotic lesions

(Figure 43). We can conclude from these data, therefore, that circulating TFAs can directly induce atherosclerosis. The mechanism whereby TFAs induce atherosclerosis is unclear. Because they resemble saturated fatty acids with respect to their linear configuration, they may act very much like them in the cardiovascular system. It is possible that TFAs may augment atherosclerosis through a stimulation of inflammatory pathways. Other dietary interventions have been shown to alter atherosclerosis in the $LDLr^{-/-}$ mouse through a modulation of the inflammatory pathways [141, 304]. TFAs adversely affect markers of inflammation in epidemiological and clinical trials [16-18, 204, 305]. Alternatively, some alternative causative factors may be ruled out. For example, the atherogenesis in the LT and HT groups was stimulated without any increases in circulating cholesterol or triglyceride levels. TFAs can exhibit detrimental cellular effects in humans unrelated to cholesterol [170, 306, 307]. Furthermore, no changes in circulating SFAs, MUFAs or PUFAs would explain the increase in atherosclerotic lesions in the LT and HT groups in comparison to their respective control groups. The atherogenic effect of TFAs on their own was also achieved without a change in weight gain.

The low dose of commercially hydrogenated vegetable shortening in this study (LT and C+LT) provided 1.4% TFAs, which is equivalent to 3.2% of caloric energy. The higher dose of hydrogenated vegetable shortening (HT and C+HT) provided 2.8% TFAs, which is equivalent to 6.4% of caloric energy. Estimates of TFA intake range from 0.7-28.7 g TFA/person/day with the average daily TFA intake in North America being 5.8g or 2.6% of calories [26]. The TFA dosage provided to the mice in this study was similar in energetic load to previous experimental reports [141, 170, 197] and similar to the average human TFA consumption values [26].

In summary, our data defines the mechanism whereby commercially hydrogenated TFAs contribute independently to cardiovascular disease. Our data have relevance to the current levels of TFA ingestion in North America today. The dose dependency of the atherogenic effects is further cause for concern and lends credence to governmental efforts to reduce its presence in foods available to the public today.

D. Effects of *trans* fatty acids from commercial and natural sources on atherosclerotic development in the LDLr$^{-/-}$ mouse

The TFA dosage provided to the mice in this study was similar in energetic load to previous clinical and experimental reports and to current human TFA consumption values [26, 170, 197]. The elaidic acid rich shortening (ES) and the vaccenic acid rich butter (VB) diets in this study each provided 1.5% TFAs, either from manufactured or dairy sources, which provided 3.2% of caloric energy. Estimates of TFA intake range from 0.7-28.7 g/person/day with the average daily *trans* fat intake in North America reported as 5.8 grams or 2.6% of calories [26]. Thus, the TFA dosages used in the present investigation are well within the appropriate range of intake to be relevant to human consumption.

The results of the present study are significant because we are the first to demonstrate that TFAs from dairy and manufactured origins have differential effects on atherosclerotic development in the LDLr$^{-/-}$ mouse. The protective effects of the vaccenic acid enriched diet in comparison to the elaidic acid supplemented diet occurred despite the former diet containing ~4x as much total TFA content (Figure 44D). It is also important to note that whenever atherosclerosis was inhibited by a vaccenic acid-enriched diet (VB<ES and CH-VB<CH-RB), only the vaccenic acid (C18:1*t* VA) was elevated. Of

all of the fatty acid species examined, it is the only one to show a relationship with the anti-atherogenic action observed in this study. This anti-atherogenic action of vaccenic acid demonstrated in the present study is consistent with other studies of the effects generally of dairy products on cardiovascular disease. Epidemiological and randomized clinical trials (RCT) suggest that dairy TFAs possess either a neutral or a potential cardioprotective benefit as compared to manufactured TFAs [172, 217, 218, 308]. The mechanism of effect remains unknown, but evidence suggests that dairy TFAs may lower CHD risk by reducing circulating total cholesterol levels and/or increasing HDL levels [217, 218]. However, the few animal studies conducted to date demonstrate contradictory effects of dairy TFAs on circulating cholesterol levels [219, 309]. One recent investigation in cholesterol-fed hamsters hypothesized that increasing the vaccenic acid content of butter results in a circulating lipoprotein cholesterol profile that is associated with a reduced risk of atherosclerosis [309]. In the present study, it is difficult to argue that vaccenic acid induced its protective effect on atherosclerosis due to a beneficial action on serum cholesterol or triglyceride levels. The serum cholesterol and triglyceride concentrations were both higher in the VB groups than the ES group and yet the extent of atherosclerotic lesions was significantly attenuated. It is important in this regard to also note that the anti-atherogenic effects of dietary vaccenic acid were observed in the presence or absence of cholesterol supplementation to the diet.

