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BIOACTIVE FATTY ACID SUPPLEMENTATION AND RISK FACTORS FOR THE METABOLIC SYNDROME

by

Patricia L. Mitchell

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
August 2010

DALHOUSIE UNIVERSITY

BIOCHEMISTRY AND MOLECULAR BIOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "BIOACTIVE FATTY ACID SUPPLEMENTATION AND RISK FACTORS FOR THE METABOLIC SYNDROME" by Patricia L. Mitchell in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: August 6 2010

External Examiner: _____

Research Supervisor: _____

Examining Committee: _____

_____

Departmental Representative: _____

DALHOUSIE UNIVERSITY

DATE: August 6, 2010

AUTHOR: Patricia L. Mitchell

TITLE: BIOACTIVE FATTY ACID SUPPLEMENTATION AND RISK FACTORS FOR THE METABOLIC SYNDROME

DEPARTMENT OR SCHOOL: Department of Biochemistry and Molecular Biology

DEGREE: Ph. D. CONVOCATION: October YEAR: 2010

In Memory of
Eleanor and Augustine Mitchell
You are never far from my thoughts

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Diet plays an important role in the development of chronic metabolic diseases (diabetes, obesity, cardiovascular disease) and as dietary fat consumption has increased, so has the incidence of these disorders. Metabolic syndrome, a clustering of risk factors that includes central obesity, increased plasma triacylglycerol (TG), elevated fasting glucose and glucose intolerance is perhaps the most notorious and aggressive. Animal and human studies indicate that bioactive fatty acids can influence cellular energy metabolism. Using susceptible rodent models (apoE$^{-/-}$ and LDLr$^{-/-}$ mice and Syrian Golden hamsters) this project investigated whether supplementation of a western type diet (WD) with bioactive fatty acids could improve hepatic lipid metabolism, plasma lipoprotein profiles or liver markers of lipogenesis. In mice, dietary supplementation with *t*-10, *c*-12 conjugated linoleic acid (CLA) decreased the weight gain induced by high fat diet compared with WD ($p<0.01$) and was accompanied by hyperinsulinemia ($p<0.05$) in the ApoE$^{-/-}$ and hypoadiponectinemia ($p<0.01$) in both mice strains. Although *t*-10, *c*-12 CLA supplementation increased plasma lipids and was associated with profound liver steatosis there was a reduction in atherosclerotic lesions in both mouse models ($p<0.05$). Analysis of mRNA and protein levels in the liver suggested that the differences in liver and plasma lipids may reflect inappropriate lipogenic response to *t*-10,*c*-12 CLA. In the high fat and fructose-fed hamster, the modulating role of fish fatty acids was investigated. The addition of DHA increased weight gain and adiposity compared to EPA and *c*-9, *t*-11 CLA supplementation. However, glucose tolerance was improved after 6 weeks of DHA supplementation ($p\leq 0.01$). Using [^{35}S]methionine radiolabelling, DHA supplementation decreased apolipoprotein B100 synthesis and secretion. Newly synthesized cellular and secreted TG, as measured by [^{3}H]glycerol incorporation, were also decreased with DHA supplementation. Although the effects of EPA were similar to those with DHA, the magnitude was generally lower. These results suggest that supplementation with fish fatty acids can improve several of the risk factors of the metabolic syndrome. Taken together, these observations indicate that some, but not all, bioactive fatty acids may be useful supplements for mediating cardiovascular risk factors.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ApoB100	apolipoprotein B100
ACO	acyl-CoA oxidase
ATF6	activating transcription factor 6
BiP/GRP78	binding immunoglobulin protein/glucose regulated protein 78
BSA	bovine serum albumin
C	cholesterol
CE	cholesterol esters
Ci	curie
CHOP	C/EBP homologous protein
CLA	conjugated linoleic acid
CO	cholesterol oleate
CRP	C-reactive protein
CVD	cardiovascular disease
DEXA	dual energy x-ray absorptiometry
DGAT	acyl-CoA:diacylglycerol acyltransferase
DHA	docosahexaenoic acid
DMEM	Dulbecco's modified Eagles medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid
eIF2α	eukaryotic translation initiation factor-2
$ApoE^{-/-}$	apoE deficient mice
ELISA	enzyme-linked-immunosorbent-assay
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
EYPC	egg yolk phosphatidylcholine

