DIETARY FLAXSEED SUPPLEMENTATION AND THE EXPRESSION OF ADIPOKINES

by

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ABSTRACT

Dietary flaxseed has cardioprotective effects that may be achieved through its rich content of the omega-3 fatty acid, alpha linolenic acid (ALA). We investigated the effects of dietary flaxseed both with and without an atherogenic cholesterol-enriched diet to determine the effects of dietary flaxseed on the expression of the adipose cytokines leptin and adiponectin.

Rabbits were fed one of four diets: a regular (RG) diet, or a regular diet with added 0.5% cholesterol (CH), or 10% ground flaxseed (FX), or both (CF) for 8 weeks. Levels of leptin and adiponectin expression were assessed by RT-PCR in visceral adipose tissue. Consumption of flaxseed significantly increased plasma and adipose levels of ALA. Leptin, but not adiponectin, mRNA expression was lower in CH animals and was elevated in CF animals. Changes in leptin expression were strongly and positively correlated with adipose ALA levels and inversely correlated with levels of *en face* atherosclerosis. Our data demonstrate that the type of fat in the diet as well as its caloric content can specifically influence leptin expression. The findings support the hypothesis that the beneficial cardiovascular effects associated with flaxseed consumption may be related to a change in leptin expression.

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For my family,

who will never read this

but are proud of me anyway.

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ABBREVIATIONS

ALA: alpha linolenic acid

ANOVA: analysis of variance

ApoE: apoprotein E

ARA: arachidonic acid

C/EBP: CCAAT-enhancer-binding protein

cDNA: complementary deoxyribonucleic acid

CF: regular diet supplemented with 0.5% cholesterol and 10% ground flaxseed

CH: regular diet supplemented with 0.5% cholesterol

CLA: conjugated linoleic acid

COX-2: cycloxygenase-2

CRP: C-reactive protein

DART: Diet and Reinfarction Trial

DGLA: dihomo-γ-linolenic acid

DHA: docosahexaenoic acid

DPA: docosapentaenoic acid

EDTA: ethylenediaminetetraacetic acid

eNOS: endothelial nitric oxide synthase

EPA: eicosapentaenoic acid

FABP: fatty acid binding protein

FAT: fatty acid translocase

FATP: fatty acid transport protein

FFA: free fatty acid

FX: regular diet supplemented with 10% ground flaxseed

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GC: gas chromatography

GLA: γ-Linolenic acid

GPR120: G-protein coupled receptor 120

HDL: high density lipoprotein

HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase

LA: linoleic acid

LDL: low density lipoprotein

LOX: 5-lipooxygenase

LSD: least significant difference

LTB: leukotrienes

MCP-1: monocyte chemoattractant protein 1

MI: myocardial infarction

PCR: polymerase chain reaction

PGI: prostaglandin

PPAR: peroxisome proliferator-activated receptor

PUFA: polyunsaturated fatty acids

RG: regular rabbit diet

RIPA: radio immunoprecipitation assay

RNA: ribonucleic acid

qRT-PCR: quantitative real time polymerase chain reaction

SDG: secoisolariciresinol diglucoside

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SREBP: sterol response element binding protein

TBS-T: tris-Buffered Saline and Tween 20

TNF-α: tumor necrosis factor α

Tx: thromboxane

US: United States

USD: United States Dollars

VCAM: vascular cellular adhesion molecules

INTRODUCTION

It is becoming increasingly evident that atherosclerotic heart disease is largely attributable to factors that can be altered or prevented by lifestyle modification. Dietary omega-3 fatty acids provide cardioprotection against ischemic heart disease and significantly reduce the incidence of myocardial infarcts and stroke. Commonly, omega-3 fatty acids are found in oils from marine animals like fish, however, flaxseed has been identified as a significant alternative source of omega-3 fatty acids.

Flaxseed is one of the richest sources of alpha linolenic acid (ALA). ALA has been identified in several epidemiological trials as having significant beneficial effects against heart disease. However, the data have been indirect and the mechanism of action for this cardioprotection is unclear. Inclusion of flaxseed or one of its derived components in the diet in animal studies has shown that flaxseed can inhibit arrhythmogenesis during ischemia/reperfusion, inhibit atherogenesis and protect against vascular dysfunction during hypercholesterolemic conditions. Although these data provide convincing evidence in support of an important action for flaxseed in protecting against the inflammation that is involved in cardiovascular disease, the full picture of its mechanism of action is not clear. It is possible that its effects may involve an action on peripheral non-cardiovascular tissue, which may alter the inflammatory profile in the body, in turn influencing vascular morphology and function.

Adipose tissue is one such possibility. Adipose actively interacts with and responds to its environment through the synthesis and release of adipokines, which

have important roles in inflammation, hunger regulation, obesity, and numerous risk factors for cardiovascular disease & type II diabetes. The diet is known to modulate the synthesis and release of these substances but there are specific questions we seek to answer. First, does dietary flaxseed have the capacity to alter the fat composition in adipose tissue?

We hypothesize that this is plausible, since the fatty acid content in adipose tissue is not normally rich in omega-3 fatty acids and alteration of dietary fat consumption should also alter storage. Secondly, can a dietary intervention like flaxseed alter the expression and release of cytokines that modulate the cardiovascular system. Finally, can inclusion of flaxseed in the diet protect against the deleterious effects of an atherogenic diet? If so, then ultimately, this action may lead to beneficial effects on the cardiovascular system, as well as metabolism. We hypothesize that a high cholesterol diet can induce detrimental effects on cardiovascular and metabolic measures, and dietary flaxseed supplementation in combination with a high cholesterol diet can inhibit some of these deleterious effects.

REVIEW OF LITERATURE

ATHEROSCLEROSIS

The term atherosclerosis comes from the Greek words *athere*, meaning gruel, and *skleros* meaning hard[1]. It describes the thick, cholesterol-based plaque that builds up inside arteries, narrowing and stiffening the vessels and rendering them prone to rupture or blockage. Cardiovascular disease represents a major burden on the heath care system, representing the leading cause of hospitalizations, and 32% of all deaths in Canada in 2004[2].

The majority of cardiovascular disease is due to ischemia, a lack of oxygen perfusion to the tissue[2]. Ischemia results in stroke in the brain, angina or myocardial infarction in the heart, and peripheral artery disease in the extremities, as well as the problems associated with the ischemia or death of cardiac tissue following a myocardial infarction such as cardiac arrhythmias and hypertrophy. These issues all stem from the development of atherosclerosis. When the vessel becomes narrowed and stiff, it is less responsive to increases in blood flow demand, creating areas of insufficient blood flow, and thus insufficient oxygen downstream[1]. Atherosclerotic tissue is also prone to rupture, resulting in the development of a thrombus. This thrombus travels in the blood stream and can entirely block narrowed arteries, such as coronary arteries, resulting in a myocardial infarction, or vessels in the brain, resulting in a stroke. Atherosclerosis also weakens the vessel walls, and vessels that are prone to high pressure, such as the aorta, may develop aneurysms and subsequent vessel ruptures.

Vessel structure

Arteries have three layers: the intima, the media, and the adventitia, as illustrated in Figure 1[1]. The intimal tissue is that which borders the vessel lumen, and consists of vascular endothelial cells, which prevent infiltration of blood components through the vessel wall (and thus initiation of clotting), and secrete vasoactive compounds such as nitric oxide, triggering relaxation of the vessel. The intimal cells also express proteins such as vascular cellular adhesion molecules (VCAM), which target the infiltration of macrophages to the sub-endothelial levels.

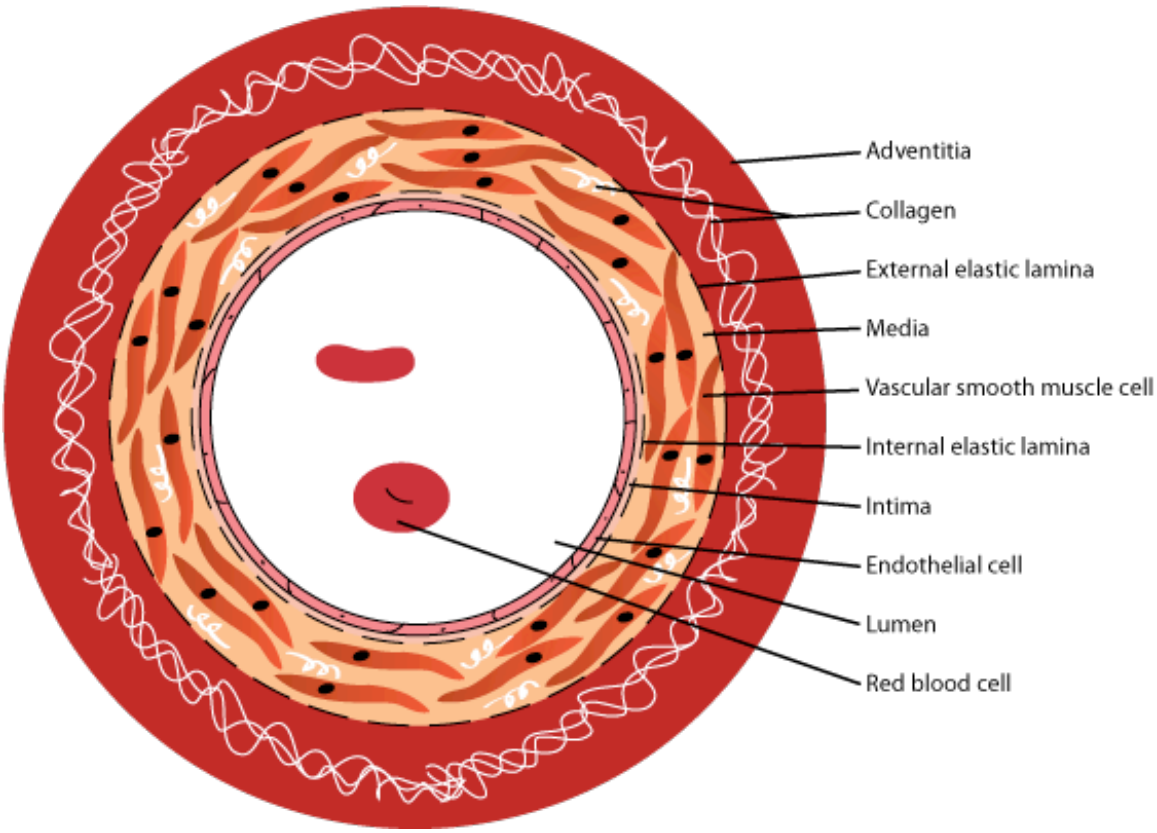

Figure 1: Structure of a large artery

Cross-sectional representation of a large artery, consisting of intimal, medial and adventitial layers. The intima consists of a single layer of endothelial cells which provide a barrier between the media and the lumen of the vessel. The media contains the vascular smooth muscle cells, which add strength to the vessel, and contract and relax to induce vasoconstriction and vasodilation, respectively. Collagen in the adventitia and media provide structural support for the vessel.

The medial layer is divided from the adventitia by the internal elastic lamina[1]. The media contains the vascular smooth muscle cells (VSMC) and elastic lamellae are arranged in concentric circles around the lumen. In smaller vessels, the majority of the media consists of VSMC, but in large vessels such as the aorta, elastic tissue contributes to much of the tissue mass. This layer is responsible for contraction and dilation of the artery, which occurs in response to physiological signals, such as those during exercise. Nitric oxide is a key vasodilator, and is produced by nitric oxide synthases in both the endothelium (eNOS) and the vascular smooth muscle cells (iNOS). The medial layer is then bordered by the external elastic lamina, which separates it from the adventitia.

The adventitial layer consists of loose connective tissue, which is composed mostly of collagen[1]. Its role is structural, as it anchors the vessel to nearby structures.

Atherogenesis and the Response to Injury Hypothesis

The development of atherosclerosis is a slow process that begins in childhood and progresses throughout life[1]. Atherosclerosis is initiated by lipid accumulation in the vessel wall, followed by vessel damage that recruits inflammatory cells. The response to injury hypothesis states that atherosclerotic lesions occur as a result of damage to the vessel[3]. This damage can come about as a result of hyperlipidemia, cytokines and hormones, oxidative stress, metabolites, infection, or mechanical force, such as the high levels of shear stress during hypertension. Circulating monocytes then bind to vascular endothelial cells via VCAM and infiltrate the vascular tissue. Once inside the vessel, the monocytes convert to macrophages, and begin the uptake of

lipids such as oxidized LDL. As the macrophages accumulate lipids, they become lipid-rich foam cells, so named for the foamy appearance of the numerous lipid droplets when observed under the microscope.

As these foam cells accumulate, they push the endothelial layer out, narrowing the lumen, as well as causing damage, which recruits more inflammatory cells[4]. The inflammatory cells begin to activate vascular smooth muscle cells, which migrate towards the plaque and proliferate, resulting in the development of a fibrous cap of the plaque. Foam cells begin to die, forming a necrotic lipid core. The plaque may become stable, or the fibrous cap may weaken, resulting in a rupture of the plaque. The ruptured contents of the plaque will result in platelet aggregation, leading to the development of a thrombus, which can restrict blood flow in the area or break off into the circulation. The thrombus has the potential to subsequently cause a heart attack, stroke, or other ischemic attack. A plaque may also accumulate calcium, which further hardens the artery and prevents it from dilating and contracting appropriately in response to vasodilators and vasoconstrictors.

Cholesterol, especially LDL cholesterol, is important for both the development and maintenance of atherosclerosis. Plaque regression is possible, and requires the reduction in circulating lipids, which can result in breaking the vicious cycle of inflammatory processes that cause atherosclerosis. Typically this effect is achieved via the use of HMG-CoA reductase inhibitors (statins) or numerous lifestyle interventions such as exercise, stopping smoking, avoiding foods that promote high cholesterol. However, consuming relevant functional foods have been shown to reduce

hypercholesterolemia, and represent an attractive alternative or supplement to statin therapy[5,6].

FUNCTIONAL FOODS & NUTRACEUTICALS

Definitions

Food products are a traditional part of folk health care and home remedies but until the late 20th century, evidence for such claims was anecdotal. Since the concept was first introduced as a research focus in Japan in the mid-1980's, functional foods have become of increasing interest to both the international scientific community and consumers[7]. Although the terms "functional food" and "nutraceutical" have become commonplace, there is little consensus on the definition of these terms or the distinction between them. As put forward by the Japanese *ad hoc* national project in 1984, functional foods are dietary components that provide a physiological benefit, such as biorhythm, or immune regulation, in addition to the base nutritional and satiety-related benefits of consuming them. However, this definition has since been broadened. Health Canada defined functional foods in 1998 as:

> ... *similar in appearance to, or may be, a conventional food, [a functional food] is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions*[8].

By this interpretation of the term, functional foods may be aspects of the diet traditionally used for treatment or prevention of illness with scientifically validated health benefits, such as ginseng as an aid in fighting infection. They may also be foods with recently discovered benefits, such red wine, which has cardioprotective benefits with moderate consumption. Functional foods also include food products that may or

may not have physiological benefits when unaltered, but which have been enriched with beneficial components, such vitamin D enriched milk, or yogurt with added probiotics.

Research into functional foods has facilitated the use of foods for disease prevention and treatment, and not just as enhancers of a normal physiological state. These were initially termed "nutraceuticals," a portmanteau of nutrition and pharmaceuticals. Stephen DeFelice coined the term nutraceutical in 1989, which he defined as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease." However, this definition leads to ambiguity in the distinction between a functional food and a nutraceutical, as both are dietary aspects defined by health benefits and nutritional value. In Japan, under the regulatory system, Foods for Specific Health Use (FOSHU), "functional foods" is an all-encompassing term[9]. Under this system, functional foods may have nutrient function claims ("Iron is necessary for hematopoiesis."), enhanced functional claims ("Milk increases bone density, promoting bone health."), or disease risk reduction claims ("A calcium-rich diet can reduce the risk of osteoporosis."). To minimize confusion, Canadian government policy makers adopted this Japanese attitude towards functional foods, and consider all food products with health claims to be functional foods[8]. Nutraceuticals, by Health Canada policy, are related to but separate from functional foods.

> *A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food... demonstrated to have a physiological benefit or provide protection against chronic disease.*

In this case, nutraceuticals are a dietary supplement derived from the functional foods, and taken purely as a health aid rather than in conjunction with nutrition. Examples of such products would be capsules of fish oil for omega-3 supplementation or psyllium fibre capsules to lower plasma cholesterol.

Both functional foods and nutraceuticals are defined by their roles as an alternative to pharmaceuticals for prevention and treatment of disease and are an emerging aspect of Canadian health care.

Economic benefits of functional foods

Functional foods and nutraceuticals may have other global benefits beyond their direct effects on health. Cost-benefit analyses from the European Union, United States and Canada have shown that nutritional interventions and education have the potential to dramatically decrease the burden of cardiovascular disease while increasing the quality and length of life. For example, mandatory supplementation of breads and other grain products in the US and Canada with folic acid (vitamin B_{12}) was implemented in 1998. Although intended to prevent neural tube defects, folic acid also decreases homocysteine levels. This has resulted in a reduction of cardiovascular events, and a projected $11 billion USD savings in health care costs in the United States alone over 15 years[10]. It has been estimated that the daily use of dietary flaxseed, omega-3 fatty acids from fish, and folic acid, even in a healthy population, could save the Canadian health care system over $6 billion annually[11]. The use of dietary n-3 polyunsaturated fatty acids (PUFAs) in a diseased population significantly decreased mortality at a cost of between $3,401 USD (in Canada) and $6,127 USD (in

Belgium) per life year gained[12]. Given that secondary prevention with statins bears a cost of $8,488 USD per life year gained and as much as $138,679 USD per life year gained when used as primary prevention[13], it seems clear that nutritional interventions used for the prevention of cardiovascular disease are an economically viable health care solution.

Savings to the health care system are not the only economic spin-off that can be realized through the translation of nutritional interventions into the general public. The use of nutritional interventions may allow nutritional companies to generate financial rewards. The farmer, the processors and the marketing firms may all benefit from the development of new nutritional products. Since the 1997 FDA approval of the marketing of oatmeal as a cholesterol-lowering dietary supplement, per capita consumption of oat products in Canada has nearly doubled leading to a significant increase in Canadian producer revenues[14]. In 2008 alone, Quaker Oats spent $50 million USD on advertising directed at the health benefits of oats[15], an investment that benefits not only the brand, but also the value of oats as a commodity and the agricultural sector that produces it. In an increasingly health-conscious consumer population, development of functional foods, including margarines like Benecol® which contain phytosterols to lower blood cholesterol levels, have become an $8.5 billion industry worldwide[16]. Despite a global economic down-turn, the functional food market is projected to reach a value of more than $90 billion by 2013[17]. Successful translation of positive medical trials examining nutritional interventions can, therefore, lead to health benefits beyond a traditional bench to bedside view of translational medicine. The institution of these nutritional modifications can produce a

more valuable translational goal of bench to bedside to better living with an additional value of economic opportunities as well.

FLAXSEED

Flax (*Linum usitatissimum*) has been cultivated for thousands of years. However, it has only recently gained popularity as a functional food. Flax is also an important Canadian export. Western Canada produces over 760,000 tonnes of flax a year on average, more than any other country, of which approximately 90% of which is exported[18]. Three main beneficial components of consuming ground flaxseed have been identified, leading to its classification as a functional food[19]. The first is the heart-healthy omega-3 fatty acid, α-linolenic acid (ALA). It is also a rich source of dietary fibre, which may lower plasma cholesterol and exhibit anti-carcinogenic properties. Finally, it is high in secoisolariciresinol glucoside (SDG), a lignan that reportedly acts as an anti-oxidant and anti-atherogenic agent. The combination of these properties could make flaxseed an excellent supplement in the prevention of cardiovascular disease.