Several other possibilities exist concerning the mechanism responsible for the differential atherogenic action of the manufactured TFA as opposed to the dairy TFA. The protective effects of dietary vaccenic acid could not be associated with the differential weight gain. Animals fed the vaccenic acid-supplemented diet gained more

weight than those fed the elaidic acid-supplemented diet. This would not contribute to a protective atherogenic effect. We could detect no change in any individual SFA, MUFA or PUFA that could reasonably interpreted as incurring the protective effect of vaccenic acid in the present investigation. Alternatively, it is known that inflammatory processes now play an important role in atherosclerosis [287]. Epidemiological and RCT evidence show that TFAs have an adverse effect on markers of inflammation and endothelial dysfunction [18, 204, 211]. However, although this may represent a mechanism for the TFAs to participate in atherogenesis, there is no evidence to suggest that dairy TFAs have any beneficial action on inflammatory processes, oxidative stress, insulin, or haemostatic variables [218, 308].

Thus, although we can rule out several factors as contributory to the protective effects of vaccenic acid in the present study, further work is required to definitively identify the mechanism of anti-atherogenic action of this TFA. Many studies have shown that fatty acid moieties with different structures have very different biological actions [147, 310-312]. Thus, it is not unprecedented that structurally different TFAs may have very distinct physiological roles to play in the body. The small difference in location of the *trans* double bond between elaidic and vaccenic acid changes the linearity of the TFA molecule. Elaidic acid is more linear than vaccenic and, therefore, may act more like a saturated fatty acid. Vaccenic would be less linear and it is this structural characteristic may be critical to its anti-atherogenic action in the present study. The linearity of the fatty acid moiety may affect its incorporation in cell membranes and its role in cellular transport and cell signaling.

In summary, consuming TFAs from a manufactured elaidic acid rich hydrogenated vegetable shortening stimulates atherosclerotic development but consuming a vaccenic acid-rich diet protected against atherosclerotic lesions. The data have important significance for the dairy industry. Currently, many dairy products contain substantial amounts of naturally-occurring TFAs, largely in the form of vaccenic acid. If vaccenic TFA stimulates atherosclerosis, this may have a negative impact upon the entire dairy industry. However, it is clear from our study that vaccenic acid is not pro-atherogenic. Conversely, consuming a vaccenic acid rich butter protects against hyperlipidemia and atherosclerosis in this model. The mechanism has yet to be determined but it is clear that it is independent of a change in circulating lipids. It is now clear that the generalization that all TFAs contribute to cardiovascular disease is false. Conversely, it appears that at least one, vaccenic acid, carries some protective effects.

E. Effects of combining dietary flaxseed and *trans* fatty acids on atherosclerotic development in the LDLr-/- mouse

The TFA dosage provided to the mice in this study was similar in energetic load to previous clinical and experimental reports and to current human TFA consumption values [26, 170, 197]. The diets providing elaidic acid rich partially hydrogenated vegetable shortening in either a low (LT) or high (HT) dose provided 1.4% and 2.8% industrial source TFAs (iTFAs), representing 3.2% and 6.4% of caloric energy, respectively. Estimates of TFA intake range from 0.7-28.7 g/person/day with the average daily *trans* fat intake in North America reported as 5.8 grams or 2.6% of calories [26]. Thus, the TFA dosages used in the present investigation are relevant to both the typical as well as a high TFA consumption levels in humans.