FA	fatty acid
FFA	free fatty acid
FBS	fetal bovine serum
FC	free cholesterol
FF	fructose diet
G6P	glucose-6-phosphotase
HDL	high density lipoprotein
HFHC	high fat high cholesterol
HFLC	high fat low cholesterol
IPGTT	intraperitoneal glucose tolerance test
IPITT	intraperitoneal insulin tolerance test
IRE1	Inositol requiring enzyme 1
LA	linoleic acid
LDL	low density lipoprotein
$LDLr^{-/-}$	LDL receptor deficient mice
LPL	lipoprotein lipase
LXR	liver X receptor
MA	myristic acid
MTP	microsomal triglyceride transfer protein
NEFA	non-esterified fatty acids
OA	oleic acid
OLETF	Otsuka Long Evans Tokushima Fatty rats
PA	palmitic acid
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidic acid phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCA	principal component analysis
q-PCR	quantitative polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PERK	protein kinase RNA (PKR)-like ER kinase

PL	phospholipids
PMSF	phenylmethylsufonyl fluoride
PPAR	peroxisome proliferation-activated receptor
PUFA	polyunsaturated fatty acid
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulphate
SCD1	stearoyl-CoA desaturase
SREBP	sterol regulatory element- binding protein
TCA	trichloroacetic acid
TE	tris EDTA buffer
TG	triglycerides
TO	triolein
TLC	thin layer chromatography
UPR	unfolded protein response
VLDL	very low density lipoprotein
WAT	white adipose tissue
WD	western diet
Wt	weight
W/V	weight per volume
W/W	weight per weight
XBP1	X-box binding protein 1

ACKNOWLEDGEMENTS

I can vividly recall sitting and leafing through the Convocation program waiting, for my name to be called, to receive my M. Sc. (first one) and reading my fellow graduates' theses titles and thinking – who would want to study these topics nevermind writing about them – the answer now is Me! Immunoblotting and [^{3}H]glycerol incorporation into TG were foreign concepts not too long ago, now they are everyday words. I have had the great fortune to be in the company of many outstanding people who have inspired me to be a life-long learner and never to become complacent. Thank you to those who have encouraged challenged and educated me.

I was truly fortunate to have been in the McLeod lab. Thanks to the supervision of Roger McLeod I feel both intellectually and technically prepared to contribute significantly to the research community. Thanks Eric for listening to my presentations and providing insightful comments. Debbie – the animal work would not have been possible without your assistance and to Brandon your mad western blotting skills were greatly appreciated.

Drs Ro and Byers, who deserve extra kudos for being on my supervisory committee not once but twice, and Dr Sinal, you were extremely helpful when the data did not seem to make sense. Thank you for being kind on the grant review; it was a humbling experience to have your work openly critiqued. Hopefully you will notice that your comments were heeded in the preparation of this thesis.

To my "Halifax family' Rob, Sarah, Alex, Mitchell and Helen what wonderful memories I would have missed if our paths had not crossed – thanks Rendez-vous 2002!

My sisters, Donna, Carol and Heather, really there are no words that describe what you mean to me – thank you for your love support and just being you.

CHAPTER ONE – INTRODUCTION

1.0. Introduction

As "westernization" of the diet becomes a global phenomenon the incidences of obesity, type II diabetes and atherosclerosis are the new epidemics in countries that once faced overwhelming infectious disease and starvation (1). The western diet, high in saturated fat, has been implicated in the development of 35% of heart attacks globally (2) and although the incidence of cardiovascular disease is declining in developed countries, it is steadily increasing in developing nations (1). Much has been made of our current diet and general deterioration in health, making it appear that this diet – disease axis is a new phenomenon. Although the extent to which metabolic diseases are being diagnosed has exponentially increased in the past few years, the connection between overeating, obesity and cardiovascular/metabolic dysfunction has long been postulated. The link between lipid deposits and cardiovascular disease was suggested in 1856 by Rudolph Virchow (3). As early as the 1950's epidemiological data was accumulating that suggested that patients admitted for myocardial infarctions had elevated levels of low density lipoprotein cholesterol (LDL-C) compared to matched controls (4). Cardiovascular disease was not the only detrimental outcome of a fat laden diet. In 1921, upon reviewing 1000 case histories, Joslin (5) made the salient observation that excess weight preceded the diagnosis of diabetes in the majority of his patients. He also pointed out that if the same number of people in one town had died of an infectious disease, health authorities would have been far more intent on finding an answer. A stronger connection between diabetes, abdominal obesity and atherosclerosis was made in the 1950's (6) and again in the 1970's (7). The idea of metabolic dysfunction was raised and in 1988 Reaven (8) provided a name for the clustering of risk factors – syndrome X. It really isn't a new problem – just