α-linolenic acid (ALA), an omega-3 fatty acid

Omega-3 fatty acids were first implicated in the prevention of cardiovascular disease in the 1970's. Bang and Dyerberg correlated the low levels of ischemic heart disease in Inuit with the high proportion of omega-3 fatty acid rich fish in the Inuit diet and the resultant high plasma levels of omega-3 fatty acids [20-22]. Omega-3 fatty acids are a type of polyunsaturated fatty acid (PUFA) that have their final double bond at the third carbon from the end of the carbon chain (the ω-3 position), as in figure 2. Other common PUFAs are the omega-6 fatty acids, which are more highly represented in the Western diet, and also have pro-inflammatory properties.[23] Both linolenic acid (C18:2

omega-6) and α-linolenic acid (C18:2 omega-3) are essential fatty acids; that is, the human body is incapable of synthesizing these polyunsaturates, and thus they must be obtained from the diet.

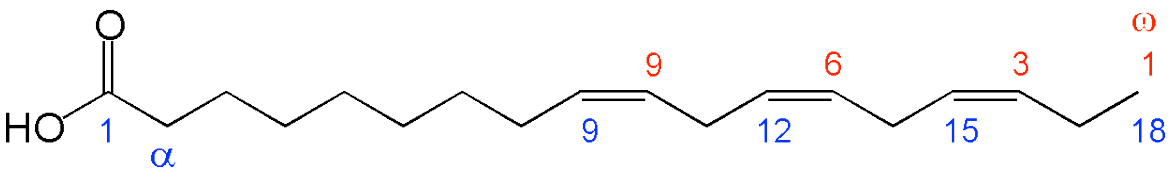

Figure 2: Chemical structure of α-linolenic acid, an omega-3 fatty acid

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(http://en.wikipedia.org/wiki/File:ALAnumbering.png)

The omega-3 fatty acids in fish are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The body may directly use these two fatty acids and both inhibit inflammatory processes by directly competing for Δ^6-desaturase with the pro-inflammatory omega-6 fatty acid, arachidonic acid [24,25]. DHA is an abundant fatty acid in the central nervous system, and is vital for normal brain and retina development and function[25-28]. EPA is a more systemic fatty acid, and has numerous biological roles including immune modulation through inhibition of eicosanoid and inflammatory cytokine production, decreased macrophage infiltration, and down-regulation of inflammatory gene expression[25]. Increased plasma EPA, in particular, has been well correlated with a decrease in high blood pressure, ischemic events[29-32] and risk factors for cardiovascular disease and metabolic syndrome, such as LDL cholesterol[31,33] triglycerides[32,33], C-reactive protein[34-36], and other inflammatory cytokines[36-39].

α-linolenic acid (ALA) is an 18 carbon omega-3 fatty acid that is unsaturated at carbons 9, 12 and 15 (Figure 2). It comprises approximately 55% of the total fatty acid content of flaxseed fatty acids and is the parent fatty acid of both EPA and DHA[19]. Conversion of dietary ALA to EPA to DHA in the human body occurs through the elongase and Δ^4-, Δ^5-, Δ^6-desaturase enzymes in the liver (Figure 3). Although ALA has been associated with many of the same health effects as EPA and DHA, the efficiency of the bioconversion process in cultured hepatocytes is 17% and 0.7%, respectively[40]. In the total organism, great variability has been reported depending on the ALA source, concentration, length of study and the age of subjects[41]. Reported efficiencies in adult humans vary from 5-8% for EPA and 0.5-4% for DHA[42-44]. As the conversion of

ALA to DHA in the body is limited and the majority of DHA is stored in neurological tissue, some have suggested that the cardiovascular benefits of consuming ALA are related more to EPA than direct effects by ALA itself[45]. However, this completely ignores the possibility that ALA may have a direct protective action itself.

ALA, once absorbed into the blood, can have systemic effects. Consumption of milled flaxseed in conjunction with a typical diet results in a modest increase in plasma ALA in both animal models[46-48] and humans[49-51]. However, if only about 10% of ALA is converted to EPA and DHA, it is reasonable to assume that some of the remaining non-metabolized ALA is stored in the adipose tissue[18]. ALA-rich diets that have been used in interventional trials reduce both fatal and non-fatal myocardial infarction[52]. Although ALA has also been associated with improved plasma lipids as a mechanism for its beneficial cardiovascular actions, these effects have been variable. In some cases, plasma LDL, total cholesterol, and triglycerides have been reduced whereas in other studies no change in these factors has been observed[52,53].

Omega-6:Omega-3 Fatty Acid Ratio

It has been hypothesized that although absolute quantities of omega-3 fatty acids such as ALA may exert beneficial effects, the ratio of omega-6 fatty acids to omega-3 fatty acids may be more important for maintenance of health[54]. Current recommendations for a healthy dietary ratio are reported to be at minimum 4:1[37]. However, conservative estimates of the ratio in the average North American diet are presently 16:1[55]. The omega-6:omega-3 ratio has increased significantly over the past century, primarily due to the incorporation of soybean oil into the North American

diet[56]. Although soybeans provide ALA, they are much higher in the omega-6 fatty acid, linoleic acid (LA), resulting in the ratio increasing from 6:1 to over 10:1 over the last 100 years. This is important not only in order to understand a potential cause of prevalence of cardiovascular disease in modern times, but to demonstrate that the incorporation of a single oil source into the food industry can dramatically affect the health properties of the entire diet of a large population.

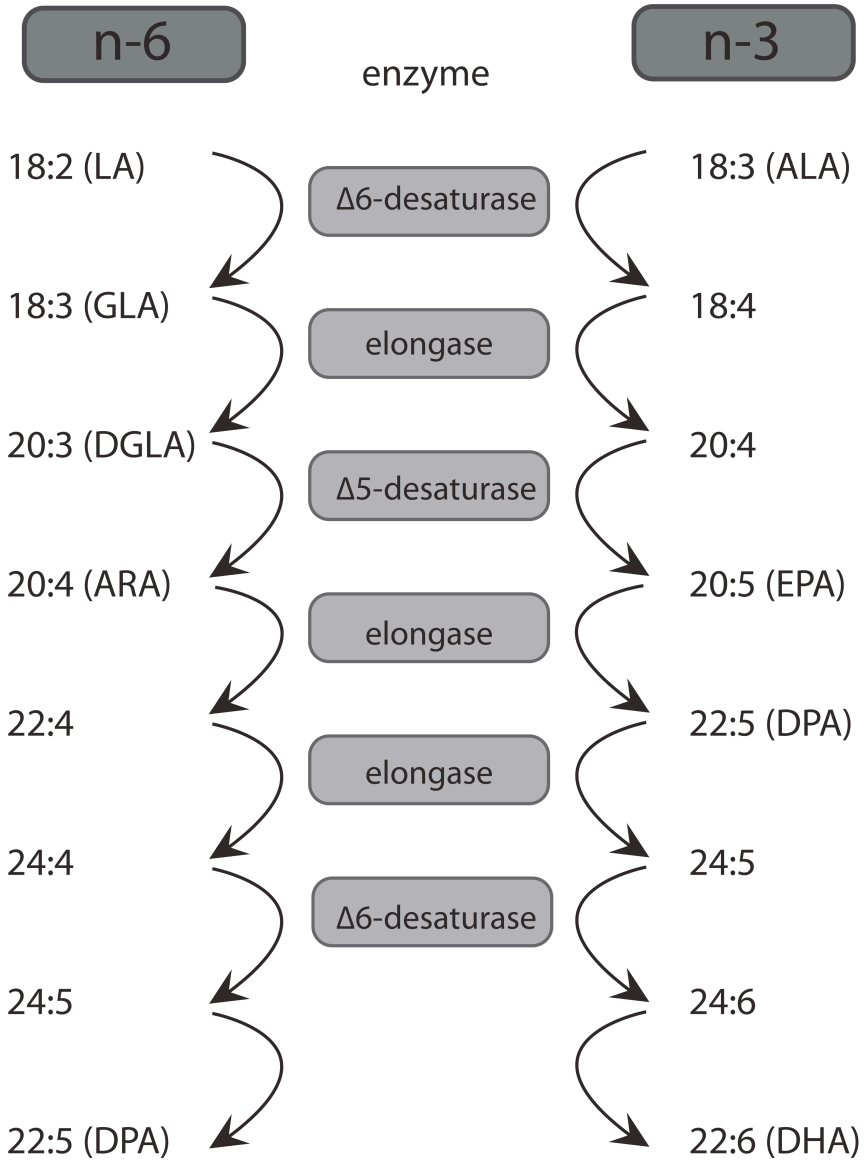

Figure 3: Production of long chain PUFAs from linoleic acid and α linolenic acid[57]

Essential omega-6 (n-6) fatty acid linoleic acid (LA) and omega-3 (n-3) alpha linolenic acid (ALA) are metabolized via desaturation and elongation reactions to form long chain PUFAs such as arachadonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Gamma-linolenic acid (GLA) and docosapentaenoic acid (DPA) are less abundant products of metabolism.

The omega-6:omega-3 ratio is believed to be important due to competition for the enzymes responsible for metabolic conversion to long chain PUFAs (polyunsaturated fatty acids). Although both classes of fatty acids interact with the same enzymes, omega-3 fatty acids are the preferred substrate[58]. Both omega-6 and omega-3 fatty acids utilize various desaturase and elongase enzymes, resulting in various PUFAs as described in Figure 3. These long chain PUFAs subsequently can be metabolized to eicosanoids such as leukotrienes, thromboxanes and prostaglandins (Figure 4). Omega-6 fatty acid arachidonic acid (ARA) produces pro-inflammatory molecules such as 4-series leukotrienes and thromboxane A_2. Conversely, omega-3 fatty acids metabolize to the less potent 5-series leukotrienes and biologically inactive thromboxane A_3[59]. Thus, the presence of omega-3 fatty acids inhibits the production of omega-6 derived pro-inflammatory products. However, a sufficient skew in the ratio of omega-6 to omega-3 fatty acids will push the reactions in favour of the products of omega-6 fatty acids[58].

Figure 4: Metabolism of long chain PUFAs[57]

Long chain omega-6 (n-6) fatty acid arachidonic acid (ARA) and omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are metabolized into leukotrienes (LT) via 5-lipoxygenase or into prostaglandins (PG) via cycloxygenase (COX). Prostaglandins can then be further metabolized into thromboxanes. N-6 fatty acids produce much more pro-inflammatory, pro-vasoconstrictive metabolites than n-3 fatty acids.

The fatty acids themselves may also have important effects. Indeed, the metabolism of ALA to EPA and DHA, or the conversion of LA to ARA and DPA is slow and incomplete[45]. Omega-3 fatty acids, particularly EPA and DHA have been shown to be potent ligands for G-protein-coupled receptor 120 (GPR120), while ALA is a weak ligand, and omega-6 fatty acids do not bind at all[60]. Oh et al demonstrated that in mice, GPR120 mediates the anti-inflammatory and insulin sensitizing effects of omega-3 fatty acids[60]. To date, no other fatty acid receptor has been shown to specifically bind a class of fatty acids[61]. However, it is probable that other similarly specific fatty acid receptor/sensors exist, and the balance between their downstream pathways may also play a key role in their subsequent effects.

Experimental design for investigating the ratio

The investigation of omega-6:omega-3 ratios is methodologically problematic, as, by the very nature of altering a ratio, confounding variables will exist. In order to maintain a constant quantity of total fatty acids provided, increasing omega-3 fatty acids must decrease omega-6 fatty acids and vice versa. This approach, however, does not distinguish between changes in total omega-3 fatty acid content and changes in the ratio of omega-6:omega-3 fatty acids. Conversely, the absolute quantity of lipids provided will vary if omega-3 fatty acids are held constant and omega-6 fatty acids are manipulated. These changes in absolute fat content are best controlled for in an in vitro setting, where purified omega-6 fatty acids can be easily replaced with monounsaturated fatty acids (MUFAs) such as the non-essential omega-9 fatty acids. Animal and clinical trials use plant or animal oils, which provide a myriad of fatty acid

types, and thus are much more difficult to manipulate precisely. Although these trials are more useful for investigating the use of functional foods and nutraceuticals in practical application, careful control of confounding dietary factors like total dietary energy, proportion of energy as fat, and changes in each fatty acid type must be taken into account before concluding that the ratio and not these other factors affected (or did not affect) the trial outcomes.

Animal Trials

Data from animal models indicate that manipulating the omega-6:omega-3 ratio may lead to cardiovascular benefit. Direct intravenous infusion of fatty acids to rats after immunologic challenge resulted in a reduction of TNF-α and IL-6 expression when a fish oil (1:7) was utilized as compared to either soy (7:1) or safflower oil (370:1)[54]. It is difficult to assess these early data in the light of the metabolism competition hypothesis, however, as the comparison of complex biological products leaves numerous variables uncontrolled, and fish oil contains the longer chain EPA and DHA, while LA in safflower oil must first undergo metabolism to ARA before the production of pro-inflammatory eicosanoids. A more recent study which manipulated quantities of plant oils in the diet found that reducing the omega-6 to omega-3 ratio from 12:1 to 3:1 lead to a significant attenuation of immune activation in the absence of any immune challenge[62]. Although some groups report that alterations in the ratio may also have effects on insulin resistance and obesity, both important factors in the development of cardiovascular disease, more appropriately balanced nutritional studies have failed to observe such changes[63,64].

Although studies of markers of cardiovascular disease have been mixed, there have been limited trials that manipulate the ratio while directly investigating the cardiovascular outcome. Sprague-Dawley rats fed a diet with a 1:1 and 1:5 omega-6:omega-3 ratio suffered a 30% smaller infarct size after left anterior descending coronary artery ligation, as compared to those fed a 5:1 ratio[65]. However, as all of these ratios remain within the healthy range, it is possible that the protective effects of altering the omega-6:omega-3 ratio are underestimated by this trial. The strongest evidence for the importance of omega-6 to omega-3 ratios comes from a 2010 study of transgenic mice by Wan et al[66]. *ApoE* -/- mice spontaneously develop atherosclerosis physiologically similar to that of humans. These mice were crossed with the *fat-1* model, which expresses n-3 fatty acid desaturase originating from *C. elegans* to produce mice that possessed the capacity to generate omega-3 fatty acids from omega-6 fatty acids, resulting in 1:1 tissue ratios. The transgenic animals were given a high omega-6 Western diet for 16 weeks, and the aortic lesion area was reduced by nearly 50% as compared to the *ApoE* -/- controls. This reduction was concurrent with reductions in inflammatory biomarkers in the plasma and the lesions such as IL-6, PGE_2, cycloxygenase-2(COX-2), and MCP-1, as well as with attenuation of monocyte infiltration into the atherosclerotic lesions. This investigation removes much of the uncertainty surrounding previous trials by controlling for variability in the diet, and indicates there is indeed merit to this course of study.

Clinical Trials

The omega-6:omega-3 ratio may be key for understanding why some randomized clinical trials of omega-3 fatty acid supplementation have failed to demonstrate appreciable benefit to cardiovascular health. In particular, it may explain the near complete lack of benefit in the recently published Alpha Omega trial, which provided EPA & DHA or ALA supplementation in the form of margarine to post-MI patients. The study's margarine also simultaneously supplemented high levels of LA, and no prevention of major cardiovascular events was seen in any of the experimental groups[67]. This trial provided ratios of 10:1 to 100:1 of omega-6:omega-3 fatty acids, and although they restricted dietary omega-3 intake in their subjects, omega-6 intake was not addressed in the patient population, nor were plasma omega-6 levels measured. Indeed, Leaf observed in 1999 that until that point in time, only interventional trials of omega-3 fatty acids, including the GISSI-Prevenzione trial, which simultaneously lowered omega-6 fatty acid consumption were effective in reducing cardiovascular and all-cause mortality[68]. These effects, much like in animal models, appear to occur independently of effects on insulin resistance[69]. By contrast, data from the Health Professionals Follow-Up Study was used in 2005 to examine the dependence of omega-3 benefits on omega-6 consumption and found that there was no distinction in benefit between low and high omega-6 consumers[70]. However, as these data originate from a prospective cohort study that relies on self-reporting of diets, a randomized controlled trial examining the interplay of omega-3 and omega-6 fatty acids is warranted. Furthermore, the question of whether absolute increases in

omega-3 fatty acids or the ratio of omega-6 to omega-3 fatty acids are the major contributing factor for cardioprotection still remains unanswered.

Fibre & SDG

Dietary fibre includes non-digestible polysaccharides such as celluose, and also numerous other plant products such as inulin, pectins, beta-glucans, dextrins, chitins and lignans. Although all dietary fibre is non-digestible, it is classified as either soluble or insoluble. These classifications are distinguished by their properties in the gut, either viscous and readily fermented by colonic bacteria, or metabolically inert and adding stool bulk, respectively.

Consumption of dietary fibre, found in fruits, vegetables, and grains, has been associated with a reduction in cardiovascular disease risk factors, incidence, and deaths in numerous epidemiological and prospective studies. Levels of fibre consumption of 10-25g/day were inversely correlated with total and cardiovascular mortality in observational trials including the Scottish Heart Health study[71], Zutphen Study[72], INTERGENE study[73], Japan Public Health Centre-based study[74], Malmö Diet and Cancer study[75] and Nurse's Health Study[76]. Furthermore, in the PREDIMED study, these high levels of dietary fibre were inversely associated with intima-media thickening, a good analogue for atherosclerosis burden[77]. These benefits do not appear to be gender specific, and show a dose-dependence response[78]. Furthermore, the NIH-AARP Diet & Health study demonstrated that in a population 50 years old and up, dietary fibre intake from grains reduced all-cause mortality, but fibre from fruits and vegetables did not, indicating the source as well as quantity of fibre is important[78].

Intervention trials, especially in humans, have been less clear. The Diet and Reinfarction Trial (DART) in 1989 found no significant effect on cardiovascular mortality after advice to increase cereal fibre intake to 18g per day, in spite of reporting good compliance and achieving an increase from 9g/day to 15g/day in the experimental group[79]. Fibre recommendations have since increased, as 15g/day may still be insufficient to achieve beneficial effects, especially in diseased populations. Smaller intervention trials providing individuals with a high-fibre Mediterranean diet have demonstrated reductions in total and LDL cholesterol[80], increases in anti-inflammatory, anti-diabetic cytokines after fibre supplementation[81,82], and decreases in pro-inflammatory cytokines[83]. However, Mediterranean diets also provide more omega-3 fatty acids, and a higher content of fruits and vegetables containing numerous other confounding bioactive compounds. Animal trials which vary fibre intake alone, however, have been successful in replicating these results[84], as well as the few small intervention trials that varied fibre intake alone[85,86].

There are multiple mechanisms by which fibre is hypothesized to beneficially affect cardiovascular disease: a) the binding of bile acids and cholesterol in the gut, leading to decreased absorption and lowering serum cholesterol, b) lowering the glycemic index of the diet resulting in improved insulin sensitivity, c) promoting weight loss, d) anti-oxidant effects and e) anti-inflammatory effects[78].

Flaxseed is approximately 27% fibre by dry weight, 40% of which is soluble fibre[87]. A small interventional trial examining the effects of partially defatted flaxseed meal, which dramatically reduced its fatty acid content, still found reductions in total and LDL cholesterol, indicating that the fibre content of flaxseed may be responsible for

these effects[88]. The major fibre component of flaxseed is a lignan, secoisolariciresinol diglucoside (SDG)[89], as illustrated in Figure 4.