Our research is the first to demonstrate that combining an anti-atherogenic dietary agent such as flaxseed with industrially hydrogenated *trans* fats (iTFAs) in the diet protects against the initiation and progression of atherosclerotic development in the $LDLr^{-/-}$ mouse. This finding is especially pertinent as the $LDLr^{-/-}$ mouse is considered an excellent model of familial hypercholesterolemia and atherosclerotic development [54]. Our study demonstrates that whole ground flaxseed and flaxseed oil completely prevented the initiation of atherosclerotic development induced by the consumption of low and high doses of iTFAs (Figure 53). This protective effect was independent of changes in circulating cholesterol and triglyceride levels. All of the flaxseed fractions (whole ground, flaxseed oil, flaxseed fibre and lignan) significantly reduced circulating cholesterol levels as compared to their respective control diets (CR: cholesterol + regular fat and CT: cholesterol + TFAs) when added to a diet with cholesterol and iTFAs. However, only whole ground flaxseed and flaxseed oil limited the development of advanced atherosclerotic lesions in the presence of the two atherogenic risk factors.

As both flaxseed oil and whole ground flaxseed provided the same benefit, the evidence suggests that the rich ω-3 ALA content of flaxseed is the responsible agent for this protective effect. The anti-atherogenic action of ALA demonstrated in the present study is consistent with other studies investigating the effects of ω-3 fatty acids on cardiovascular disease. Epidemiological, randomized clinical trials and experimental investigations suggest that ω-3 fatty acids may be anti-atherogenic via a host of cardioprotective actions [80-82]. Contrary to our findings, the only other study directly investigating the role of flaxseed oil on atherosclerosis in hypercholesterolemic conditions reported no effect [142]. The protective effects of ALA extend beyond

inhibiting the initiation of atherosclerosis caused by iTFAs alone. We have demonstrated that dietary ALA from flaxseed offers preventative benefits in the presence of two atherogenic risk factors (cholesterol and TFAs). Surprisingly, the partially defatted flaxseed meal poor in ALA and the lignan SDG provided little benefit on atherosclerotic development induced by feeding cholesterol and iTFAs, further confirming that the ALA rich content of dietary flaxseed is the responsible beneficial agent.

Previous clinical and experimental research indicates that although ALA supplementation often does not alter circulating lipid levels, it offers cardioprotection by hypotensive, anti-aggregatory, anti-arrhythmic and anti-inflammatory mechanisms [147, 153, 162-164]. Our study indicates that ALA from flaxseed oil or from dietary flaxseed prevented atherosclerosis without a corresponding lipid lowering effect, suggesting that ALA from flaxseed offers protection via other cellular mechanisms. Dietary ALA in whole ground flaxseed and flaxseed oil offered protection despite causing a small, although not always significant, increase in circulating triglyceride and cholesterol levels in the presence of iTFAs. The CTF group had limited advanced atherosclerotic lesions regardless of having the highest circulating triglyceride levels. This is surprising as a recent systematic review of 14 studies investigating the cardiovascular benefits of ALA reports that ALA has little effect on circulating lipids [313]. This is also contrary to the majority of published clinical findings that provide strong scientific evidence that ω-3 fatty acids from fish sources significantly and dose-dependently reduce circulating triglyceride levels [314]. Although the reason for the elevated triglyceride levels in this group is unknown, it cannot be solely attributed to the higher fat content of the CTF diet, as it contained a similar fat content as the other diets containing flaxseed (TF, TALA,

HTF, HTALA and CTALA). Surprisingly, the addition of any of the three flaxseed fractions (ALA, fibre and lignan) all reduced circulating triglyceride levels to a greater extent than whole ground flaxseed which contains all three known cardioprotective agents.

Although some experts have encouraged the replacement of saturated and *trans* fatty acids with ω-6 and ω-3 PUFAs [315], no studies have been published investigating the effects of combining flaxseed in an atherogenic diet containing TFAs on cardiovascular markers. This research provides useful information regarding the therapeutic potential of dietary flaxseed. It is apparent that adding whole ground flaxseed or flaxseed oil to the typical Western diet containing TFAs and cholesterol offers protection against atherosclerotic CVD. These results warrant further clinical investigation.