a larger one. Given the evidence accumulated over the years from epidemiological studies and now clinical interventions it has been asserted that, in addition to genetic factors, diet represents the most important determinant of the development and progression of metabolic dysfunction (9). It is also becoming clear that it is not simply the overload of fats in the diet that results in increased metabolic disease, but that specific fatty acids act as signalling molecules and participate in the overall metabolic homeostasis of the cell (10;11). When cellular homeostasis is disrupted, an inflammatory response or cellular stress response can be triggered leading to the development of obesity, insulin resistance and atherosclerotic plaque. It behoves us to learn more about the actions of dietary fatty acids in metabolic function and dysfunction so we can use this knowledge to not only develop medical treatments but also to ultimately prevent the development of metabolic dysfunction and chronic disease.

1.1. Atherosclerosis

Atherosclerosis is the progressive narrowing of the lumen of large arteries by the continual accumulation of lipid-enriched macrophages in the intimal layer of the vessel wall. Although early hypotheses suggested that the accumulation of excess lipid from the plasma lipoproteins in the artery wall was responsible for the disease, it is now known that atherogenesis is a much more complex inflammatory response to injury (12;13) with lipoprotein particles involved in either the injury itself or in the response.

A number of risk factors, both genetic and environmental, have been identified through numerous epidemiological studies (Table 1) (http://www.heartandstroke.ca/risk). Although many risk factors, such as hyperlipidemia, metabolic syndrome and obesity,

Table 1. Risk factors for the development of atherosclerosis.

Non-Modifiable Risk factors – Significant Genetic Component	Potentially Modifiable Risk Factors – Possible Genetic Component	Minor Risk Factors – Possible Genetic Component
Increasing Age	Hyperlipidemia (VLDL/LDL)	Obesity
Male Gender	Low levels of HDL	Elevated Homocysteine
Family History	Hypertension	Stress
Genetic Abnormalities	Smoking	Elevated Lipoprotein (a)
	Physical Inactivity	High Fat Diet
	Diabetes	Infectious Agent
	Insulin Resistance	Alcohol
	Metabolic Syndrome	

have a genetic component they are still strongly influenced by environmental factors.

Elevated serum cholesterol was one of the first risk factors to be identified, in both humans and rodent models, as being associated with development of atherosclerosis without the presence of any other risk factors (14). Insights from studies of patients with homozygous familial hypercholesterolemia have led to a greater understanding of how abnormal cholesterol homeostasis can result in atherosclerosis. Even within familial hypercholesterolemia patients, the clinical manifestations of cardiovascular disease can be widely variable indicating that many other factors play a role in the development and progression of atherosclerosis. One such factor is dietary fat. The diet–health hypothesis was initiated from the belief that saturated fat in the diet was the major cause of lipid build-up in the artery wall. A review of 27 randomized controlled trials concluded that, under isocaloric conditions, favourable lipoprotein profiles could be achieved if saturated fatty acids were replaced with unsaturated fatty acids with no reduction in overall fat intake (10). The authors, however, advised caution when applying the results to free living individuals or if fat content was partially replaced with carbohydrate. Although the evidence to support this hypothesis has not been as conclusive as the public health messages may imply, it is emerging that dietary fat is not simply a nutrient, where excess causes disease, but fatty acids within the dietary fat may act as signalling molecules that impact many components of atherosclerosis (15).

Early lesions or fatty streaks occur at locations in the artery where laminar flow is disrupted. What starts as a fatty streak may eventually develop into an intermediate

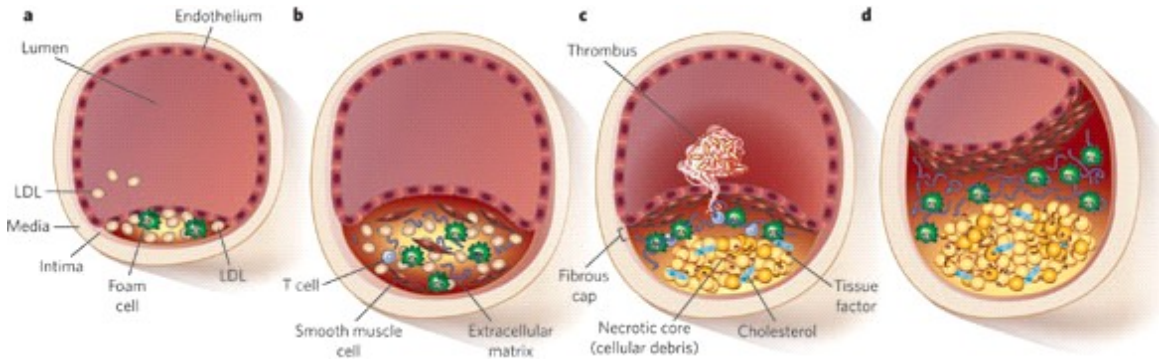


Figure 1. Initiation and progression of atherosclerosis.

Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. A lesion begins as a fatty streak (a) and can progress into an intermediate lesion (b), and then into a lesion that is vulnerable to rupture (c) and finally, into an advanced obstructive lesion (d). Rader, D.J. and Daugherty A. (2008) Nature 451:904-13.

lesion, which is vulnerable to rupture, and finally to a luminal obstruction (Figure 1). Fatty streak formation is a result of atherogenic lipoprotein particles, like low density lipoprotein (LDL), becoming oxidized and entrapped in the intimal layer of the vessel wall (16). During the course of normal circulation the LDL particles transit through the intimal space where oxidative or enzymatic modification may occur (Figure 2). If the normal exit of the particle from the intimal space is prevented, by aggregation of the modified lipoprotein, phagocytosis by the resident macrophages may be initiated. Initially, the recruitment of monocytes to the arterial wall is a defence mechanism meant to alleviate the build-up of cytotoxic and proinflammatory particles. Endothelial cells express cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), in response to inflammatory stimuli (Figure 2)(13;17). Once the monocyte has adhered to the endothelial layer, it can migrate into the intima via several mechanisms. Oxidized LDL can directly attract monocytes (18) or various chemokines such as monocyte chemotactic protein (MCP-1)(Figure 2) can form a chemoattractant gradient (16). Intriguingly, monocyte expression of (C-C motif) receptor 2 (CCR2), the receptor for MCP-1, is stimulated by hypercholesterolemia and monocytes derived from hypercholesterolemic patients exhibit increased chemotactic responses to MCP-1 (19). If unregulated accumulation of lipid-laden macrophages continues, foam cells will begin to appear. Foam cells are macrophages that contain massive amounts of cholesterol esters in cytoplasmic lipid droplets and are a marker of early and late lesion development. Along with other proteins, two of the major scavenger receptors, SR-A and CD36, have been shown to play a significant role in recognizing oxidized phospholipids. Atherosclerosis was significantly diminished in ApoE-deficient mice lacking both SR-A and CD36

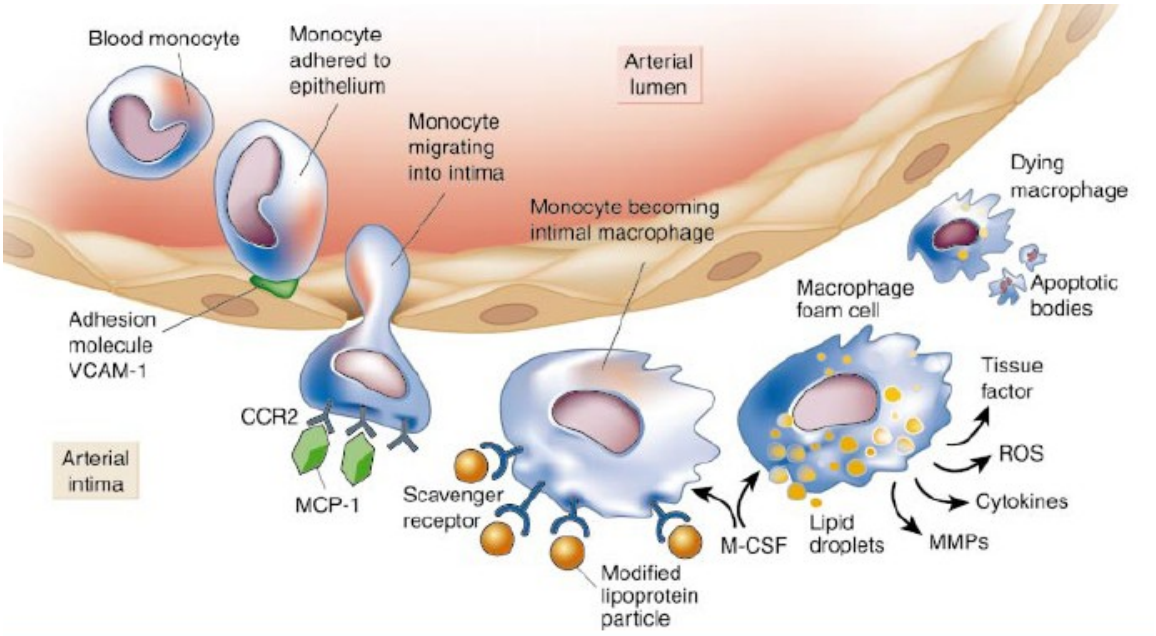


Figure 2. Initiating events in the formation of a fatty streak lesion.