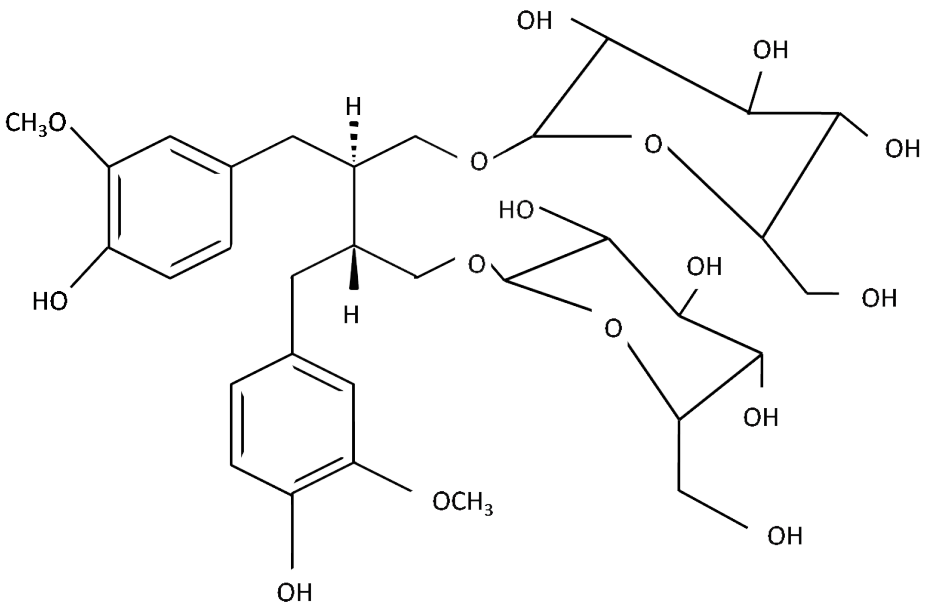

Figure 4: Secoisolariciresinol diglucoside (SDG), a flaxseed lignan

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(http://en.wikipedia.org/wiki/File:Secoisolarisiresinol_diglucoside.svg)

SDG is converted via microbes in the gut to the phytoestrogens enterolactone and enterodiol. Phytoestrogens are readily absorbed into the plasma, and are capable of exerting cardiovascular protective effects similar to those seen by endogenous estrogens[90]. These effects, however, occur without the feminizing effects of human estrogen[91], and so indicate that their mechanism of action may be due to weak binding of estrogen receptors[92]. Although the precise mechanisms may be unknown, numerous trials examining partially defatted flaxseed, as well as purified SDG, have demonstrated lipid-lowering effects[93,94,95]. Zhang et al. demonstrated that SDG may by a useful nutraceutical, as a 600mg/day dose decreased total, HDL, and LDL cholesterol, as well as triglycerides, indicating a generalized, rather than a specific lipid lowering effect[96]. This study also found benefits to fasting blood glucose levels, which may have a further impact on cardiovascular health. Furthermore, although partially defatted flaxseed meal was observed to have anti-thrombotic and anti-atherogenic effects in high-fat fed ApoE and LDL receptor deficient mice, SDG did not, indicating that flaxseed's effects on atherosclerosis and thrombosis are due to its fatty acid content, or another fibre component which has not yet been identified[89].

ADIPOSE TISSUE AND CARDIOVASCULAR DISEASE

Adipose tissue and adipokines

Adipocytes, the cells that store lipids and make up the bulk of adipose tissue stores in the body, are now known to have an important physiological role beyond their lipid storage capacity. Adipocytes, as well as the stromal-vascular cells and macrophages in adipose tissue, secrete a number of important cellular signaling molecules, termed adipokines. These adipokines have consequences ranging from autocrine and paracrine effects, to systemic endocrine actions. Adipokines also vary widely in both their function and mechanisms of control. One such mechanism of control is the fatty acid composition of adipose tissue, which can affect cellular signaling, fatty acid trafficking, gene expression and, consequently, metabolism[97]. Adipose tissue composition varies based on three main effectors: i) energy balance, which regulates the metabolism of free fatty acids within the adipose tissue, ii) diet, which will alter the fatty acid profile of the adipose tissue, and iii) physical location, as local factors can influence fatty acid storage and lipolysis. When an individual consumes more calories than they can immediately metabolize, storage of energy as lipids in the adipose increases. A person who is consistently in a state of caloric excess will gain fat tissue, and consequently become obese. During obesity, the adipokine profile shifts dramatically from an anti-inflammatory state to a pro-inflammatory state. Although these changes with caloric intake are well documented, the effects of the variations in dietary composition on the endocrine function of adipose tissue have only recently begun to be investigated.

Adipose tissue sources and their roles

Adipokines are expressed in both brown and white adipose tissue, however, brown adipose tissue's impact on systemic adipokine activity is believed to negligible in most cases[98]. White adipose tissue is the primary source of adipokines in circulation[99,100]. White adipose tissue is deposited both subcutaneously and viscerally, and these two depots exhibit differential expression of adipokines[100]. Subcutaneous white adipose tissue is located beneath the derma, and primarily functions for thermoregulation, expressing uncoupling proteins and preventing heat loss through the skin. Generally, it expresses low levels of most adipokines, having little effect on systemic levels of these adipokines, although it may have important paracrine effects on insulin sensitivity in skeletal muscle. Subcutaneous adipose tissue is also the primary source of the beneficial, anti-inflammatory adipokine, adiponectin[101]. Visceral adipose tissue is located in the abdomen, and in adult humans, by mass, consists mainly of retroperitoneal, perirenal, mesenteric, and omental tissue. Smaller visceral sources such as epicardial adipose tissue have special significance for cardiovascular disease due to the close proximity to the myocardium as well as the coronary left anterior descending artery[102]. Perivascular adipose tissue also surrounds most vessels. From this location, adipocytes may infiltrate into the adventitia, where they can have paracrine actions on endothelial and vascular smooth muscle cells without affecting circulating adipokine levels[102,103]. In general, visceral sources of adipose tissue highly express both anti- and pro-inflammatory adipokines, while thermoregulatory activities play a minor role in their function.

Fatty acid metabolism

After being consumed, fatty acids cross the intestinal wall in lipid-rich chylomicrons, which are then taken up by the lymphatic system and shunted into the blood stream[104]. Once in the blood, free fatty acids bind to albumin or circulate in lipoproteins, and can be used to provide for immediate energy needs. However, over 95% of consumed fatty acids are stored for later use[104]. In addition to the consumption of fats, the adipose tissue can produce many required fatty acids *de novo* from carbohydrates. However, animals cannot create omega-3 or omega-6 unsaturations, and therefore these key polyunsaturated fatty acids are termed essential fatty acids, as they must originate from the diet.

In the adipose tissue, chylomicrons and very low density lipoproteins (VLDL) have the lipids extracted by lipoprotein lipase[104]. The fats are then transferred into the adipocytes by one of two methods. The fatty acids can insert into the lipid bilayer of the adipocyte cell membrane, when they can be flipped across the bilayer to the intracellular side of the membrane. Although long believed to be the primary means of entry into the cell, the efficiency of this method is a subject of debate. Lipids may also be transferred into cells enzymatically, a much more rapid and chemically favourable situation than a lipid "flip-flop." Adipocytes produce numerous proteins to facilitate the uptake of lipids. Fatty acid transport protein (FATP) and fatty acid translocase (FAT) are responsible for the transfer of the lipids from the external environment to the cytoplasm, while fatty acid binding proteins (FABP) bind lipids in order to shuttle them to the translocators[104]. Once inside the cell, the fatty acids are then stored in the form of triacylglycerols – a three-carbon chain with three fatty acid

ester groups. Changes in the quantity as well as the type of lipids stored in adipocytes can dramatically affect the cell's function and thus the signal it secretes.

The release of free fatty acids (FFA) from adipose into the plasma, is a highly regulated event under normal conditions[105]. This process ensures fatty acids are systemically available for beta-oxidation, a major energy source for most tissues, including the heart. However, high BMI has been associated with increased FFA levels as lipolysis in the adipose increases. High FFA levels are an independent risk factor for atherosclerosis, hypertension, and sudden cardiac death[106-108]. Increased levels of FFAs are also known to directly contribute to hepatic steatosis and insulin resistance, through inhibition of glucose uptake[109,110]. It is also important to note, however, that FFAs are in a continuous state of flux within the body, and vary based on hormones, activity, fitness levels, and diet[109]. Therefore, changes in FFA levels without establishment of baseline fluctuations should be interpreted with caution.

Adiponectin

Adiponectin (Acrp30) is the most highly expressed and secreted adipokine, with beneficial effects on metabolism, inflammation, and vascular function[111]. The sole source of circulating adiponectin is the adipose tissue. In healthy individuals, adiponectin acts as an insulin-sensitizing agent and antioxidant, suppresses proinflammatory cytokines like TNF-α and IL-6, and increases eNOS activation, causing vasodilation. However, adiponectin has a paradoxical expression – as adiposity increases, adiponectin expression and secretion decreases within the adipose tissue[112]. This dichotomy is a function of the inhibitory effects of TNF-α on adiponectin

expression[113]. Adiponectin suppresses TNF-α expression in the adipose, but if TNF-α up-regulation signals are stronger than adiponectin's down-regulative effects, as in obesity, the system can rapidly switch to a pro-inflammatory phenotype (Figure 5). It is believed that these changes play an important role in the initiation of pathological obesity, and are symptomatic of dysfunctional adipose tissue. Adiponectin also has roles in inhibiting LDL oxidation, and increasing fatty acid catabolism. Thus, hypoadiponectinemia is of interest as a biomarker of cardiovascular disease and metabolic syndrome.

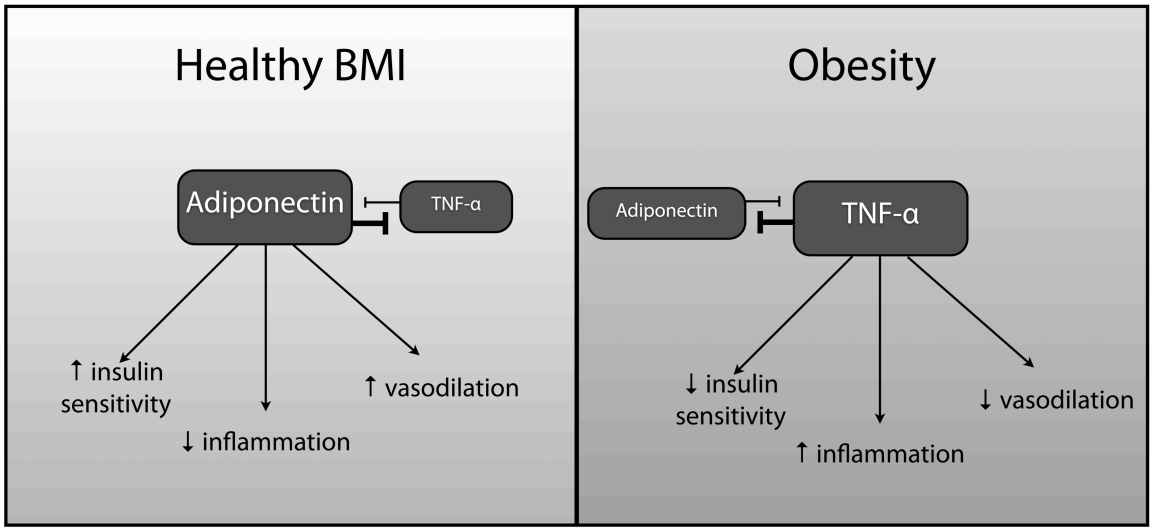

Figure 5: The adiponectin paradox.

When individuals are a healthy weight, the adipose tissue produces enough adiponectin to suppress the expression of the of tumor necrosis factor α (TNF-α)[113]. This suppression of TNF-α leads to dominance of adiponectin production, causing a decrease in systemic inflammation, and increases in insulin sensitivity and vasodilation capacity of the blood vessels. During obesity, TNF-α is up regulated, causing increased suppression of adiponectin. Consequently, the adipose tissue of an obese individual produces far less adiponectin, shifting to a pro-inflammatory, damaging cytokine profile.

Polyunsaturated fats have diverse impacts on adiponectin production in the adipose tissue. Omega-3 fatty acid accumulation in the adipose tissue can increase levels of adiponectin in the healthy children of persons with type II diabetes, as well as in obese humans, mice and rats[114-116]. Omega-3 fatty acids inhibit TNF-α production through enzymatic competition with arachidonic acid, a precursor to pro-inflammatory eicosanoids. Thus the consumption of omega-3 polyunsaturates can ameliorate the down-regulation of adiponectin production, leading to a recovery of adiponectin levels in the plasma[115]. Conversely, adiponectin expression may be suppressed by the same pathway, through the bioconversion of omega-6 fatty acids to pro-inflammatory eicosanoids, leading to TNF-α production. In this way, a diet rich in omega-3 fatty acids may suppress adiponectin production. Other polyunsaturates that affect adiponectin include the numerous isomers of conjugated linoleic acid, such as *trans*-10, *cis*-12-CLA. CLA has variable effects on adiponectin production. In one human study looking at a population of healthy post-menopoausal women, CLA suppressed adiponectin via unknown mechanisms, despite its other beneficial effects on the cardiovascular function and obesity[117]. However, in hypertensive patients, CLA had the opposite effect on adiponectin expression, while still showing cardiovascular benefits[118]. In rats, dietary CLA alternatively increased or did not affect adiponectin production[117,119]. As the effects of dietary supplementation of CLA are variable, its mechanism for adiponectin regulation should be identified.

Leptin

Leptin was first discovered by Zhang *et al* in 1994 as the protein encoded by

the *obese* gene, named for the phenotype of the double knockout mouse[120]. These mice experience no satiety, and thus eat continuously when fed *ad libitum*, leading to severe diet-induced obesity. After a meal, the body metabolizes the nutrients that were consumed in order to meet its current energy needs. Once these needs have been met, excess calories are stored in the form of lipids in the adipose tissue. This storage process induces the release of leptin to the blood stream, where it can cross the blood-brain barrier to the hypothalamus and suppresses hunger. In humans, genetic or metabolic leptin deficiencies have been implicated in cases of morbid obesity, especially in children[121]. Leptin's canononical role of hypothalamic appetite suppression in response to caloric intake, however, is not its only function. It is expressed in numerous other tissues, although the only other source of circulating leptin is the placenta, where it plays a role in the suppression of uterine contraction and is vital for early pregnancy maintenance[122,123].

Leptin receptors are present in the brain, liver, lung, heart, skeletal muscle, spleen, kidney and testes[124]. Plasma leptin levels have clinically been associated with body fat and short-term energy balance, due to its important roles in hunger suppression. However, chronic leptin expression may also be important in modulation of T cell activity in the early stages of atherosclerotic development, as well as other immune cells[125]. Obesity can affect leptin in one of two ways: leptin may be under-expressed by the adipose in response to a consistent high-caloric diet, or, more commonly, leptin receptors in the periphery may be down-regulated, leading to elevated plasma leptin and leptin resistance[126]. Leptin resistance is widespread in obesity-associated cardiovascular diseases, such as hypertension, atherosclerosis,

myocardial infarction, and heart failure[127]. *Ex vivo*, leptin treatments at pathophysiologically high levels have been shown to induce endothelial dysfunction through the suppression of endothelial nitric oxide synthase (eNOS)[128]. Although in general, leptin appears to exacerbate cardiovascular issues in disease-state individuals, it is important to note that this adipokine is still highly expressed in the healthy state.

The effects of consumption of polyunsaturated fatty acids on leptin expression are not well understood. When evaluating changes in leptin, a key point to consider is that leptin expression is also affected by caloric intake. Therefore, the effects of dietary components in the absence of an isocaloric control should be interpreted with caution. Early work on the influence of diet on adipokine expression indicated that the addition of fish oils (EPA and DHA) to the diet had no effect on leptin[129]. In diet-induced obese rats, addition of conjugated linoleic acid (CLA) to a diet high in saturated fats decreased leptin expression in both brown and white visceral adipose tissue[130]. Clinical investigations have identified a leptin receptor polymorphism which puts individuals more at risk for insulin resistance and metabolic syndrome[127]. In individuals with this leptin receptor polymorphism, a low omega-3, high omega-6 diet increases their risk for insulin resistance. However, when these individuals ate a high omega-3, low omega-6 diet, there was an apparent masking of the deleterious genotype. Consumption of monounsaturated fatty acids, in contrast, had no effect on risk of metabolic syndrome. Although the specific role of polyunsaturated fatty acids in the regulation of leptin expression and signaling has not been fully elucidated, it is clear that omega-3 PUFAs have an important role in leptin expression and signaling.

Resistin

Resistin was first identified in 2001 after a screen for adipocyte genes that were down regulated after treatment with anti-diabetic drugs[131]. It is an important obesity-related adipokine in rodents, but seems to play an alternative role in humans, as it is expressed primarily in macrophages and may or may not be related to insulin resistance in human beings[132]. In mice and rats, it is expressed robustly and almost exclusively in visceral white adipose tissue, and serum concentrations correlate positively with insulin resistance and obesity[131]. It acts as an insulin antagonist in the circulation, negating insulin's hypoglycemic effects, thus maintaining hyperglycemia and insulin resistance in diabetic rodents. Furthermore, resistin promotes the activation of vascular endothelial cells through VCAM-1 and MCP-1 up-regulation, and has been associated with increased severity of atherosclerosis[133]. Unlike rodent models, human resistin is produced in lymphocytes, monocytes, and macrophages in the adipose tissue, but not primary adipocytes[134]. Although some evidence indicates that resistin may be of use as a biomarker for obesity in human beings[135,136], clinical data generally do not support a direct role for resistin in insulin resistance or cardiovascular disease in human beings[137-139].

In culture, exposure of murine adipocytes to both short- and long-chain omega-3 and omega-6 polyunsaturated fatty acids reduced resistin expression[140]. However, in animal models, no effects on resistin have been observed with either type of dietary PUFA[115]. Despite the effects of PUFAs on insulin resistance, there appears to be no *in vivo* correlation with resistin. However, dietary interventions that assess resistin are limited, and therefore it may be premature to assert that they are

unrelated.

TNF-α

Tumor necrosis factor (TNF)-α is most notable for its roles in inflammation. TNF-α is acutely secreted in large quantities by macrophages as a key mediator of local immune response to infection. However, it is also produced in low doses by numerous tissues, including the adipose. The production of TNF-α can lead to a chronic low-grade state of inflammation, one of the hallmarks of obesity, and a key player in the progression of atherosclerosis[141]. In adipose, TNF-α plays a major role in obesity through its autocrine and paracrine actions. TNF-α leads to the activation of the MAPK pathway, and downstream second messenger NF-κB, which directly opposes the beneficial effects of adiponectin[142]. Futhermore, as discussed previously, TNF-α inhibits the production of adiponectin, exacerbating the pro-inflammatory state of the adipose (Figure 1). It is hypothesized that this shift from anti-inflammatory to pro-inflammatory endocrine activities leads to the pathogenesis of obesity. Although TNF-α has clinical importance for numerous reasons, it is notable in the context of obesity, diet and cytokines for its associations with endothelial dysfunction, insulin resistance, and macrophage infiltration.

Omega-3 PUFAs inhibit the production of inflammatory cytokines, including TNF-α[143]. Numerous studies have associated dietary omega-3 fatty acids with reductions in plasma TNF-α[144], and omega-3 treatment of macrophages with decreases in TNF-α secretion. *In vitro*, adipocytes treated with docosahexaenoic acid (DHA), one of the major omega-3 fatty acids in fish, did not exhibit any changes in

TNF-α secretion. Furthermore, TNF-α has been correlated with changes in BMI after PUFA supplementation[145].