CHAPTER VII: CONCLUSIONS

Through a series of studies, we have identified that:

1. Dietary flaxseed alters the circulating fatty acid profile favouring an increase in the ω-3 PUFA ALA and to a smaller extent EPA

2. Dietary flaxseed inhibits atherosclerotic development

3. Dietary flaxseed protects against cholesterol-induced impairment of endothelium-dependent vascular relaxation

4. Dietary flaxseed partially inhibits atherosclerosis through an anti-proliferative and anti-inflammatory mechanism of action

5. Consuming *trans* fatty acids (TFAs) from an industrially produced hydrogenated vegetable shortening source (iTFA) stimulates atherosclerotic development in a dose dependent manner independent of dietary cholesterol

6. Consuming iTFAs does not advance atherosclerotic development beyond that induced by cholesterol feeding

7. Consuming TFAs from a naturally occurring ruminant source (rTFAs) protects against hyperlipidemia and atherosclerosis

8. Dietary flaxseed protects against atherosclerotic development induced by iTFAs and cholesterol feeding

CHAPTER VIII: IMPLICATIONS FOR CANADIANS

Dietary interventions with flaxseed and *trans* fatty acids (TFAs) have relevance to Canadians. In addition to costing lives, cardiovascular disease costs the Canadian economy more than $18 billion a year [316]. However, it is becoming increasingly evident that atherosclerotic coronary heart disease (CHD) is largely attributable to factors that can be altered or prevented by lifestyle modification, including dietary choices [48]. Increasing the consumption of ω-3 fatty acids has been suggested as one dietary strategy to provide cardioprotection against CHD and significantly reduce the incidence of myocardial infarcts and stroke [78, 79]. The most common way to consume ω-3 fatty acids has been in the form of marine oils like fish. However, dietary compliance for fish supplementation is often an issue due to concerns about environmental toxins, palatability and eructation [115]. Sadly, no matter how good a product is for you, if the general population will not ingest it, it will never provide the expected benefits. Finding an alternative source of ω-3 PUFAs, therefore, could be an important issue for cardiovascular health. The use of flaxseed as a palatable, natural, affordable and readily available dietary supplement may prove to be an important modality to ensure that ω-3 PUFAs are delivered to the body in doses that have clear cardioprotective effects. The level of 10% flaxseed supplementation used in these studies represents ~13% of the total energetic intake and is comparable to that used in a clinical trials where human subjects consumed 50 g flaxseed/day [132]. Thus, our level of dietary flaxseed supplementation has physiological relevance to humans. Our data lends further support to the hypothesis that nutritional interventions that elevate circulating ω-3 fatty acid levels are a simple and

effective measure to maintain good cardiovascular health. This research will hopefully provide Canadians with scientific evidence of the beneficial effects of consuming flaxseed and ALA to improve their health by preventing or reducing the development of atherosclerosis, therefore, directly reducing the risk of heart disease and stroke. As the world's largest producer and exporter of flaxseed [317], Canada is in a position to supply flaxseed and ALA to its population and abroad with significant economical benefits, both through increased product commercialization and through savings in health care costs that would follow a decline in CVD [318].

TFAs have also been a topic of interest for Health Canada, the food industry, health professionals, the media and the general population, as more information becomes available concerning the negative health effects of these fatty acids. The intake of *trans* fats in Canada is amongst the highest in the world [27]. In light of the concerns regarding the TFA content of the Canadian food supply and the related health implications, the Canadian government has recently made several efforts to reduce the levels of *trans* fats in Canadian food. Canada was the first country in the world to introduce mandatory labeling of *trans* fat on prepackaged foods. Health Canada and the Heart and Stroke Foundation of Canada have also created a task force that has developed recommendations and strategies for reducing the TFA content of the Canadian food supply. The mandatory labeling of *trans* fat is intended to assist consumers in making healthy food choices enabling them to limit their intake of *trans* fats. It has already prompted a number of food companies to reduce or even eliminate *trans* fat in their products.

The epidemiological evidence for TFAs is reasonably clear: Higher intakes of *trans* fats are associated with elevated rates of cardiovascular morbidity and mortality

[19-23, 183]. However there is little understanding of how *trans* fats contribute to CVD. This research provides direct scientific evidence on the effects of *trans* fat consumption on atherosclerosis, the main underlying pathology leading to CVD. We have provided scientific evidence of the effects of TFAs on atherosclerosis at a time when the use of *trans* fats in the diet is being questioned. Our data supports the concept that iTFAs contribute independently to CVD. However, our data also demonstrates that TFA isomers have differential effects on the cardiovascular system. We would recommend that rTFAs should not be grouped together with iTFAs on nutrition information labels. Further research on the mechanism(s) of action is required to support labelling guidelines and recommended limits imposed on TFAs.

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