LDL is subject to oxidative modifications in the subendothelial space, progressing from minimally modified LDL (mmLDL), to extensively oxidized LDL (oxLDL). Monocytes attach to endothelial cells that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate into the subendothelial space and differentiate into macrophages. Uptake of oxLDL via scavenger receptors leads to foam cell formation. OxLDL cholesterol taken up by scavenger receptors is subject to esterification and storage in lipid droplets, is converted to more soluble forms, or is exported to extracellular HDL acceptors via cholesterol transporters, such as *ABC-A1*. Libby, P. Nature (2002) 420:868-874.

scavenger receptors (20;21) suggesting that the uptake of damaged lipoproteins via these receptors is central to atherogenesis.

Once taken up by the macrophage the cholesterol has two possible fates: conversion to insoluble CE in the cytoplasmic lipid droplet or efflux via membrane transporters. Once transported to the membrane High Density Lipoprotein (HDL) is the most likely extracellular acceptor. The reverse cholesterol transport pathway can maintain a balance between influx into the intima and efflux through the circulatory system back to the liver.

Indicative of a disease that may take decades to progress from initiation to clinical pathology, fatty streaks have been identified in the aortas of infants and coronary arteries of adolescents (22). Progression and regression of these fatty streaks may continue without clinical significance for years until finally the sustained assault by an increased lipid load exacerbates the inflammatory response leading to a more complicated lesion. An advanced lesion (Figure 1) is characterized as having vascular smooth muscle cells, either recruited from the media into the intima or proliferating within the intima. The increased number of vascular smooth muscle cells results in further aggregation of lipoproteins and recruitment of inflammatory mediators like monocytes, leukocytes and T-cells to perpetuate the inflammatory response (13;17). The lesion will continue to be modified by the necrosis of the foam cell, leaving cellular debris, further recruitment of inflammatory mediators, and further development of a fibrous cap, all of which can lead to a lesion that is slowly encroaching on the lumen diameter, leading ultimately to a clinical event such as myocardial infarction or a stroke.

Regulation of the atherosclerosis process is complex, as metabolic and immunologic factors combine to up-regulate or suppress normal function in the

endothelium and surrounding tissue. Members of the peroxisome proliferation-activated receptor (PPAR) family play a major role in influencing the development of atherosclerosis (23). Many aspects of fatty acid metabolism are regulated by PPARα such as β-oxidation and fatty acid uptake from triglyceride-rich lipoproteins. There is also evidence to support the expression of PPARα in endothelial, smooth muscle cells and macrophages which exert anti-inflammatory effects (24). PPARγ, known primarily as an adipogenesis regulator has also been shown to be expressed in macrophage foam cells and can exert both pro- and anti-inflammatory effects (25;26). When macrophages were treated with the PPARγ agonist, troglitazone, a member of the class of compounds called thiazolidinediones (TZD), an increased expression of scavenger receptor CD36 was observed, suggesting a pro-inflammatory response leading to the formation of foam cells (25;26). In other situations TZDs have been shown to inhibit expression of tissue necrosis factor α (TNFα) and other cell mediators of inflammation suggesting an anti-inflammatory role in atherosclerosis (27;28). Consistent with these opposing results $LDLr^{-/-}$ mice fed a western diet and treated with a PPARγ agonist had up-regulation of the CD36 mRNA but showed strong antiatherogenic effects. There was down-regulation of TNFα and 60% less aortic lesion area than vehicle treated mice, suggesting an overall anti-atherogenic response (29). These differences are likely a result of the variety of PPARγ ligands that exist within each type of tissue.