C-reactive protein

Like TNF-α, C-reactive protein (CRP) is an important biomarker for inflammation, mainly secreted from the liver, but also from adipocytes[146]. CRP production is stimulated by interleukin-6 production by macrophages and adipocytes. It has roles in innate immunity, binding foreign or damaged cells and targeting them for phagocytosis. It is dramatically up regulated in response to acute infection, but it is a current marker of interest due to its use as a cardiovascular risk factor. In a healthy population, chronically elevated CRP levels predict future metabolic syndrome and cardiovascular disease, from stroke to peripheral artery disease to myocardial infarction[147]. Furthermore, its predictive value is independent of other well-established biomarkers. In animal research, CRP appears to play an active role in atherosclerosis. CRP treatment reduces nitric oxide production by the endothelial cells, up-regulates monocyte infiltration, increases the production of reactive oxygen species, and stimulates vascular smooth muscle cell proliferation and migration within the vessel walls[148]. Elevated CRP levels also have been shown to be associated with cancer risk in health individuals, and with increased mortality after cancer diagnosis, especially in non-metastatic cancers[149].

As CRP is an inflammatory marker, and omega-3 PUFAs act as anti-inflammatories, it can be deduced that omega-3 PUFAs will reduce CRP levels. This is usually, but not always, the case. Several human and animal trials showed a significant

reduction of circulating CRP with the addition of fish oil to the diet[150,151]. In other clinical trials, CRP did not appear to vary as a direct result of the addition of fatty acids to the diet[152,153]. Instead, starting CRP concentrations predicted the benefits of a high PUFA diet. Individuals with high CRP levels (i.e. those most at risk for cardiovascular disease) gain more benefit from the lipid lowering effects of omega-3 polyunsaturates[153]. The disparity between these trials, which gave similar levels of omega-3 fatty acids, can be explained by the omega-3/omega-6 ratio[23]. If the experimental diet provided a high omega-6 content in conjunction with the omega-3s, emulating the ratio of a Western Diet (16 omega-6: 1 omega-3), no effects on CRP were seen. However, if the relative omega-6 content was reduced to 4:1 or less, plasma CRP decreased[154]. Whether this change is due to a decrease in omega-6 content, or the relative increase in omega-3 content has not yet been elucidated. Furthermore, the source of the plasma CRP in these studies – the hepatocytes or adipocytes – is unclear.

HYPOTHESIS

1. Supplementation of the diet of rabbits with cholesterol will initate atherosclerosis, alter the lipid composition of adipocytes and decrease the expression of protective adipokines and increase the expression of pro-inflammatory adipokines in these animals.
2. Supplementation with dietary flaxseed to provide the omega-3 fatty acid ALA will alter the lipid composition of adipocytes and increase the expression of protective adipokines and decrease the expression of pro-inflammatory adipokines in these animals.
3. Supplementation of the diet with flaxseed will protect against the deleterious effects of simultaneous dietary supplementation with cholesterol by altering the lipid composition of adipocytes and ameloration of the detrimental alterations in adipokine expression in these animals.

METHODS, RESULTS AND DISCUSSION

The methods, results and the discussion of our results are presented as a manuscript, which was published in Lipids (2011) 46:1043–1052. My contributions were as follows:

i) development of methods for RNA extraction, quantitative RT-PCR, and Western Blotting methods for fatty tissues,

ii) planning and implementation of all two-step qRT-PCR experiments, including design of primers, RNA extraction, spectrophotometry, cDNA production, PCR, data collection, analysis and statistics,

iii) planning and assistance in the design of gas chromatography studies, and the analysis and statistics of gas chromatography data,

iv) writing the manuscript and producing all figures and tables.

I also am responsible for the experimental design, tissue collection, experiments, and data analysis of the appended figures.

THE ALPHA LINOLEIC ACID CONTENT OF FLAXSEED IS ASSOCIATED WITH AN INDUCTION OF ADIPOSE LEPTIN EXPRESSION

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Running Title: Adipokines and dietary flaxseed

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KEYWORDS: flaxseed, alpha linolenic acid, adipokine, leptin, adiponectin, cholesterol, atherosclerosis, adipose tissue

Abstract

Dietary flaxseed has cardioprotective effects that may be achieved through its rich content of the omega-3 fatty acid, alpha linolenic acid (ALA). Because ALA can be stored in adipose tissue, it is possible that some of its beneficial actions may due to effects it has on the adipose tissue. We investigated the effects of dietary flaxseed both with and without an atherogenic cholesterol-enriched diet to determine the effects of dietary flaxseed on the expression of the adipose cytokines leptin and adiponectin. Rabbits were fed one of four diets: a regular (RG) diet, or a regular diet with added 0.5% cholesterol (CH), or 10% ground flaxseed (FX), or both (CF) for 8 weeks. Levels of leptin and adiponectin expression were assessed by RT-PCR in visceral adipose tissue. Consumption of flaxseed significantly increased plasma and adipose levels of ALA. Leptin mRNA expression was lower in CH animals and was elevated in CF animals. Changes in leptin expression were strongly and positively correlated with adipose ALA levels and inversely correlated with levels of *en face* atherosclerosis. Adiponectin expression was not significantly affected by any of the dietary interventions. Our data demonstrate that the type of fat in the diet as well as its caloric content can specifically influence leptin expression. The findings support the hypothesis that the beneficial cardiovascular effects associated with flaxseed consumption may be related to a change in leptin expression.

Introduction

Adipocytes, the cells that make up the bulk of adipose tissue in the body, have an important physiological role beyond their lipid storage capacity. They secrete a number of important cellular signaling molecules, termed adipokines. These adipokines have consequences ranging from local autocrine and paracrine effects to systemic endocrine actions. Adipokines also vary widely in both their function and mechanisms of control. One such mechanism of control is the fatty acid composition of adipose tissue which can affect cellular signaling, fatty acid trafficking, gene expression and, consequently, metabolism [1]. Adipose tissue composition varies based on two main effectors: energy balance, which regulates the metabolism of free fatty acids within the adipose tissue, and diet, which will alter the fatty acid profile of the adipose tissue. Although the former has been examined extensively, particularly with regard to leptin expression and adipocyte differentiation, the effects of the latter on endocrine function have only recently begun to be investigated.

Adiponectin is the most highly expressed and secreted adipokine, with beneficial effects on metabolism, inflammation, and vascular function. Adiponectin has a paradoxical expression pattern. As adiposity increases, adiponectin expression and secretion decreases within the adipose tissue [2]. It is believed that this paradox is part of the pathology of obesity, and is symptomatic of dysfunctional adipose tissue. Adiponectin plays a role in insulin sensitivity, LDL oxidation, eNOS activation, inflammation suppression and fatty acid catabolism [3-5]. Thus, hypoadiponectinemia is of interest as a biomarker of both cardiovascular disease and metabolic syndrome.

Another important adipokine that could be stimulated by changes in fatty acid profile is leptin. It was first discovered as the protein encoded by the *obese* gene, named for the phenotype of the double knockout mouse. These mice experience no satiety, and thus eat continuously when fed ad libidum, leading to severe diet-induced obesity. In humans, leptin deficiency has been implicated in cases of morbid obesity, as either a genetic factor or a metabolic insufficiency [6,7]. Leptin's role in hypothalamic-mediated appetite suppression in response to caloric intake is not its only function. Leptin may also be important in the modulation of T cell activity in the early stages of atherosclerotic development as well as other immune cells [8]. In obesity, leptin may be under-expressed by the adipose tissue in response to a consistently high caloric diet, or, leptin receptors may be down regulated, thus leading to high plasma leptin levels and leptin resistance [9].

Flaxseed has recently gained popularity as a functional food. Alpha-linolenic acid [ALA] comprises approximately 55% of the total fatty acid content of flaxseed fatty acids [10]. ALA-rich diets, including diets enriched with ground flaxseed, have been shown in interventional and experimental trials to reduce both fatal and non-fatal myocardial infarction [11, 12], cardiac arrhythmias [12-15], and the incidence of atherosclerotic lesions [12, 14, 16, 17]. However, the mechanism whereby ALA and flaxseed induce this cardio-protective action is unclear. Previous data have indicated that ALA from a flaxseed enriched diet is deposited in adipose tissue[18]. It is possible, therefore, that this change in adipose tissue fatty acid content may influence adipose tissue function. We hypothesize that the change in lipid composition in adipose tissue in response to a flaxseed supplemented diet may affect the adipokine signaling from

the adipocytes. It is possible, therefore, that the beneficial cardiovascular actions of flaxseed previously observed may be associated with changes in adipokine expression.

Materials and Methods

Diet and feeding

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Sixteen male New Zealand White rabbits [2.8 ± 0.1 kg, Southern Rose Rabbitry] were randomly assigned to receive one of four diets. Diets were prepared as previously described [15, 17] by the addition of components to a regular [RG] rabbit diet [CO-OP Complete Rabbit Ration, Federated Co-operatives]: 0.5% cholesterol [CH], or 10% ground flaxseed [FX], or both [CF] for 8 weeks [n=4]. The chow was stored at 4°C and protected from light. The diets differed only in total fat content due to the inclusion of the naturally ALA-rich ground flaxseed [Tables 1 and 2]. The diet fatty acid composition is outlined in Table 2. Addition of flaxseed to the diet significantly increased the amount of C16:0, C18:0, C18:1 (oleic acid) and C18:3 (ALA) provided. The addition of cholesterol had no significant effect on dietary fatty acids provided in comparison to the RG diet. Rabbits were fed 125 g/day of the diet.

Blood sampling and analysis

Blood was drawn from the left marginal ear vein of rabbits that were fasted overnight before starting their experimental diets and at 8 weeks. It was collected in vacutainer tubes containing EDTA [Becton Dickinson]. Blood samples were centrifuged at 4500 x g at room temperature for 10 min, and plasma was then stored at –80°C. Before analysis, plasma samples were thawed and centrifuged at 6800 x g. Plasma levels of cholesterol and triglycerides were analyzed using a VetTest 8008 blood chemistry analyzer [IDEXX Laboratories]. Fatty acids were extracted from plasma and derivatized, as described previously [15, 18].

Table 1. Crude dietary composition and total daily caloric intake

	RG	FX	CH	CF
Crude Protein (%)	21.3	20.5	20.4	20.5
Carbohydrates (%)	51.4	51.7	52.5	50.6
Crude Fat (%)	5.4	8.1	5.2	8.9
Crude Fibre (%)	13.5	11.7	13.6	12.4
Ash (%)	8.4	8.1	8.1	7.7
ALA (mg/g of diet)	2.0	20.0	2.1	22.5
Digestible Energy (kcal/g)	3.38	3.56	3.37	3.60

RG: regular diet; FX: 10% flaxseed supplemented diet; CH: 0.5% cholesterol supplemented diet; CF: 10% flaxseed and 0.5% cholesterol supplemented diet

Table 2. Fatty acid composition of rabbit diets

FAME (mg/g)	RG	FX	CH	CF
C14:0	0.293 ± 0.014	0.371 ± 0.013	0.267 ± 0.002	0.337 ± 0.016
C14:1	0.131 ± 0.001	t	0.033 ± 0.033	t
C16:0	6.502 ± 0.247	9.567 ± 0.349*	6.338 ± 0.044	9.364 ± 0.232*
C16:1	0.403 ± 0.024	0.540 ± 0.031	0.354 ± 0.001	0.442 ± 0.004
C18:0	2.200 ± 0.147	3.942 ± 0.157*	2.048 ± 0.006	3.643 ± 0.186*
C18:1 Ol	10.693 ± 0.325	17.055 ± 0.862*	10.292 ± 0.010	16.361 ± 0.840*
C18:1 Vac	1.719 ± 0.148	2.775 ± 0.002*	1.644 ± 0.064	2.611 ± 0.067
C18:2 LA	11.191 ± 0.343	11.781 ± 0.513	12.328 ± 0.170	13.713 ± 0.490
C20:0	0.113 ± 0.011	0.165 ± 0.009	0.117 ± 0.001	0.159 ± 0.025
C18:3n-6 GLA	t	0.115 ± 0.002	t	0.125 ± 0.004
C20:1	t	t	t	t
C18:3n-3 ALA	1.993 ± 0.120	20.077 ± 0.841*	2.119 ± 0.036	22.535 ± 0.679*^
C20:2	0.092 ± 0.012	0.125 ± 0.009	0.087 ± 0.002	0.106 ± 0.007
C22:0	0.160 ± 0.012	0.196 ± 0.002	0.166 ± 0.006	0.199 ± 0.036
C22:1	0.112 ± 0.032	0.292 ± 0.009	0.095 ± 0.009	0.094 ± 0.042
C20:3	t	t	t	0.193 ± 0.003
C22:6 DHA	t	t	0.105 ± 0.105	0.115 ± 0.115

Values are means ± SE, as mg of lipid/g of diet. Fatty acids were extracted from rabbit chow. RG, regular fed; FX, 10% flaxseed fed; CH, 0.5% cholesterol fed; CF, 0.5% cholesterol plus 10% flaxseed fed; t, trace amounts present (<0.010 mg/g of diet); * $p<0.05$ vs. RG, ^ $p<0.05$ vs. FX

Tissue collection

After 8 weeks of dietary treatment, animals were euthanized by 5% isoflurane gas delivered by facemask, followed by cardiac extraction. Retroperitoneal and epididymal adipose tissue were collected. To prevent RNase contamination, the animal and tools were sprayed with RNaseZap [Ambion] both before and during tissue collection. Adipose tissue was immediately placed in RNAlater, and kept overnight at 4°C, as indicated in the manufacturer's instructions [Ambion]. Preliminary testing indicated that there was successful stabilization of mRNA compared to flash freezing or maintenance overnight at 4°C , (as assessed by agarose gel electrophoresis and subsequent qRT-PCR) despite the high lipid content of this tissue. RNAlater was removed from the tissue by suction, and the samples were then flash frozen in liquid nitrogen and stored at -80°C.

qRT-PCR

RNA was extracted from adipose in an RNase-free environment. Adipose tissue was homogenized in Trizol reagent (Invitrogen), and fat was removed. Phenol was separated from the solution by washing the solution twice with chloroform. The RNA was precipitated from solution with ethanol, and added to RNeasy columns for further purification (Qiagen). Extracted RNA was quantified and assessed for quality by spectrophotometer and agarose gel electrophoresis. It was then used for qRT-PCR (Quanta Biosystems) using a iQ5 Real-Time PCR Detection System (Bio-Rad). Primers designed using BLAST software (NCBI) and were as follows: Adiponectin: *(Forward, 5' ACCAGGACAAGAACGTGGAC 3', Reverse, 5' TGGAGATGGAATCGTTGACA 3)]* ;

Leptin: (*Forward, 5′ GTCGTCGGTTTGGACTTCATC 3′, Reverse, 5′ CGGAGGTTCTCCAGGTCGTTG 3′)* [19];

GAPDH: [*Forward, 5′ GATGGTGAAGGTCGGAGTGAA 3′, Reverse, 5′ GGTGAAGACGCCAGTGGATT 3′)* [20].

Primers were validated using NCBI's BLAST software [21]. Unused samples were stored at -80°C. cDNA was synthesized from 1 ug of RNA with qScript cDNA Supermix (Quanta) via the manufacturer's directions. qPCR proceeded for 2 minutes at 50°C, 95°C for 8.5 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, at which point data was captured. A melt curve was obtained after cycling with 95°C for 1 minute followed by 55°C for 1 minute, and eighty 10 second capture cycles of 55°C+ 0.5°C/cycle. Results were normalized by GAPDH expression and analyzed by delta-delta-Ct method using iCycler Real-Time Detection Software.

Fatty Acid Extraction and Methylation

Plasma fatty acids were directly extracted and derivatized using a modification of the original method described by Lepage and Roy [22] and later modified by Garg *et al* [23]. Briefly, 100 µL of plasma was combined with 2 mL of 4:1 (*v*/*v*) methanol:toluene in a borosilicate glass tube. The methanol:toluene solution contained 0.5 mg/ml of the internal standard, C19:0 (Nu-Chek Prep. Inc.)**.** While vortexing, 200 µL of acetyl chloride was slowly added. Tubes were capped with a teflon lined lid, weighed and then heated at 100 °C for one hour. Once cooled to room temperature, tubes were re-weighed to ensure no sample loss had occurred. Five milliliters of an aqueous 6% K_2CO_3 solution was then added to terminate and neutralize the reaction. The sample tube was then centrifuged at 5000 rpm x 5 mins at

room temperature after which the upper toluene layer was removed and subjected to gas chromatographic analysis using flame ionization detection (GC-FID). Methylation was verified by thin layer chromatography. Fatty acids from approximately 15 mg of adipose tissue were extracted and derivatized using the method outlined by Lepage and Roy [24].

Gas Chromatography

Fatty acid methyl esters (FAME's) were injected onto a Varian CP 3800 gas chromatographic system using a Varian CP 8400 autosampler. Analytes were detected using flame ionization detection and analyzed on a Varian MS Workstation (vrs. 6.9.1). One microlitre of sample was injected at 250 °C at a split ratio of 50:1 onto a Varian CP-Sil 88 capillary column (60 m x 0.25 mm x 0.20 µm). Helium gas (ultra pure) was used as the carrier gas at a constant flow rate of 1.5 mL/min. The oven temperature was maintained at 111 °C for 1 min then rapidly increased by 20 °C/min to 170 °C. It was then slowly increased at a rate of 5 °C /min to 190 °C and finally by 3 °C/min up to 225 °C where it was maintained for 10 mins. FAMES were quantified against an external standard, GLC 462 (Nu-Chek Prep, Inc.).

Quantification of aortic atherosclerosis

The aorta from the ascending arch to the iliac bifurcation was isolated from peripheral tissues and washed in cold PBS, then opened longitudinally and pinned flat. The aortic lumen was digitally photographed and luminal images were analyzed with Silicon Graphics Imaging software. Fatty streaks and complicated lesions were expressed as a percentile of total luminal surface area.

Statistics

Results were reported as mean ± SE, and analyzed with Sigma-Stat software by one-way ANOVA, using Fishers LSD test. A significant correlation was identified by a t-test. $p \leq 0.05$ was considered statistically significant.

Results

Body Weight

After eight weeks of dietary treatment, mean body weight significantly increased from 2.8 ± 0.06 kg to 3.7 ± 0.09 kg. However, there was no effect on weight with the experimental diets as compared to control diets (data not shown).

Plasma lipids

There was no significant change in the plasma cholesterol of animals fed a regular or flaxseed supplemented diet after 8 weeks. Supplementation of the diet with cholesterol for 8 weeks induced severe hypercholesterolemia (Figure 1A). Addition of dietary flaxseed to a cholesterol-enriched diet did not lower the plasma cholesterol values from those observed in animals fed a diet supplemented with cholesterol alone.

Plasma triglycerides were not significantly affected by any of the diets (Figure 1B). Addition of milled flaxseed to the diet, providing ALA, induced a 17-fold increase in the percent composition of ALA in the plasma (Figure 1C). Simultaneous consumption of flaxseed and cholesterol doubled the amount of ALA in the plasma as compared to the consumption of flaxseed alone, comprising 21% of all plasma fatty acids., Animals fed dietary cholesterol in the absence of flaxseed exhibited a significant 8-fold increase in plasma ALA, despite being provided with only 2 mg of ALA/gram, supporting the observation that cholesterol aids in absorption of ALA (15-18).

Atherosclerosis

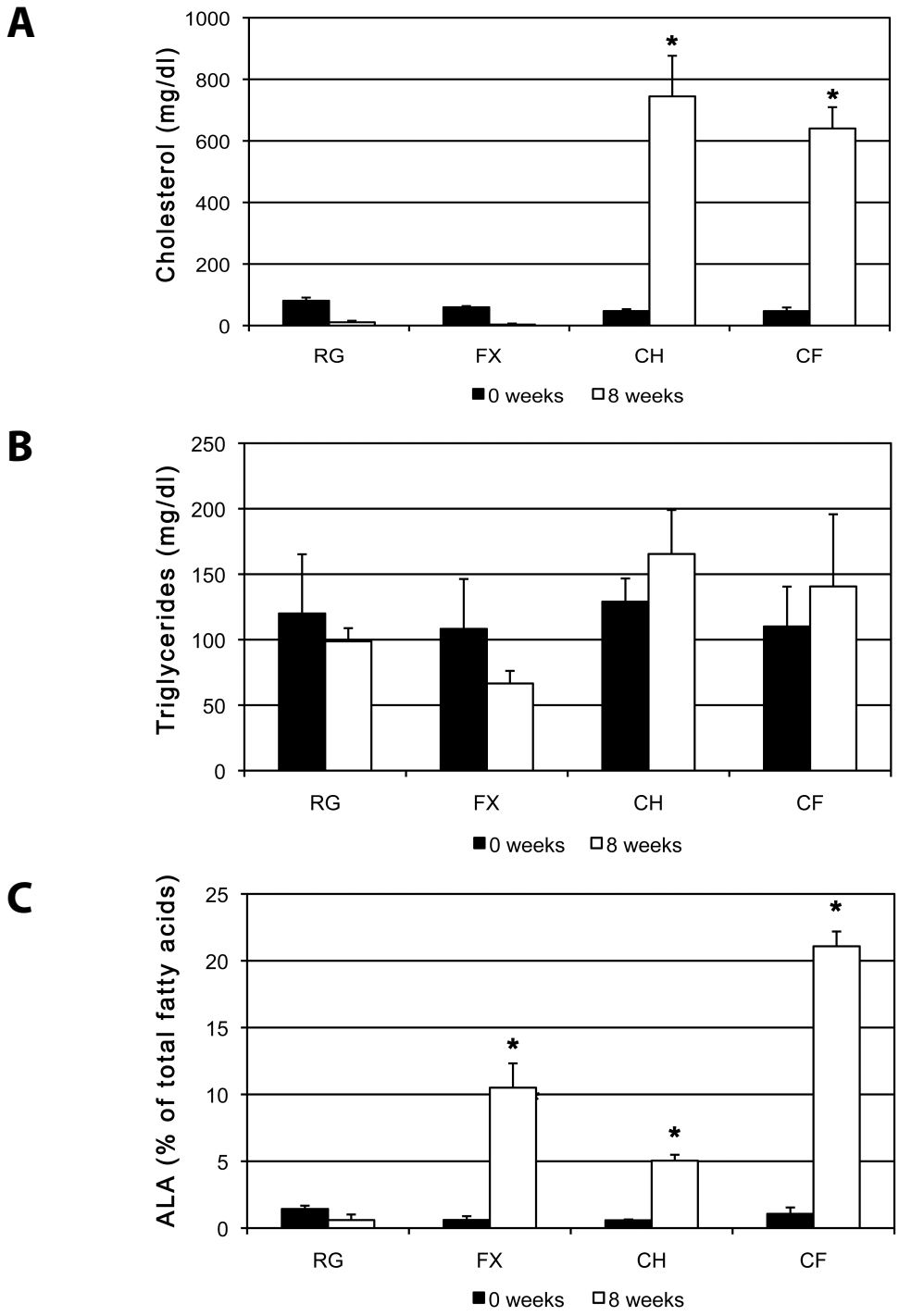

Figure 1. Plasma cholesterol (A), triglycerides (B), and alpha-linolenic acid (C) levels in male New Zealand White rabbits at baseline and after 8 weeks of dietary treatment. *P <0.05 vs. RG; n=3-4. RG, regular chow; FX, chow supplemented with 10% flaxseed; CH, 0.5% cholesterol-supplemented diet; CF, 10% flaxseed and 0.5% cholesterol-supplemented diet.

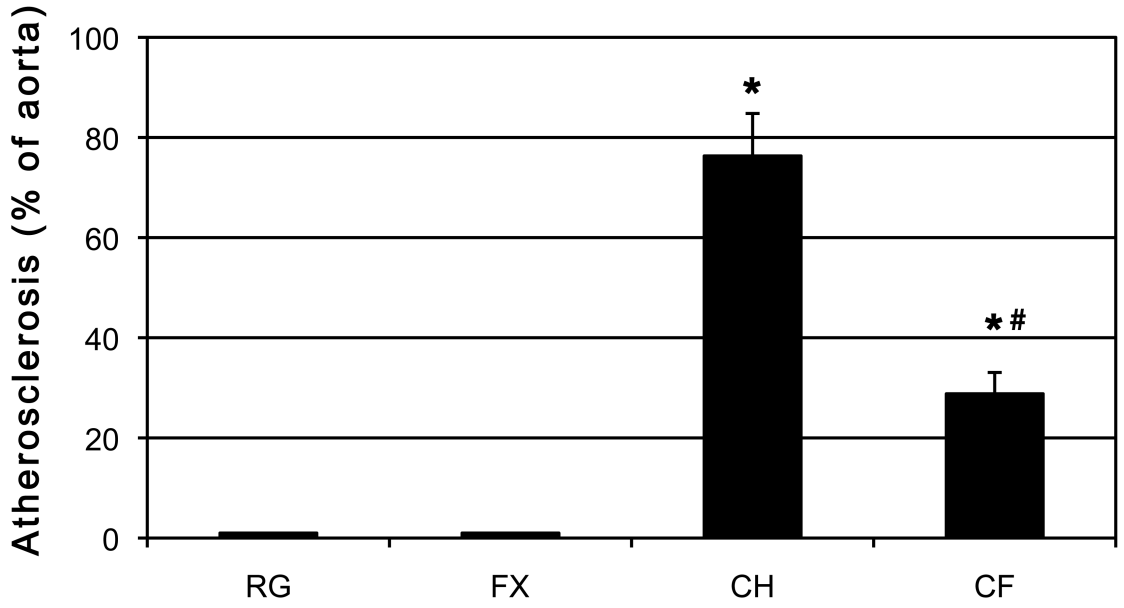

Figure 2. Development of atherosclerotic lesions on the aorta of New Zealand White rabbits after 8 weeks of dietary treatment. Values are means ± SE; n=3. *P ≤0.05 vs RG; #P ≤0.05 vs. CH. RG, regular chow; FX, chow supplemented with 10% flaxseed; CH, 0.5% cholesterol-supplemented diet; CF, 10% flaxseed and 0.5% cholesterol-supplemented diet.

Animals fed a regular or flaxseed-supplemented diet for 8 weeks did not develop any quantifiable atherosclerosis in the aortic arch (Figure 2). Inclusion of 0.5% cholesterol in the diet induced atherosclerotic lesions in the aorta, covering 76.3 ± 8.5% of the aortic lumen ($p<0.05$ vs. RG, n=3). Addition of ground flaxseed to the cholesterol-supplemented diet ameliorated the atherogenic effects of cholesterol, significantly reducing lesions to 28 ± 4.3% of the aortic lumen ($p<0.05$ vs. CH, n=3).

Fatty acid composition of adipose tissue

Total lipids were extracted from two primary sources of visceral fat. Epididymal and retroperitoneal fat consisted of 92.24% lipid by wet weight (range: 83.3-99.5%), with no significant changes in total lipid between different dietary treatments, or either adipose source (Tables 3 and 4).

The main component of adipose tissue was C18:1, oleic acid, which composed 32.3 ± 1.9% of total fatty acids in the retroperitoneal adipose of RG-fed animals (Table 3). C16:0, palmitic acid, and C18:2, linoleic acid, were also common, comprising 25.2 ± 1.4% and 26.7 ± 1.3% of the total retroperitoneal adipose tissue fatty acid content, respectively. Also stored in appreciable quantities in the retroperitoneal adipose tissue of animals fed a regular diet were ALA (6.34 ± 0.34%), steric acid (6.14 ± 0.57%), palmitoleic acid (2.12 ± 0.08%), vaccenic acid (1.98 ± 0.21%), and myristic acid (1.77 ± 0.12%) (Table 3).

The fatty acid proportions in the epididymal tissue of the animals fed a regular diet were not significantly different than the proportions of fatty acid in retroperitoneal tissue (Table 4). Dietary cholesterol did not significantly affect either

Table 3. Fatty acids in retroperitoneal adipose tissue from rabbits fed diets for 8 weeks

FAME (µmol/g)	Retroperitoneal adipose tissue			
	RG	FX	CH	CF
10:0	ND	ND	ND	ND
12:0	ND	ND	1.54 ± 0.94	1.45 ± 0.87
12:1	ND	ND	ND	ND
14:0	74.40 ± 4.00	50.91 ± 6.76*	66.17 ± 2.12	60.90 ± 1.84
14:1	ND	ND	ND	ND
16:0	945.68 ± 37.82	699.88 ± 15.45*	905.45 ± 40.17	773.21 ± 25.07*
16:1	81.17 ± 7.50	46.95 ± 6.42	90.90 ± 10.74	83.39 ± 13.85
18:0	195.76 ± 12.98	169.31 ± 8.66	179.48 ± 6.93	169.35 ± 4.22
18:1 n-9	1,104.79 ± 75.25	821.93 ± 49.73*	981.88 ± 20.87	962.26 ± 21.53
18:1 *trans*	67.80 ± 7.42	42.39 ± 3.38*	55.96 ± 1.62	49.30 ± 1.26*
18:2 n-6	878.82 ± 26.06	705.14 ± 35.87*	938.47 ± 69.49	778.75 ± 19.54
20:0	0.51 ± 0.38	0.45 ± 0.24	0.88 ± 0.45	ND
18:3 n-6	ND	ND	ND	ND
18:3 n-3	218.96 ± 7.95	895.96 ± 63.33*	184.22 ± 6.98	672.52 ± 78.99*^
20:1	14.49 ± 1.69	11.83 ± 1.16	12.46 ± 1.29	11.14 ± 1.40
20:2	1.68 ± 0.57	0.27 ± 0.18	1.31 ± 0.51	0.60 ± 0.23
22:0	ND	ND	ND	ND
20:3 n-6	ND	ND	ND	ND
20:3 n-3	ND	5.76 ± 0.89	ND	3.32 ± 0.80
22:1	0.68 ± 0.28	0.22 ± 0.15	0.43 ± 0.26	0.83 ± 0.62
20:4 n-6	0.76 ± 0.44	0.38 ± 0.89	1.28 ± 0.75	0.83 ± 0.48
C22:2	ND	ND	ND	ND
C20:5 n-3	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
C24:1	ND	ND	ND	ND
C22:4	ND	ND	ND	ND
C22:5	ND	0.58 ± 0.45	ND	0.48 ± 0.2
C22:6	ND	ND	ND	ND
TL (%)	90.5 ± 0.23	91.4 ± 3.70	95.7 ± 2.02	94.4 ± 1.35

Values are means ± SE. Fatty acids were extracted from retroperitoneal adipose tissue following 8 weeks of feeding ($n = 4$)

RG regular fed, *FX* 10% flaxseed fed, *CH* 0.5% cholesterol fed, *CF* 0.5% cholesterol plus 10% flaxseed fed, *ND* not detectable amounts present, *TL* total mg lipids extracted per mg of tissue

* $p < 0.05$ versus RG

^ $p < 0.05$ versus FX

Table 4. Fatty acids in epididymal adipose tissue from rabbits fed diets for 8 weeks

FAME (µmol/g)	Epididymal adipose tissue			
	RG	FX	CH	CF
10:0	ND	ND	ND	ND
12:0	ND	1.13 ± 0.85	2.48 ± 1.70	ND
12:1	ND	ND	ND	ND
14:0	79.12 ± 3.28	59.37 ± 4.65	68.03 ± 4.33	57.85 ± 7.95
14:1	0.99 ± 0.52	ND	ND	ND
16:0	919.51 ± 32.27	676.35 ± 20.48*	862.90 ± 46.25	743.00 ± 44.11*
16:1	101.26 ± 11.89	64.76 ± 10.31	89.76 ± 14.79	92.60 ± 21.24
18:0	193.31 ± 7.60	162.54 ± 6.79	169.93 ± 11.23	158.20 ± 7.84
18:1 n-9	1,122.28 ± 38.65	889.86 ± 29.71*	925.11 ± 25.28	917.13 ± 73.87*
18:1 *trans*	69.78 ± 6.12	48.23 ± 1.50*	54.66 ± 3.68	49.07 ± 5.21*
18:2 n-6	893.65 ± 36.57	694.95 ± 22.56*	887.65 ± 17.06	789.54 ± 40.46
20:0	0.88 ± 0.45	ND	1.16 ± 0.61	1.27 ± 0.30
18:3 n-6	ND	ND	ND	ND
18:3 n-3	219.61 ± 7.33	916.35 ± 27.84*	176.76 ± 6.12	569.80 ± 96.29*^
20:1	15.06 ± 1.53	12.81 ± 0.92	11.97 ± 0.45	12.26 ± 0.26
20:2	1.13 ± 0.40	0.48 ± 0.28	0.98 ± 0.57	ND
22:0	ND	ND	ND	ND
20:3 n-6	ND	ND	ND	ND
20:3 n-3	ND	6.58 ± 2.16	ND	0.96 ± 0.81
22:1	1.67 ± 0.64	ND	1.83 ± 0.54	1.78 ± 0.34
20:4 n-6	ND	ND	ND	ND
C22:2	ND	ND	ND	ND
C20:5 n-3	ND	ND	ND	ND
C24:0	ND	ND	ND	ND
C24:1	ND	ND	ND	ND
C22:4	ND	ND	ND	ND
C22:5	ND	ND	ND	ND
C22:6	ND	ND	ND	ND
TL (%)	93.2 ± 2.87	90.5 ± 4.11	88.1 ± 3.36	94.1 ± 2.18

Values are means ± SE. Fatty acids were extracted from epididymal adipose tissue following 8 weeks of feeding ($n = 4$)

RG regular fed, *FX* 10% flaxseed fed, *CH* 0.5% cholesterol fed, *CF* 0.5% cholesterol plus 10% flaxseed fed, *ND* not detectable amounts present, *TL* total mg lipids extracted per mg of tissue

* $p < 0.05$ versus RG

^ $p < 0.05$ versus FX

total lipids or individual fatty acids as compared to RG. Addition of flaxseed to a regular diet significantly reduced levels of C16:0, C18:1-*cis*, C18:1-*trans*, and C18:2 in adipose tissue, both in absolute concentrations and relative to total fatty acid content (Tables 3 and 4). When consumed in conjunction with 0.5% cholesterol, dietary flaxseed induced a 3.1-fold increase in adipose tissue ALA, a significant increase as compared to RG, but significantly less than animals supplemented only with flaxseed. Dietary cholesterol did not affect any of the other fatty acids observed.

Adipokine expression

Adiponectin and leptin are the two most highly expressed adipokines in adipose tissue. In epididymal adipose tissue, there was no significant change in either leptin or adiponectin mRNA expression after addition of either flaxseed or cholesterol to the diet (Figure 3A). Adiponectin expression did not vary with dietary treatment in retroperitoneal adipose tissue (Figure 3B). However, in retroperitoneal adipose tissue, dietary flaxseed induced a two-fold increase in leptin mRNA ($p<0.05$ vs. RG). Conversely, dietary cholesterol reduced leptin mRNA expression by about one-half in comparison to control, and the addition of flaxseed to the cholesterol-supplemented diet induced a recovery of leptin expression, increasing expression beyond that of flaxseed alone.

These changes in leptin expression were positively correlated with plasma ALA and adipose ALA levels (Figure 4). Plasma ALA correlated with leptin expression in the retroperitoneal adipose tissue ($p<0.05$), but not in epididymal adipose (Figure 4A).

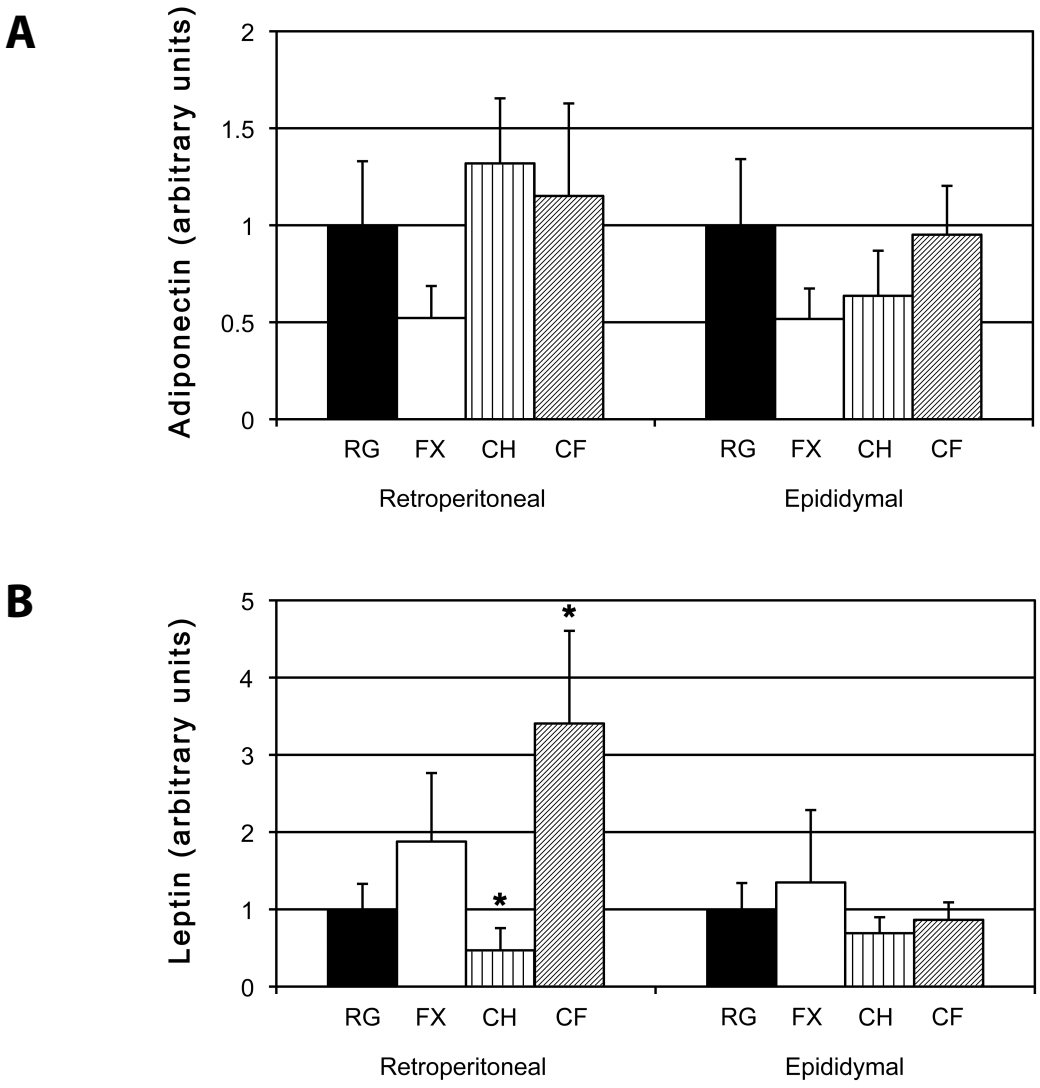

Figure 3. mRNA expression of adiponectin (A) or leptin (B) in epididymal and retroperitoneal adipose tissue of New Zealand White rabbits after 8 weeks of dietary treatment, measured by quantitative real-time PCR. Values were normalized by GAPDH expression and expressed as mean ± SE; n=3-4. * $P<0.05$. RG, regular chow; FX, chow supplemented with 10% flaxseed; CH, 0.5% cholesterol-supplemented diet; CF, 10% flaxseed and 0.5% cholesterol-supplemented diet.