More recently, LXRs have been identified as transcription factors that regulate, or co-regulate with SREBPs (30), a number of genes controlling cholesterol homeostasis (31). While elevated cellular levels of cholesterol and oxysterols suppress the transcription of SREBP target genes, they stimulate transcription of LXR target genes. LXR target genes include the 7α-hydroxylase gene in rodents, which encodes an enzyme

catalyzing the rate-limiting step required for bile acid synthesis and thus cholesterol excretion from liver. In addition, the *ABC-A1* gene is regulated in the intestine and in macrophages by LXRs (32). Mice lacking LXRα develop massive hepatomegaly when placed on a high cholesterol diet due to increased cholesterol absorption in the gut and impaired excretion from liver (32).

Given the complexity of atherosclerosis, which often results from a combination of lipid metabolism abnormalities and inflammatory processes, many potential targets for treatment exist. Two current treatment strategies are aimed at altering plasma lipoprotein metabolism or cellular cholesterol, and reducing the inflammatory process. Nutraceuticals and function foods may be able to play a role in either or both of these strategies.

1.2. Metabolic Syndrome

The interrelationship between diet and disease plays a prominent role in our health as nutrient availability increases and lifestyle changes put added pressure on the body's physiological regulatory systems to maintain homeostasis (9;33). When this balance, between energy intake and energy expenditure, is no longer maintained, the capacity to store energy is overloaded and lipids are deposited ectopically in visceral fat, liver, skeletal muscle and the vessel wall. The cellular changes associated with abnormal fat deposition can lead to non-alcoholic fatty liver disease, insulin resistance and cardiovascular disease, respectively. Nutrient overload is handled first by the liver and if unable to adequately deal with the excess carbohydrate, fat and protein changes in gene expression can be initiated in an attempt to prevent pathological changes (34;35). Of the metabolic distresses that have increased within the population in recent years, metabolic syndrome is perhaps the most notorious and aggressive. First proposed in 1988 by Reaven

(8) and termed “syndrome X”, this grouping of risk factors, hypertension, hyperlipidemia, insulin resistance, low plasma levels of high density lipoprotein cholesterol (HDL-C), elevated fasting glucose and central obesity, has undergone numerous “re-definitions” by various organizations (36-38). Although disagreement still exists as to the existence of the metabolic syndrome and its treatment, there is no doubt that each of these risk factors is a result of dramatically altered homeostasis (39). Regardless of definition, the underlying principle is to identify the individuals at increased risk of developing cardiovascular disease. The most common definition utilized for population studies is the National Cholesterol Education Program (36) which showed a 2-fold increase of CVD risk in individuals with metabolic syndrome compared to those who do not have the syndrome (40-42). All definitions are in agreement that visceral obesity and insulin resistance are major causes of the metabolic syndrome, both of which are independent risk factors for cardiovascular disease.

The liver is emerging as the frontline defence to metabolic syndrome and understanding how hepatic physiology is altered at the cellular level may lead to a further understanding of the molecular mechanisms that signal the transition from liver stress to distress.

1.3. Biomarkers of Metabolic Dysfunction

Measuring numbers of lives saved or number of disease-related morbidities prevented requires long term measurements and is often impossible to determine empirically. Therefore, biomarkers of clinical endpoints can be utilized as surrogate measures for diseases such as atherosclerosis or metabolic syndrome. It is important to understand whether the relationship between biomarker and disease is causative or simply

an association. A number of biomarkers have been suggested for following the risk of developing atherosclerosis, some causative, like plasma LDL-C and insulin, or some simply associated such as adiponectin and obesity.

1.3.1. Lipoproteins

Lipoproteins, the plasma transporters of lipids including cholesterol, are integral to the development of atherosclerosis. An increased plasma concentration of LDL-C is a well established risk factor for developing cardiovascular disease. Initially the lipoprotein particle, containing apolipoprotein B, triglycerides, phospholipids and minor amounts of cholesterol esters is assembled in the liver as very low density lipoprotein (VLDL) (Figure 3). The VLDL particle is then secreted into the plasma where it is metabolized by lipoprotein lipase, releasing free fatty acids to be used by various tissues for energy or storage. The VLDL remnant, with approximately equal amounts of TG and cholesterol, is further metabolized leaving a LDL particle containing a primarily CE as its lipid component. LDL is a potentially atherogenic particle that circulates in the plasma and also has access to the extra-vascular space of the intimal layer of the vessel wall. Retention of LDL in the intima can trigger an inflammatory response leading to the early stages of atherosclerosis. Therefore monitoring the level of circulating LDL particles provides an indication of atherosclerotic risk. Further, reducing VLDL production or enhancing the removal of LDL-C from the plasma are potential treatments for cardiovascular disease.