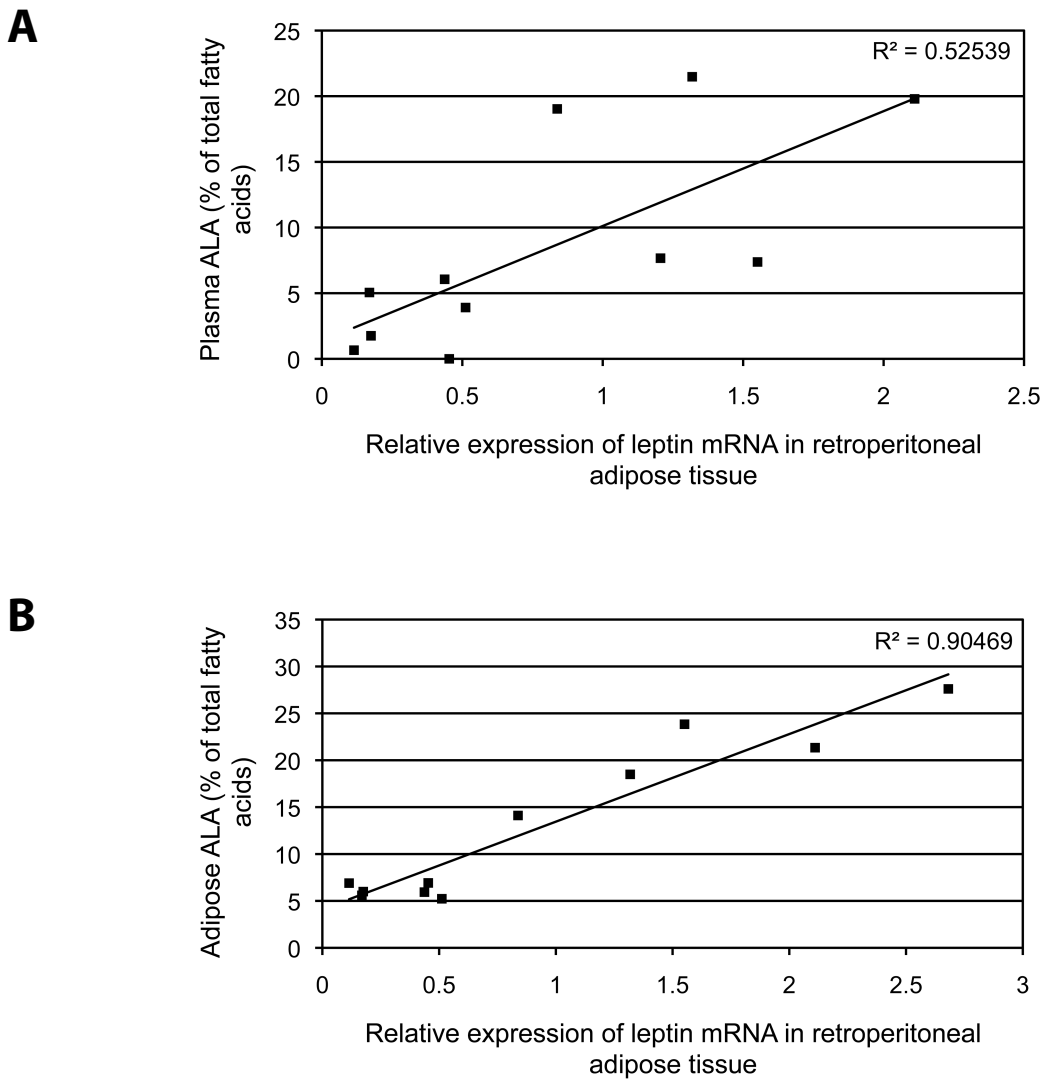

Figure 4. Correlational analysis of expression of leptin mRNA from retroperitoneal adipose tissue with plasma alpha-linolenic acid (A), and adipose alpha-linolenic acid (B) of New Zealand White rabbits after 8 weeks of dietary treatment. A: $P<0.05$; B: $P<0.05$. White rabbits after 8 weeks of dietary treatment.

Adipose tissue ALA exhibited a stronger relationship than plasma ALA with leptin expression in retroperitoneal adipose tissue (Figure 4B).

Relationship of leptin expression to atherosclerosis

All animals that were not fed cholesterol did not exhibit any atherosclerosis. Consequently, in order to determine if there was a significant relationship between leptin expression and the development of atherosclerotic lesions, linear regression was performed only on data obtained from animals fed a cholesterol-supplemented diet. Increased leptin expression in the retroperitoneal adipose tissue significantly correlated ($p<0.05$) with decreased atherosclerosis (Figure 5).

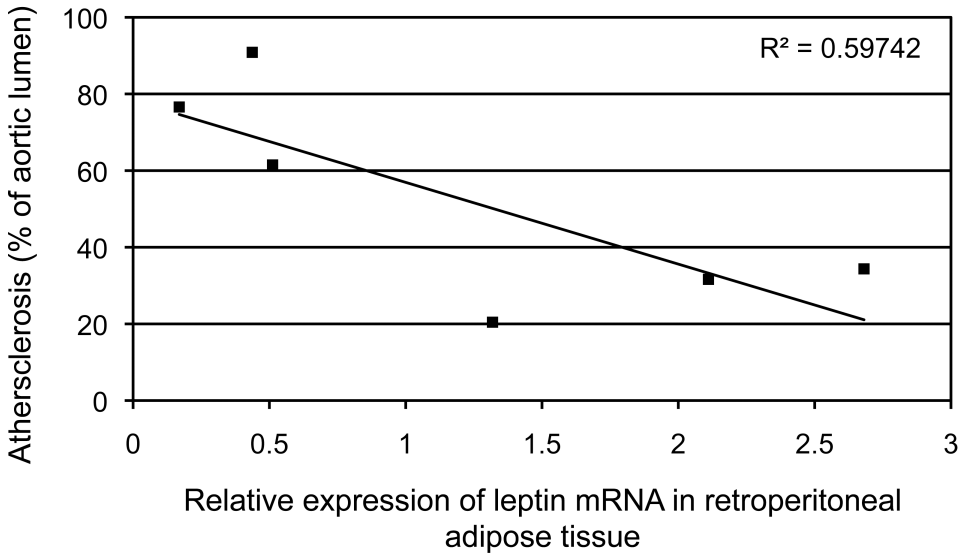

Figure 5. Correlational analysis of the percentage of the aortic lumen covered with atherosclerotic plaque with leptin mRNA from retroperitoneal adipose tissue of hypercholesterolemic New Zealand White rabbits after 8 weeks of atherogenic dietary treatment. $P<0.05$.

Discussion

One of the primary purposes of this study was to determine if diets of very different lipid composition could influence the expression of leptin or adiponectin in adipose tissue when caloric balance was maintained. It is known that leptin expression varies with caloric balance and adiposity [26-28]. The present study was designed to avoid these potential variables by maintaining the diets in a manner which maintained a consistent level of caloric intake while at the same time insuring diversity in lipid composition. The differences in caloric values between the diets were so minor that they did not induce a significant change in body weight. The data obtained in the present study demonstrates that the lipid composition of the diet can have an important role to play in adipokine expression. The changes in adipokine expression were sensitive to the type of lipid in the diet, the adipose tissue examined and the adipokine studied as well. A high cholesterol diet suppressed leptin expression whereas a diet rich in the omega-3 fatty acid ALA increased leptin expression. Leptin expression was influenced by the diets whereas adiponectin was not. Retroperitoneal but not epididymal adipose tissue was affected by the diets. It is known that the location of the different adipose tissues will influence circulating adipokine levels [29].

The mechanism whereby leptin mRNA expression is regulated by dietary lipids is presently unclear. Although SREBP1c mRNA expression if directly related to polyunsaturated fatty acid content in adipose tissue [30] and an SREBP-like binding element is present in the leptin promoter, it is not responsive to SREBP itself [31]. However, polyunsaturated fatty acids may act as ligands for PPAR gamma to alter adipokine expression [32]. This may occur through an increase in translation [33].

Mason *et al* have demonstrated a novel binding site for an adipocyte-specific transcription factor in the -87 position of the leptin promoter which is conserved in both mice and humans, however, the consensus sequence does not match any known transcription factor [28]. This region, termed LP1, presents an interesting possibility for a novel transcription factor which may regulate the response of leptin to dietary lipids in addition to PPAR-gamma.

The observation that the adipose tissue is responding to these diets in a very specific manner suggests that the changes are physiologically important. Increased leptin levels have been identified previously as a risk factor for atherosclerosis [34]. However, data correlating leptin and atherosclerosis have been derived from obese humans and animal models of obesity [35-39]. Our data indicates that in a non-obese population, leptin may have a previously unidentified role in cardioprotection. In support of this hypothesis, leptin expression was significantly correlated in a negative fashion with atherosclerosis. When leptin levels were high, atherosclerosis was low and when leptin expression was depressed by circulating cholesterol, atherogenesis was stimulated.

Consumption of flaxseed has previously been shown to improve insulin resistance, hyperlipidemia, atherosclerosis and hypertension and decrease the incidence of cardiac arrhythmias [15-17, 40]. These effects of dietary flaxseed have been attributed, in part, to the rich ALA content of flaxseed [15-17, 40]. Dietary flaxseed provided a source of ALA in the present study which subsequently increased ALA both in the circulation and in the adipose tissue. With the addition of cholesterol to the flaxseed-supplemented diet, plasma ALA increased substantially but adipose

tissue levels of ALA did not exhibit further increases beyond those observed when the diet was supplemented with flaxseed alone (Table 3). This could be due to one of several factors. ALA may be deposited in other organs preferentially, such as the heart and liver, as previously observed in the hypercholesterolemic rabbit [18]. Alternatively, a cholesterol-rich diet may direct the ALA to be used directly for beta-oxidation, thus reducing the amount of ALA available for storage [41]. Cholesterol may also induce an efflux of ALA from the adipose tissue, which is greater than the influx of ALA from the plasma, leading to a net decrease in ALA [42]. Further study is required to understand the relative lack of storage of ALA in the adipose tissue when it is presented with such high levels of circulating ALA.

Both beneficial and deleterious cytokines from the adipose tissue may be responsible for many of the links between diet, BMI and cardiovascular disease. The present data demonstrates that dietary cholesterol and flaxseed have the capacity to alter leptin expression. The cardioprotective effects of flaxseed are thought to be provided in part by its delivery of ALA to the body [16, 40]. However, the mechanism to explain the induction of these effects by ALA remains elusive. In the present study, ALA in the adipose tissue was strongly associated with increased leptin expression and the subsequent reduction of atherosclerosis. Our data, therefore, suggests that flaxseed may induce its anti-atherogenic effects in part via an ALA-mediated modulation of the expression of leptin.

Acknowledgements

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References

1 Gertow K, Rosell M, Sjogren P, Eriksson P, Vessby B, de Faire U, Hamsten A, Hellenius ML, Fisher RM (2006) Fatty acid handling protein expression in adipose tissue, fatty acid composition of adipose tissue and serum, and markers of insulin resistance. Eur J Clin Nutr 60:1406-1413.

2 Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G (2003) Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes 52:1779-1785.

3 Haluzik M, Parizkova J, Haluzik MM (2004) Adiponectin and its role in the obesity-induced insulin resistance and related complications. Physiol Res 53:123-129.

4 Halleux CM, Takahashi M, Delporte ML, Detry R, Funahashi T, Matsuzawa Y, SrBrichard SM (2001) Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. Biochem Biophys Res Commun 288:1102-1107.

5 Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G, Gallo P, di Gioia CR (2005) Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine 29:251-255.

6 Paz-Filho G, Esposito K, Hurwitz B, Sharma A, Dong C, Andreev V, Delibasi T, Erol H, Ayala A, Wong ML, Licinio J (2008) Changes in insulin sensitivity during leptin replacement therapy in leptin-deficient patients. Am J Physiol Endocrinol Metab 295: E1401-1408.

7 Bluher S, Mantzoros CS (2009) Leptin in humans: lessons from translational research. Am J Clin Nutr 89:991S-997S.

8 Taleb S, Herbin O, Ait-Oufella H, Verreth W, Gourdy P, Barateau V, Merval R, Esposito B, Clement K, Holvoet P, Tedgui A, Mallat Z (2007) Defective leptin/leptin receptor signaling improves regulatory T cell immune response and protects mice from atherosclerosis. Arterioscler Thromb Vasc Biol 27:2691-2698.

9 Dubey H, Das SK, Panda T (2006) Numerical simulation of a fully baffled biological reactor: the differential circumferential averaging mixing plane approach. Biotechnol Bioeng 95:754-766.

10 Flax Council 2015 (2009) Flax - A Healthy Food, Flax Council of Canada.

11 de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin J L, Monjaud I, Guidollet J, Touboul P, Delaye J (1994) Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet 343:1454-1459.

12 Albert CM, Oh K, Whang W, Manson JE, Chae CU, Stampfer MJ, Willett WC, Hu FB (2005) Dietary alpha-linolenic acid intake and risk of sudden cardiac death and coronary heart disease. Circulation 112:3232-3238.

13 Smith PJ, Blumenthal JA, Babyak MA, Georgiades A, Sherwood A, Sketch MH, Jr, Watkins LL (2009) Association between n-3 fatty acid consumption and ventricular ectopy after myocardial infarction. Am J Clin Nutr, 89:1315-1320.

14 Tziomalos K, Athyros VG, Karagiannis A, Mikhailidis DP (2008) Omega-3 fatty acids: how can they be used in secondary prevention? Curr Atheroscler Rep, 10:510-517.

15 Ander BP, Weber AR, Rampersad PP, Gilchrist JSC, Pierce GN, Lukas A (2004) Dietary flaxseed protects against ventricular fibrillation induced by ischemia-reperfusion in normal and hypercholesterolemic Rabbits. J Nutr 134:3250-3256.

16 Dupasquier CM, Dibrov E, Kneesh AL, Cheung PK, Lee KG, Alexander HK, Yeganeh BK, Moghadasian MH, Pierce GN (2007) Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative and anti-inflammatory actions. Am J Physiol Heart Circ Physiol 293:H2394-2402.

17 Dupasquier CM, Weber AM, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, Mitchell RW, Kroeger EA, Gilchrist JS, Moghadasian MM, Lukas A, Pierce GN (2006) Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. Am J Physiol Heart Circ Physiol 291:H2987-2996.

18 Ander BP, Edel AL, McCullough RS, Rodriguez-Leyva D, Rampersad P, Gilchrist JSC, Lukas A, Pierce GN (2010) Distribution of omega-3 fatty acids in tissues of rabbits fed a flaxseed-supplemented diet. Metabolism 59: 620-627.

19 Zhao SP, Wu ZH (2005) Atorvastatin reduces serum leptin concentration in hypercholesterolemic rabbits. Clin Chim Acta 360:133-140.

20 Rajagopalan S, Duquaine D, King S, Pitt B, Patel P (2002) Mineralocorticoid receptor antagonism in experimental atherosclerosis. Circulation 105:2212-2216.

21 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

22 Lepage G and Roy CC (1986). Direct transesterification of all classes of lipids in a one step reaction. Journal of Lipid Research. 27: 114-120.

23 Garg ML, Leitch J, Blake RJ, Garg R (2006). Long-chain n-3 polyunsaturated fatty acid incorporation into human atrium following fish oil supplementation. Lipids. 41: 1127-1132.

24 Lepage G and Roy CC (1984). Improved recovery of fatty acid through direct transesterification without prior extraction or purification. Journal of Lipid Research. 25: 1391-1396.

25 Farooqi IS and O'Rahilly S (2009) Leptin: a pivotal regulator of human energy homeostasis. Am J Clin Nutr 89:980S-984S.

26 Havel PJ (2000) Role of adipose tissue in body-weight regulation: mechanisms regulating leptin production and energy balance. Proc Nutr Soc 59:s359-371.

27 Ingvartsen KL, Boisclair YR (2001) Leptin and the regulation of food intake, energy homeostasis and immunity with special focus on periparturient ruminants. Domest Anim Endocrinol 21:215-250.

28 Mason MM, He Y, Chen H, Quon MJ, Reitman M (1998) Regulation of leptin promoter function by Sp1, C/EBP, and a novel factor. Endocrinology 139:1013-1022.

29 Turer AT, Khera A, Ayers CR, Turer CB, Grundy SM, Vega GL, Scherer PE (2011) Adipose tissue mass and location affect circulating adiponectin levels. Diabetologia July 22 Epub ahead of print.

30 Muhihauser BS, Cook-Johnson R, James M, Miljkovic D, Duthoit E, Gibson R (2010) Opposing effects of omega-3 and omega-6 long chain polyunsaturated

fatty acids on the expression of lipogenic genes in omental and retroperitoneal adipose depots in the rat. J Nutr Metab pii 927836 Epub.

31 Rahmouni K, Sigmund CD (2008) Id3, E47, and SREBP-1c: fat factors controlling adiponectin expression. Circ Res 103:565-567.

32 Yu YH, Wu SC, Cheng WT, Mersmann HJ, Shen TL, Ding ST (2011) The function of porcine PPARy and dietary fish oil effect on the expression of lipid and glucose metabolism related genes. J Nutr Biochem 22:179-186.

33 Banga A, Unal R, Tripathi P, Pokrovskaya I, Owens RJ, Kern PA, Ranganathan G (2009) Adiponectin translation is increased by the PPARgamma agonists pioglitazone and omega-3 fatty acids. Am J Physiol 296:E480-489.

34 Correia ML, Rahmouni K (2006) Role of leptin in the cardiovascular and endocrine complications of metabolic syndrome. Diabetes Obes Metab 8:603-610.

35 Bartels ED, Bang CA, Nielsen LB (2009) Early atherosclerosis and vascular inflammation in mice with diet-induced type 2 diabetes. Eur J Clin Invest 39:190-199.

36 Ukkola O, Poykko S, Paivansalo M, Kesaniemi YA (2008) Interactions between ghrelin, leptin and IGF-I affect metabolic syndrome and early atherosclerosis. Ann Med 40: 465-473.

37 Hoefle G, Saely CH, Risch L, Rein P, Koch L, Schmid F, Aczel S, Marte T, Langer P, Drexel H (2007) Leptin, leptin soluble receptor and coronary atherosclerosis. Eur J Clin Invest 37:629-636.

38 Ogawa T, Hirose H, Yamamoto Y, Nishikai K, Miyashita K, Nakamura H, Saito I, Saruta T (2004) Relationships between serum soluble leptin receptor level and serum leptin and adiponectin levels, insulin resistance index, lipid profile, and leptin receptor gene polymorphisms in the Japanese population. Metabolism 53:879-885.

39 Chiba T, Shinozaki S, Nakazawa T, Kawakami A, Ai M, Kaneko E, Kitagawa M, Kondo K, Chait A, Shimokado K (2008) Leptin deficiency suppresses progression of atherosclerosis in apoE-deficient mice. Atherosclerosis 196:68-75.

40 Bassett CM, Rodriguez-Leyva D, Pierce GN (2009) Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. Appl Physiol Nutr Metab 34: 965-974.

41 Barcelo-Coblijn G, Murphy EJ (2009) Alpha-linolenic acid and its conversion to longer chain n-3 fatty acids: Benefits for human health and a role in maintaining tissue n-3 fatty acid levels. Prog Lipid Res 48:355-7.

42 Tang AB, Nishimura KY, Phinney SD (1993) Preferential reduction in adipose tissue alpha-linolenic acid (18:3 omega 3) during very low calorie dieting despite supplementation with 18:3 omega 3. Lipids 28:987-993.

APPENDIX 1: VALIDATION OF RNALATER USE FOR ADIPOSE TISSUE

Methods

Because the manufacturer's directions cautions against the use of RNAlater in fatty tissues, before utilizing it, we validated that it would improve mRNA stabilization beyond simple flash freezing. To minimize variability, all samples were obtained from adjacent cuts to the retroperitoneal adipose tissue of one control-fed New Zealand White rabbit. All samples and tools were sprayed with RNaseZap (Ambion), and placed in sterile tubes. Samples were assigned to one of four conditions: a) immediate RNA extraction with no RNA preservation treatment, b) flash freezing in liquid nitrogen and storage at -80˚C for at least 24 hours, followed by RNA extraction, c) storage overnight in at 4˚C, flash freezing in liquid nitrogen and storage at -80˚C for at least 24 hours, followed by RNA extraction, or d) storage in RNAlater over night at 4˚C, flash freezing in liquid nitrogen and storage at -80˚C for at least 24 hours, followed by RNA extraction (as per the manufacturer's directions).

Sample concentrations and quality were then quantified by spectrophotometer, and analyzed by qRT-PCR as described previously on page 46, using GAPDH and adiponectin primers.

Results

Although no benefit was achieved in stabilization of GAPDH mRNA, there was demonstrable benefit in adiponectin stabilization with the use of RNAlater (Figure A1-1).

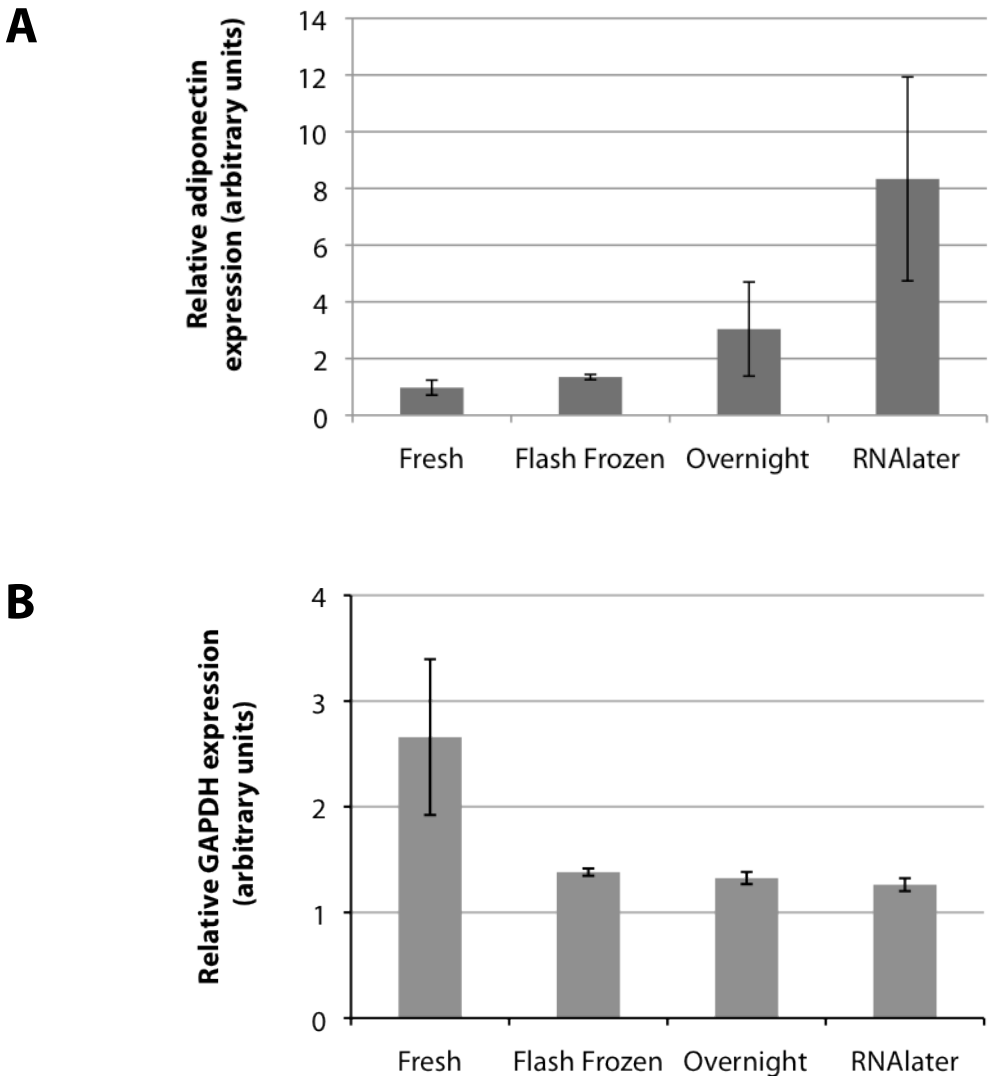

Figure A1-1. Validation of RNAlater for adipose tissue mRNA. mRNA expression from one sample of retroperitoneal adipose tissue of a healthy, control diet-fed male New Zealand White rabbit was examined for adiponectin (A) or GAPDH expression (B). Fresh, freshly excised tissue was used for immediate RNA extraction; Flash Frozen, sample frozen in liquid nitrogen after being excised; Overnight, samples kept in 4˚C overnight before being flash frozen for storage; RNAlater, samples were stored in RNAlater (Ambion) overnight in 4˚C before being flash frozen for storage. Values are means ± SE (n=3).

APPENDIX 2: CIRCULATING C-REACTIVE PROTEIN IS REDUCED BY THE ADDITION OF FLAXSEED TO THE DIET

Methods

Plasma was collected from fasted rabbits after eight weeks of dietary treatment as described previously on page 46. Plasma was flash frozen in liquid nitrogen and stored at -80˚C until use. Samples were pre-cleared of IgGs before Western blotting using Protein A beads. 20 ul of plasma was added to a 10% protein A bead solution in RIPA buffer. Samples were rotated at 4˚C for four hours, then, centrifuged, collected, and stored in sample buffer at -20˚C until ready for Western blotting.

2.5 ul of pre-cleared plasma was run on a 10% gel via SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 10% milk powder(Carnation) in TBS-T, then incubated overnight with chicken anti-CRP primary antibody (1:1000) in 1% milk powder in TBS-T. Appropriate horseradish perodixase conjugated secondary antibody was applied and the signal was developed using West Pico chemiluminescence substrate (Pierce) and quantified by densitometric analysis using Quantity One software on a Bio-Rad GS-670 imaging densitometer. Equal loading and transfer of protein were verified using Ponceau S and Coomassie blue staining of the membrane and gel, respectively. Protein levels were normalized via albumin expression and represented as percent expression of control (RG).

Results

Figure A2-1 demonstrates that flaxseed is capable of dramatically reducing CRP expression, reducing it by 50% even in the absence of the pro-inflammatory stimulus of cholesterol. As adipose tissue is a major source of pro-inflammatory cytokines like CRP, these data warrants further investigation into the anti-inflammatory effects of flaxseed in adipose tissue.

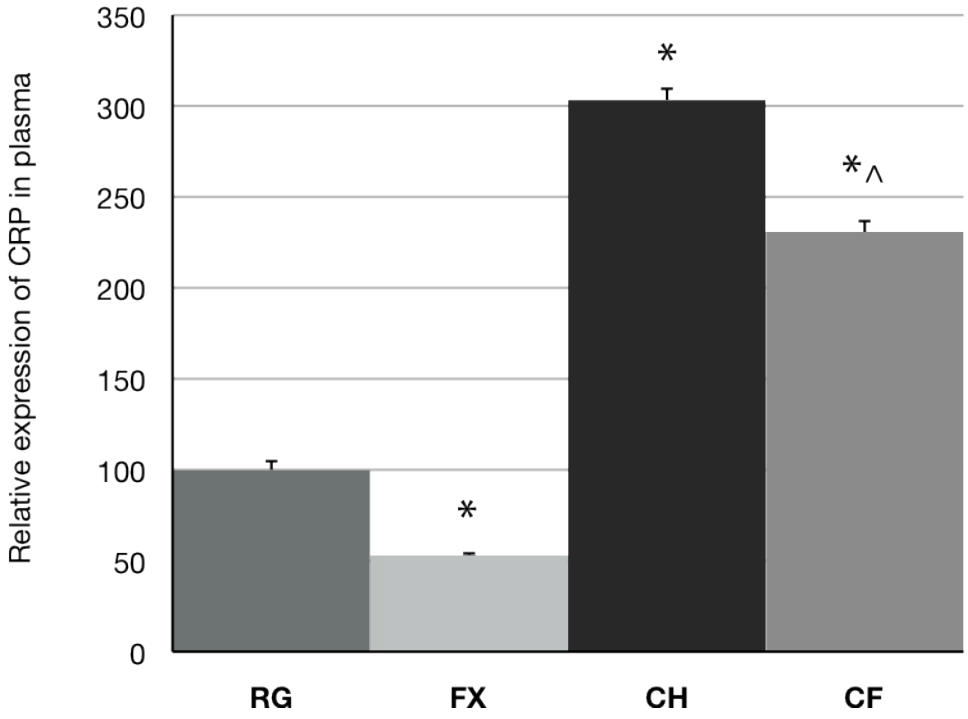

Figure A2-1. Expression of C-reactive protein in the plasma of New Zealand White rabbits after 8 weeks of dietary treatment.

Values are means ± SE; n=4. *P ≤0.05 vs RG; ^P ≤0.05 vs. CH.

RG, regular chow; FX, chow supplemented with 10% flaxseed; CH, 0.5% cholesterol-supplemented diet; CF, 10% flaxseed and 0.5% cholesterol-supplemented diet.

CONCLUSIONS

We have demonstrated that dietary flaxseed exhibits anti-atherogenic, anti-inflammatory effects in the hypercholesterolemic rabbit model of atherosclerosis. Furthermore, we have demonstrated that ALA from dietary flaxseed localizes to the adipose tissue, and that these changes in ALA are associated with leptin expression in the retroperitoneal adipose tissue. Finally, we have shown leptin mRNA expression levels in the retroperitoneal adipose tissue are correlated with decreases in atherosclerosis in this model, establishing a potential causative link between ALA and cardioprotection.

These conclusions lend further understanding to the mechanism whereby flaxseed exerts is beneficial effects. Given the social and economic impacts of cardiovascular disease, and the rising obesity epidemic worldwide, further research into this link may result in important knowledge for the promotion easily accessible interventions for health maintenance.

LITERATURE CITED

1. Levick RJ. *An Introduction to Cardiovascular Pathophysiology*. 5th ed. Oxford University Press, USA; 2010:1–328.

2. Cardiovascular Disease Morbidity, Mortality and Risk Factors Surveillance Information - Public Health Agency Canada. *phac-aspc.gc.ca*.:1–16. Available at: http://www.phac-aspc.gc.ca/cd-mc/cvd-mcv/cvdmmrf-mmmcvfr-eng.php. Accessed July 7, 2011.

3. R Ross JGLH. Response to injury and atherogenesis. *The American Journal of Pathology*. 1977;86(3):675.

4. Weissberg PL. Coronary disease: Atherogenesis: current understanding of the causes of atheroma. *Heart*. 2000;83(2):247–252.

5. Cesar L, Suarez SV, Adi J, et al. An essential role for diet in exercise-mediated protection against dyslipidemia, inflammation and atherosclerosis in ApoE$^{-/-}$ mice. *PLoS ONE*. 2011;6(2):e17263.

6. Chan SY, Mancini GBJ, Burns S, et al. Dietary measures and exercise training contribute to improvement of endothelial function and atherosclerosis even in patients given intensive pharmacologic therapy. *J Cardiopulm Rehabil*. 2006;26(5):288–293.

7. Shimizu T. Health claims on functional foods: the Japanese regulations and an

international comparison. *Nutr Res Rev*. 2003;16(2):241–252.

8. Canada H. *Nutraceuticals/Functional Foods and Health Claims on Foods*. Health Canada; 1998.

9. Shimizu T. Health claims on functional foods: the Japanese regulations and an international comparison. *Nutr Res Rev*. 2003;16(2):241–252.

10. Hornberger J. A cost-benefit analysis of a cardiovascular disease prevention trial, using folate supplementation as an example. *Am J Public Health*. 1998;88(1):61–67.

11. Holub B. *Potential benefits of functional foods and nutraceuticals to reduce the risk and costs of disease in Canada*. 2002.

12. Lamotte M, Annemans L, Kawalec P, Zoellner Y. A multi-country health economic evaluation of highly concentrated N-3 polyunsaturated fatty acids in secondary prevention after myocardial infarction. *Pharmacoeconomics*. 2006;24(8):783–795.

13. Fidan D, Unal B, Critchley J, Capewell S. Economic analysis of treatments reducing coronary heart disease mortality in England and Wales, 2000-2010. *QJM*. 2007;100(5):277–289.

14. Bouphasiri C V. *A case study of the Canadian oat market: The evolution from the central desk system to the open market*. 2005.

15. Advertising Expenditure Measurement: Quaker Oats. 2009.

16. Food and Ingredient Trends Addressing Specific Diseases and Other Health

Conditions. 2008.

17. Global Market Review of Functional Foods - Forecasts to 2013. 2008.

18. *Canada: Grains and oilseeds outlook 2009-10*. Agriculture and Agrifood Canada; 2009.

19. 2015 F. Flax - A Healthy Food. 2009.

20. Bang H, Dyerberg J. The bleeding tendency in Greenland Eskimos. *Dan Med Bull*. 1980;27(4):202–205.

21. Bang H, Dyerberg J, Hjoorne N. The composition of food consumed by Greenland Eskimos. *Acta Med Scand*. 1976;200(1-2):69–73.

22. Bang H, Dyerberg J, Sinclair H. The composition of the Eskimo food in north western Greenland. *Am J Clin Nutr*. 1980;33(12):2657–2661.

23. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med (Maywood)*. 2008;233(6):674–688.

24. Calder PC. Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. *Braz. J. Med. Biol. Res.* 1998;31(4):467–490.

25. Calder PC. Dietary modification of inflammation with lipids. *Proc Nutr Soc*. 2002;61(3):345–358.

26. Singh M. Essential fatty acids, DHA and human brain. *Indian J Pediatr*. 2005;72(3):239–242.

27. Ellis EF, Police RJ, Dodson LY, McKinney JS, Holt SA. Effect of dietary n-3 fatty acids on cerebral microcirculation. *Am J Physiol*. 1992;262(5 Pt 2):H1379–86.

28. Chung W-L, Chen J-J, Su H-M. Fish oil supplementation of control and (n-3) fatty acid-deficient male rats enhances reference and working memory performance and increases brain regional docosahexaenoic acid levels. *J Nutr*. 2008;138(6):1165–1171.

29. Matsuzaki M, Yokoyama M, Saito Y, et al. Incremental effects of eicosapentaenoic acid on cardiovascular events in statin-treated patients with coronary artery disease. *Circ. J.* 2009;73(7):1283–1290.

30. Tanaka K, Ishikawa Y, Yokoyama M, et al. Reduction in the recurrence of stroke by eicosapentaenoic acid for hypercholesterolemic patients: subanalysis of the JELIS trial. *Stroke*. 2008;39(7):2052–2058.

31. Yokoyama M, Origasa H, Matsuzaki M, et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. *Lancet*. 2007;369(9567):1090–1098.

32. Saito Y, Yokoyama M, Origasa H, et al. Effects of EPA on coronary artery disease in hypercholesterolemic patients with multiple risk factors: sub-analysis of primary prevention cases from the Japan EPA Lipid Intervention Study (JELIS). *Atherosclerosis*. 2008;200(1):135–140.

33. Egert S, Kannenberg F, Somoza V, Erbersdobler H, Wahrburg U. Dietary alpha-linolenic acid, EPA, and DHA have differential effects on LDL fatty acid composition but similar effects on serum lipid profiles in normolipidemic humans. *J Nutr*. 2009;139(5):861–868.

34. Micallef M, Munro I, Garg M. An inverse relationship between plasma n-3 fatty acids and C-reactive protein in healthy individuals. *Eur J Clin Nutr*. 2009.

35. Madsen T, Schmidt E, Christensen J. The effect of n-3 fatty acids on C-reactive protein levels in patients with chronic renal failure. *J Ren Nutr*. 2007;17(4):258–263.

36. Farzaneh-Far R, Harris W, Garg S, Na B, Whooley M. Inverse association of erythrocyte n-3 fatty acid levels with inflammatory biomarkers in patients with stable coronary artery disease: The Heart and Soul Study. *Atherosclerosis*. 2008.

37. Simopoulos A. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med (Maywood)*. 2008;233(6):674–688.

38. Mickleborough T, Tecklenburg S, Montgomery G, Lindley M. Eicosapentaenoic acid is more effective than docosahexaenoic acid in inhibiting proinflammatory mediator production and transcription from LPS-induced human asthmatic alveolar macrophage cells. *Clin Nutr*. 2009;28(1):71–77.

39. Li H, Ruan X, Powis S, et al. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: evidence for a PPAR-gamma-dependent mechanism. *Kidney*

Int. 2005;67(3):867–874.

40. Harnack K, Andersen G, Somoza V. Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. *Nutr Metab (Lond)*. 2009;6:8.

41. Gerster H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vitam Nutr Res*. 1998;68(3):159–173.

42. Plourde M, Cunnane S. Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. *Appl Physiol Nutr Metab*. 2007;32(4):619–634.

43. Brenna J, Salem N, Sinclair A, Cunnane S. alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins Leukot Essent Fatty Acids*. 2009;80(2-3):85–91.

44. Kris-Etherton PM, Harris WS, Appel LJ, for the Nutrition Committee. Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. *Circulation*. 2002;106(21):2747–2757.

45. Williams C, Burdge G. Long-chain n-3 PUFA: plant v. marine sources. *Proc Nutr Soc*. 2006;65(1):42–50.

46. Dupasquier CMC, Dibrov E, Kneesh AL, et al. Dietary flaxseed inhibits atherosclerosis in the LDL receptor-deficient mouse in part through antiproliferative

and anti-inflammatory actions. *Am J Physiol Heart Circ Physiol*. 2007;293(4):H2394–402.

47. Dupasquier CMC, Weber A-M, Ander BP, et al. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *Am J Physiol Heart Circ Physiol*. 2006;291(6):H2987–96.

48. Cohen S, Ward W. Flaxseed oil and bone development in growing male and female mice. *J Toxicol Environ Health A*. 2005;68(21):1861–1870.

49. Austria JA, Richard MN, Chahine MN, et al. Bioavailability of alpha-linolenic acid in subjects after ingestion of three different forms of flaxseed. *J Am Coll Nutr*. 2008;27(2):214–221.

50. Harper C, Edwards M, DeFilippis A, Jacobson T. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J Nutr*. 2006;136(1):83–87.

51. Bloedon L, Balikai S, Chittams J, et al. Flaxseed and cardiovascular risk factors: results from a double blind, randomized, controlled clinical trial. *J Am Coll Nutr*. 2008;27(1):65–74.

52. de Lorgeril M, Renaud S, Mamelle N, et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet*. 1994;343(8911):1454–1459.

53. Li D, Sinclair A, Wilson A, et al. Effect of dietary alpha-linolenic acid on thrombotic risk factors in vegetarian men. *Am J Clin Nutr*. 1999;69(5):872–882.