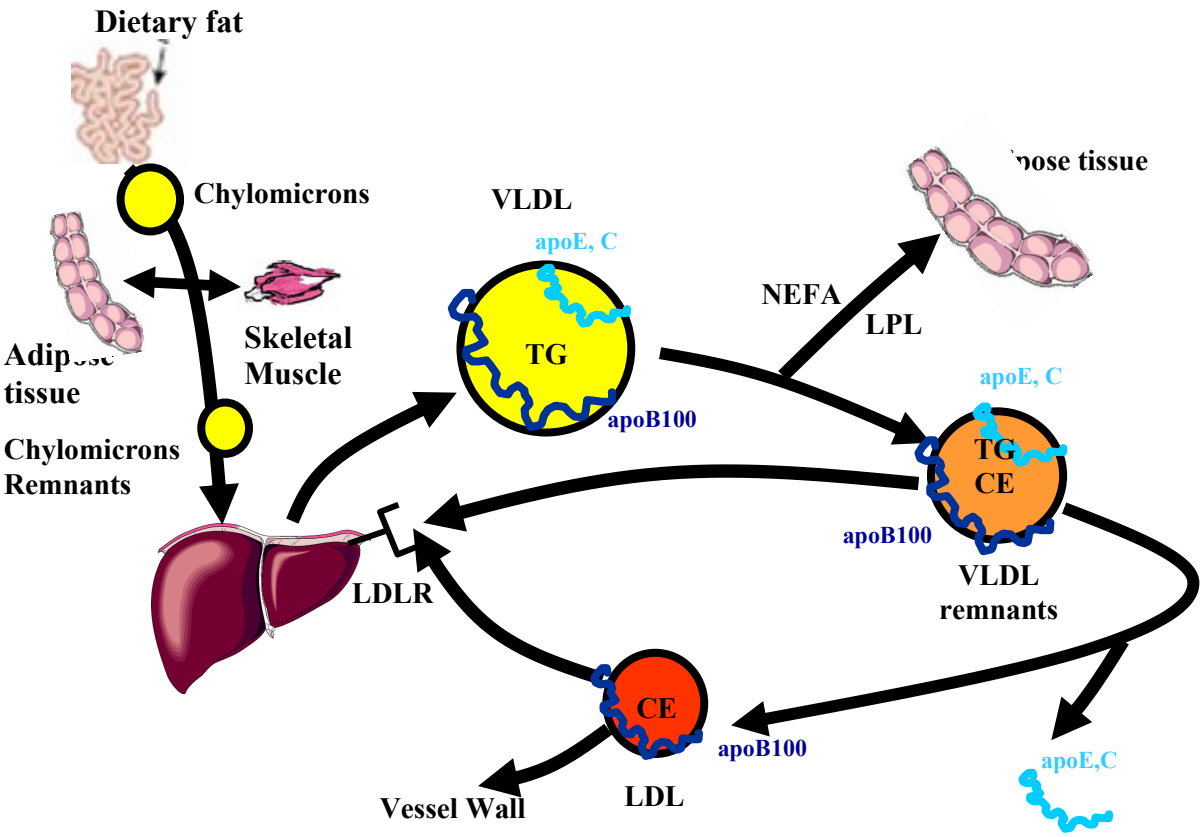


Figure 3. Lipoprotein metabolism.

Lipoprotein metabolism plays a major role in the development of lesions and the progression of atherosclerosis. Lipoproteins are responsible for the transport of lipids, specifically TG and cholesterol, within the circulatory system. Dietary fat is absorbed by enterocytes in the intestines, packaged as TG- rich chylomicrons which are carried by the blood to the adipose tissue and muscles. Fatty acids are released by LPL to the tissue. The remnant particle is removed by the liver. The liver then produces very-low-density lipoproteins (VLDL) by adding lipid to the apolipoprotein B backbone. Once secreted into the circulatory system the VLDL are metabolized by LPL leaving LDL which are predominately cholesterol containing particles. LDL particles are primarily taken up by the LDL receptor. LDL are the atherogenic particles that can transit the artery wall. (LPL – lipoprotein lipase; NEFA – non-esterified fatty acid).