54. Grimm H, Tibell A, Norrlind B, et al. Immunoregulation by parenteral lipids: impact of the n-3 to n-6 fatty acid ratio. *JPEN J Parenter Enteral Nutr*. 1994;18(5):417–421.

55. de Lorgeril M, Salen P. Dietary prevention of coronary heart disease: the Lyon diet heart study and after. *World Rev Nutr Diet*. 2005;95:103–114.

56. Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *Am J Clin Nutr*. 2011;93(5):950–962.

57. Rodruigez-Leyva D, McCullough RS, Pierce GN. Medicinal use of hempseeds (Cannibis sativa L.): effects on platelet aggregation. In: Preedy V, Watson R, Patel V, eds. *Nuts & Seeds in Health and Disease Prevention*. 1st ed. Burlington, San Diego: Academic Press; 2011:637–646.

58. Martinelli N, Consoli L, Olivieri O. A "desaturase hypothesis" for atherosclerosis: Janus-faced enzymes in omega-6 and omega-3 polyunsaturated fatty acid metabolism. *J Nutrigenet Nutrigenomics*. 2009;2(3):129–139.

59. Fischer S, Weber PC. Thromboxane (TX)A3 and prostaglandin (PG)I3 are formed in man after dietary eicosapentaenoic acid: identification and quantification by capillary gas chromatography-electron impact mass spectrometry. *Biomed. Mass Spectrom.* 1985;12(9):470–476.

60. Oh DY, Talukdar S, Bae EJ, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell*.

2010;142(5):687–698.

61. Talukdar S, Olefsky JM, Osborn O. Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases. *Trends Pharmacol Sci*. 2011.

62. Wang MZ, Ding LY, Wang JF, Wang HR, Yu LH. Effects of n-6:n-3 polyunsaturated fatty acid ratio on heterophil: lymphocyte ratio and T lymphocyte subsets in the peripheral blood of the Yangzhou gosling. *Poult Sci*. 2011;90(4):824–829.

63. Costa CASD, Carlos AS, Gonzalez G de PL, et al. Diet containing low n-6/n-3 polyunsaturated fatty acids ratio, provided by canola oil, alters body composition and bone quality in young rats. *Eur J Nutr*. 2011.

64. Gillam M, Noto A, Zahradka P, Taylor CG. Improved n-3 fatty acid status does not modulate insulin resistance in fa/fa Zucker rats. *Prostaglandins Leukot Essent Fatty Acids*. 2009;81(5-6):331–339.

65. Rondeau I, Picard S, Bah TM, et al. Effects of different dietary omega-6/3 polyunsaturated fatty acids ratios on infarct size and the limbic system after myocardial infarction. *Can J Physiol Pharmacol*. 2011;89(3):169–176.

66. Wan J-B, Huang L-L, Rong R, et al. Endogenously decreasing tissue n-6/n-3 fatty acid ratio reduces atherosclerotic lesions in apolipoprotein E-deficient mice by inhibiting systemic and vascular inflammation. *Arterioscler Thromb*. 2010;30(12):2487–2494.

67. Kromhout D, Giltay EJ, Geleijnse JM, Alpha Omega Trial Group. n-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med*. 2010;363(21):2015–2026.

68. Leaf A. Dietary prevention of coronary heart disease: the Lyon Diet Heart Study. *Circulation*. 1999;99(6):733–735.

69. Brostow DP, Odegaard AO, Koh W-P, et al. Omega-3 fatty acids and incident type 2 diabetes: the Singapore Chinese Health Study. *Am J Clin Nutr*. 2011.

70. Mozaffarian D, Ascherio A, Hu FB, et al. Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation*. 2005;111(2):157–164.

71. Bolton-Smith C, Woodward M, Tunstall-Pedoe H. The Scottish Heart Health Study. Dietary intake by food frequency questionnaire and odds ratios for coronary heart disease risk. II. The antioxidant vitamins and fibre. *Eur J Clin Nutr*. 1992;46(2):85–93.

72. Kromhout D, Bosschieter EB, de Lezenne Coulander C. Dietary fibre and 10-year mortality from coronary heart disease, cancer, and all causes. The Zutphen study. *Lancet*. 1982;2(8297):518–522.

73. Berg CM, Lappas G, Strandhagen E, et al. Food patterns and cardiovascular disease risk factors: the Swedish INTERGENE research program. *Am J Clin Nutr*. 2008;88(2):289–297.

74. Kokubo Y, Iso H, Saito I, et al. Dietary fiber intake and risk of cardiovascular disease

in the Japanese population: the Japan Public Health Center-based study cohort. *Eur J Clin Nutr*. 2011.

75. Hlebowicz J, Persson M, Gullberg B, et al. Food patterns, inflammation markers and incidence of cardiovascular disease: the Malmö Diet and Cancer study. *J Intern Med*. 2011.

76. Baer HJ, Glynn RJ, Hu FB, et al. Risk factors for mortality in the nurses' health study: a competing risks analysis. *Am J Epidemiol*. 2011;173(3):319–329.

77. Buil-Cosiales P, Irimia P, Ros E, et al. Dietary fibre intake is inversely associated with carotid intima-media thickness: a cross-sectional assessment in the PREDIMED study. *Eur J Clin Nutr*. 2009;63(10):1213–1219.

78. Park Y, Subar AF, Hollenbeck A, Schatzkin A. Dietary Fiber Intake and Mortality in the NIH-AARP Diet and Health Study. *Arch Intern Med*. 2011;171(12):1061–1068.

79. Burr ML. Secondary prevention of CHD in UK men: the Diet and Reinfarction Trial and its sequel. *Proc Nutr Soc*. 2007;66(1):9–15.

80. Estruch R, Martínez-González MA, Corella D, et al. Effects of dietary fibre intake on risk factors for cardiovascular disease in subjects at high risk. *J Epidemiol Community Health*. 2009;63(7):582–588.

81. Esposito K, Nappo F, Giugliano F, et al. Meal modulation of circulating interleukin 18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. *Am J Clin Nutr*. 2003;78(6):1135–1140.

82. Esposito K, Marfella R, Ciotola M, et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. *JAMA*. 2004;292(12):1440–1446.

83. Esposito K, Pontillo A, Di Palo C, et al. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. *JAMA*. 2003;289(14):1799–1804.

84. Venkatesan N, Devaraj SN, Devaraj H. A fibre cocktail of fenugreek, guar gum and wheat bran reduces oxidative modification of LDL induced by an atherogenic diet in rats. *Mol Cell Biochem*. 2007;294(1-2):145–153.

85. Slavin JL, Savarino V, Paredes-Diaz A, Fotopoulos G. A review of the role of soluble fiber in health with specific reference to wheat dextrin. *J. Int. Med. Res.* 2009;37(1):1–17.

86. Chandalia M, Garg A, Lutjohann D, et al. Beneficial effects of high dietary fiber intake in patients with type 2 diabetes mellitus. *N Engl J Med*. 2000;342(19):1392–1398.

87. Ratnayake WMN, Behrens WA, Fischer PWF, et al. Chemical and nutritional studies of flaxseed (variety Linott) in rats. *The Journal of Nutritional Biochemistry*. 1992;3(5):232–240.

88. Jenkins DJ, Kendall CW, Vidgen E, et al. Health aspects of partially defatted flaxseed, including effects on serum lipids, oxidative measures, and ex vivo androgen and progestin activity: a controlled crossover trial. *Am J Clin Nutr*. 1999;69(3):395–402.

89. Sano T, Oda E, Yamashita T, et al. Antithrombic and anti-atherogenic effects of

partially defatted flaxseed meal using a laser-induced thrombosis test in apolipoprotein E and low-density lipoprotein receptor deficient mice. *Blood Coagul Fibrinolysis*. 2003;14(8):707–712.

90. Saleh F, Afnan F, Ara F, et al. Phytoestrogen intake and cardiovascular risk markers in bangladeshi postmenopausal women. *Mymensingh Med J*. 2011;20(2):219–225.

91. Hamilton-Reeves J, Vazquez G, Duval S. Clinical studies show no effects of soy protein or isoflavones on reproductive hormones in men: results of a meta-analysis. *Fertility and sterility*. 2010.

92. Carreau C, Flouriot G, Bennetau-Pelissero C, Potier M. Enterodiol and enterolactone, two major diet-derived polyphenol metabolites have different impact on ERalpha transcriptional activation in human breast cancer cells. *J Steroid Biochem Mol Biol*. 2008;110(1-2):176–185.

93. Prasad K. Flax lignan complex slows down the progression of atherosclerosis in hyperlipidemic rabbits. *J Cardiovasc Pharmacol Ther*. 2009;14(1):38–48.

94. Fukumitsu S, Aida K, Shimizu H, Toyoda K. Flaxseed lignan lowers blood cholesterol and decreases liver disease risk factors in moderately hypercholesterolemic men. *Nutr Res*. 2010;30(7):441–446.

95. Felmlee MA, Woo G, Simko E, et al. Effects of the flaxseed lignans secoisolariciresinol diglucoside and its aglycone on serum and hepatic lipids in hyperlipidaemic rats. *BJN*. 2009;102(3):361–369.

96. Zhang W, Wang X, Liu Y, et al. Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *BJN*. 2008;99(6):1301–1309.

97. Gertow K, Rosell M, Sjogren P, et al. Fatty acid handling protein expression in adipose tissue, fatty acid composition of adipose tissue and serum, and markers of insulin resistance. *Eur J Clin Nutr*. 2006;60(12):1406–1413.

98. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev*. 2004;84(1):277–359.

99. Trayhurn P, Wood I. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr*. 2004;92(3):347–355.

100. Wajchenberg B. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev*. 2000;21(6):697–738.

101. Lihn A, Bruun J, He G, et al. Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Mol Cell Endocrinol*. 2004;219(1-2):9–15.

102. Sacks H, Fain J. Human epicardial adipose tissue: a review. *Am Heart J*. 2007;153(6):907–917.

103. Henrichot E, Juge-Aubry C, Pernin A, et al. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler Thromb*. 2005;25(12):2594–2599.

104. Large V, Peroni O, Letexier D, Ray H, Beylot M. Metabolism of lipids in human white adipocyte. *Diabetes Metab*. 2004;30(4):294–309.

105. Koutsari C, Jensen M. Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. *J Lipid Res*. 2006;47(8):1643–1650.

106. Fagot-Campagna A, Balkau B, Simon D, et al. High free fatty acid concentration: an independent risk factor for hypertension in the Paris Prospective Study. *Int J Epidemiol*. 1998;27(5):808–813.

107. Sarafidis P, Bakris G. Non-esterified fatty acids and blood pressure elevation: a mechanism for hypertension in subjects with obesity/insulin resistance? *J Hum Hypertens*. 2007;21(1):12–19.

108. Jouven X, Charles M, Desnos M, Ducimetiere P. Circulating nonesterified fatty acid level as a predictive risk factor for sudden death in the population. *Circulation*. 2001;104(7):756–761.

109. Roden M. How free fatty acids inhibit glucose utilization in human skeletal muscle. *News Physiol Sci*. 2004;19:92–96.

110. Bays H, Mandarino L, DeFronzo R. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J Clin Endocrinol Metab*. 2004;89(2):463–478.

111. Scherer P, Williams S, Fogliano M, Baldini G, Lodish H. A novel serum protein

similar to C1q, produced exclusively in adipocytes. *J Biol Chem*. 1995;270(45):26746–26749.

112. Arita Y, Kihara S, Ouchi N, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun*. 1999;257(1):79–83.

113. Sowers J. Endocrine functions of adipose tissue: focus on adiponectin. *Clin Cornerstone*. 2008;9(1):32–8; discussion 39–40.

114. Rizza S, Tesauro M, Cardillo C, et al. Fish oil supplementation improves endothelial function in normoglycemic offspring of patients with type 2 diabetes. *Atherosclerosis*. 2009;206(2):569–574.

115. Gonzalez-Periz A, Horrillo R, Ferre N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J*. 2009;23(6):1946–1957.

116. Kuda O, Jelenik T, Jilkova Z, et al. n-3 fatty acids and rosiglitazone improve insulin sensitivity through additive stimulatory effects on muscle glycogen synthesis in mice fed a high-fat diet. *Diabetologia*. 2009;52(5):941–951.

117. Raff M, Tholstrup T, Toubro S, et al. Conjugated linoleic acids reduce body fat in healthy postmenopausal women. *J Nutr*. 2009;139(7):1347–1352.

118. Zhao W, Zhai J, Wang Y, et al. Conjugated linoleic acid supplementation enhances antihypertensive effect of ramipril in Chinese patients with obesity-related hypertension. *Am J Hypertens*. 2009;22(6):680–686.

119. Lopes P, Martins S, Pinho M, et al. Diet supplementation with the cis-9,trans-11 conjugated linoleic acid isomer affects the size of adipocytes in Wistar rats. *Nutr Res*. 2008;28(7):480–486.

120. Zhang Y, Proenca R, Maffei M, et al. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372(6505):425–432.

121. Gibson W, Farooqi I, Moreau M, et al. Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy. *J Clin Endocrinol Metab*. 2004;89(10):4821–4826.

122. Toth B, Fischl A, Scholz C, et al. Insulin and leptin receptors as possible new candidates for endocrine control in normal and disturbed human pregnancy. *Mol Hum Reprod*. 2009;15(4):231–239.

123. Moynihan A, Hehir M, Glavey S, Smith T, Morrison J. Inhibitory effect of leptin on human uterine contractility in vitro. *Am J Obstet Gynecol*. 2006;195(2):504–509.

124. Kawachi H, Yang S, Hamano A, et al. Molecular cloning and expression of bovine (Bos taurus) leptin receptor isoform mRNAs. *Comp Biochem Physiol B Biochem Mol Biol*. 2007;148(2):167–173.

125. Taleb S, Herbin O, Ait-Oufella H, et al. Defective leptin/leptin receptor signaling improves regulatory T cell immune response and protects mice from atherosclerosis. *Arterioscler Thromb*. 2007;27(12):2691–2698.

126. Dubey L, Hesong Z. Role of leptin in atherogenesis. *Exp Clin Cardiol*. 2006;11(4):269–275.

127. Phillips C, Goumidi L, Bertrais S, et al. Leptin Receptor Polymorphisms Interact with Polyunsaturated Fatty Acids to Augment Risk of Insulin Resistance and Metabolic Syndrome in Adults. *J Nutr*. 2009.

128. Procopio C, Andreozzi F, Laratta E, et al. Leptin-stimulated endothelial nitric-oxide synthase via an adenosine 5'-monophosphate-activated protein kinase/Akt signaling pathway is attenuated by interaction with C-reactive protein. *Endocrinology*. 2009;150(8):3584–3593.

129. Flachs P, Mohamed-Ali V, Horakova O, et al. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia*. 2006;49(2):394–397.

130. Gudbrandsen O, Rodriguez E, Wergedahl H, et al. Trans-10, cis-12-conjugated linoleic acid reduces the hepatic triacylglycerol content and the leptin mRNA level in adipose tissue in obese Zucker fa/fa rats. *Br J Nutr*. 2009;102(6):803–815.

131. Steppan C, Bailey S, Bhat S, et al. The hormone resistin links obesity to diabetes. *Nature*. 2001;409(6818):307–312.

132. Nogueiras R, Novelle M, Vazquez M, Lopez M, Dieguez C. Resistin: Regulation of Food Intake, Glucose Homeostasis and Lipid Metabolism. *Endocr Dev*. 17:175–184.

133. Verma S, Li S, Wang C, et al. Resistin promotes endothelial cell activation: further

evidence of adipokine-endothelial interaction. *Circulation*. 2003;108(6):736–740.

134. Nagaev I, Bokarewa M, Tarkowski A, Smith U. Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes. *PLoS ONE*. 2006;1:e31.

135. Pischon T. Use of obesity biomarkers in cardiovascular epidemiology. *Dis Markers*. 2009;26(5-6):247–263.

136. Wang H, Chen D, Cao J, et al. High serum resistin level may be an indicator of the severity of coronary disease in acute coronary syndrome. *Chin Med Sci J*. 2009;24(3):161–166.

137. Lee J, Chan J, Yiannakouris N, et al. Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. *J Clin Endocrinol Metab*. 2003;88(10):4848–4856.

138. Won J, Park C, Lee W, et al. Association of plasma levels of resistin with subcutaneous fat mass and markers of inflammation but not with metabolic determinants or insulin resistance. *J Korean Med Sci*. 2009;24(4):695–700.

139. Aeberli I, Spinas G, Lehmann R, et al. Diet determines features of the metabolic syndrome in 6- to 14-year-old children. *Int J Vitam Nutr Res*. 2009;79(1):14–23.

140. Haugen F, Zahid N, Dalen K, et al. Resistin expression in 3T3-L1 adipocytes is reduced by arachidonic acid. *J Lipid Res*. 2005;46(1):143–153.

141. McKellar G, McCarey D, Sattar N, McInnes I. Role for TNF in atherosclerosis? Lessons from autoimmune disease. *Nat Rev Cardiol*. 2009;6(6):410–417.

142. Zhao T, Hou M, Xia M, et al. Globular adiponectin decreases leptin-induced tumor necrosis factor-alpha expression by murine macrophages: involvement of cAMP-PKA and MAPK pathways. *Cell Immunol*. 2005;238(1):19–30.

143. Rudkowska I, Marcotte B, Pilon G, et al. Fish nutrients decrease expression levels of tumor necrosis factor alpha in cultured human macrophages. *Physiol Genomics*. 2009;40:189–194.

144. Zeitlin L, Segev E, Fried A, Wientroub S. Effects of long-term administration of N-3 polyunsaturated fatty acids (PUFA) and selective estrogen receptor modulator (SERM) derivatives in ovariectomized (OVX) mice. *J Cell Biochem*. 2003;90(2):347–360.

145. Mehra M, Lavie C, Ventura H, Milani R. Fish oils produce anti-inflammatory effects and improve body weight in severe heart failure. *J Heart Lung Transplant*. 2006;25(7):834–838.

146. Clos du T, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol Res*. 2004;30(3):261–277.

147. Ridker P, Buring J, Cook N, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. *Circulation*. 2003;107(3):391–397.

148. Venugopal S, Devaraj S, Jialal I. Macrophage conditioned medium induces the

expression of C-reactive protein in human aortic endothelial cells: potential for paracrine/autocrine effects. *Am J Pathol*. 2005;166(4):1265–1271.

149. Allin K, Bojesen S, Nordestgaard B. Baseline C-reactive protein is associated with incident cancer and survival in patients with cancer. *J Clin Oncol*. 2009;27(13):2217–2224.

150. Zhang L, Geng Y, Yin M, et al. Low omega-6/omega-3 polyunsaturated fatty acid ratios reduce hepatic C-reactive protein expression in apolipoprotein E-null mice. *Nutrition*. 2009.

151. Saifullah A, Watkins B, Saha C, et al. Oral fish oil supplementation raises blood omega-3 levels and lowers C-reactive protein in haemodialysis patients--a pilot study. *Nephrol Dial Transplant*. 2007;22(12):3561–3567.

152. Pischon T, Hankinson S, Hotamisligil G, et al. Habitual dietary intake of n-3 and n-6 fatty acids in relation to inflammatory markers among US men and women. *Circulation*. 2003;108(2):155–160.

153. St-Onge M, Zhang S, Darnell B, Allison D. Baseline serum C-reactive protein is associated with lipid responses to low-fat and high-polyunsaturated fat diets. *J Nutr*. 2009;139(4):680–683.

154. Zampelas A, Paschos G, Rallidis L, Yiannakouris N. Linoleic acid to alpha-linolenic acid ratio. From clinical trials to inflammatory markers of coronary artery disease. *World Rev Nutr Diet*. 2003;92:92–108.