Apolipoprotein B is an ideal marker for the study of circulating VLDL and LDL as each particle contains exactly one molecule of apoB and it is non-exchangeable. The protein becomes associated with lipids during initial assembly and remains associated with the same particle until the LDL is removed from the plasma. It is now thought that the level of apoB is a better marker for risk of cardiovascular disease than plasma or LDL cholesterol (43;44). Recent epidemiological studies, such as the multiethnic Insulin Resistance Atherosclerosis Study (45), found that apoB levels correlated significantly with insulin sensitivity and LDL size. The Third National Health and Nutrition Examination Survey (NHANES) study, involving 7000 participants, reported that metabolic syndrome was associated with high apoB levels after adjustment for age, sex and body mass index (BMI). Sniderman et al (46) reported that 25% of 2103 subjects whose apoB concentrations were surprisingly higher than that predicted by LDL-C levels, were obese and exhibited many of the features of metabolic syndrome. In the Quebec Cardiovascular Study, lipid and lipoprotein measurements from more than 2000 men who were free of coronary heart disease (CHD) at baseline, showed that elevated apo B had an increased risk of 1.4 for developing CHD over 5 years (47). In a population with a high prevalence of metabolic syndrome, serum apoB concentrations significantly predicted new diagnosis of metabolic syndrome in both men and women independent of waist circumference and C-reactive protein (48). A recent consensus statement by the American Diabetes Association and the American College of Cardiology recommended that apoB be added as a therapeutic target given the evidence that the dyslipidemia associated with insulin resistance—high triglycerides, low levels of high-density lipoprotein cholesterol (HDL-C), and small, dense low-density lipoprotein (LDL) particles—is exceptionally atherogenic. Optimal CVD risk reduction in diabetic or metabolic syndrome patients

likely requires even more accurate risk assessment and focused treatment. Evidence suggests that apolipoprotein (apo) B and apo A-I predict CVD risk more accurately than conventional lipid measures, either separately, or together as a calculated apo B/apo A-I ratio (49).

1.3.2. Adiponectin

Adiponectin, is a circulating adipokine, and has also been suggested to be a biomarker that is inversely correlated with the development of cardiovascular disease. Adiponectin, a 247 amino acid polypeptide, is synthesized and secreted predominantly by adipocytes (50). Adiponectin circulates in the plasma at high concentrations (3 -30 μg/ml) (51) in at least three multimer complexes: trimers, hexamers and higher molecular weight oligomers (50;52). Whereas there is no consensus concerning the biological significance of the various adiponectin isoforms, it is currently believed that circulating adiponectin complex distribution, specifically higher proportions of high-molecular-weight multimers, and not the absolute amount of plasma adiponectin, may be more clinically relevant, at least with respect to diabetes and coronary artery diseases (53-55). However, this hypothesis remains largely unproven since, until recently, the main means of profiling the individual adiponectin components was limited to semiquantitative methods. Molecular assays that are capable of differentiating between the various adiponectin multimers are currently being developed as investigators endeavour to determine which, if any, of the adiponectin complexes are more biologically active.

Two adiponectin receptors, AdipoR1 and AdipoR2, have been identified each with different affinity for the multimer complexes (56). AdipoR1 is thought to mediate adiponectin actions in skeletal muscle (56) while both AdipoR1 and AdipoR2 are present

in the liver (57). Although the entire signalling cascade has yet to be elucidated, it is known that AdipoR1 activates AMP kinase signalling and AdipoR2 acts through PPARα pathways to increase insulin sensitivity (Figure 4)(57;58). Reduced ATP or increasing AMP levels, indicators of cellular energy status, activate AMP kinase, up-regulating ATP generating processes such as fatty acid oxidation and glycolysis while suppressing lipogenesis. An amino terminal Cys-Ser mutation in adiponectin which blocked the formation of complexes larger than trimers abolished the effect of adiponectin on the AMP kinase pathway in hepatocytes (53). Other pathways through which adiponectin mediates its actions are the peroxisome proliferator-activated receptor (PPAR)-α, and the p38 mitogen-activated protein kinase (MAPK)-signalling pathways (59).

Paradoxically, adiponectin, although secreted from the adipocyte, decreases with obesity (50;51) and this inverse relationship is strongest with visceral fat (60) strengthening the association with metabolic syndrome. Other obesity-related states such as type 2 diabetes, cardiovascular disease and hepatic dysfunction (61-65) are also associated with hypoadiponectinemia. Mice lacking adiponectin show delayed clearance of plasma free fatty acids and diet-induced insulin resistance through decreased insulin receptor signalling and the insulin resistance is reversed with viral mediated adiponectin expression (66). Over-expression of adiponectin greatly improves insulin sensitivity (67). The study of Pima Indians of Arizona, a population at high risk of developing the metabolic syndrome, revealed that plasma adiponectin and insulin sensitivity were highly

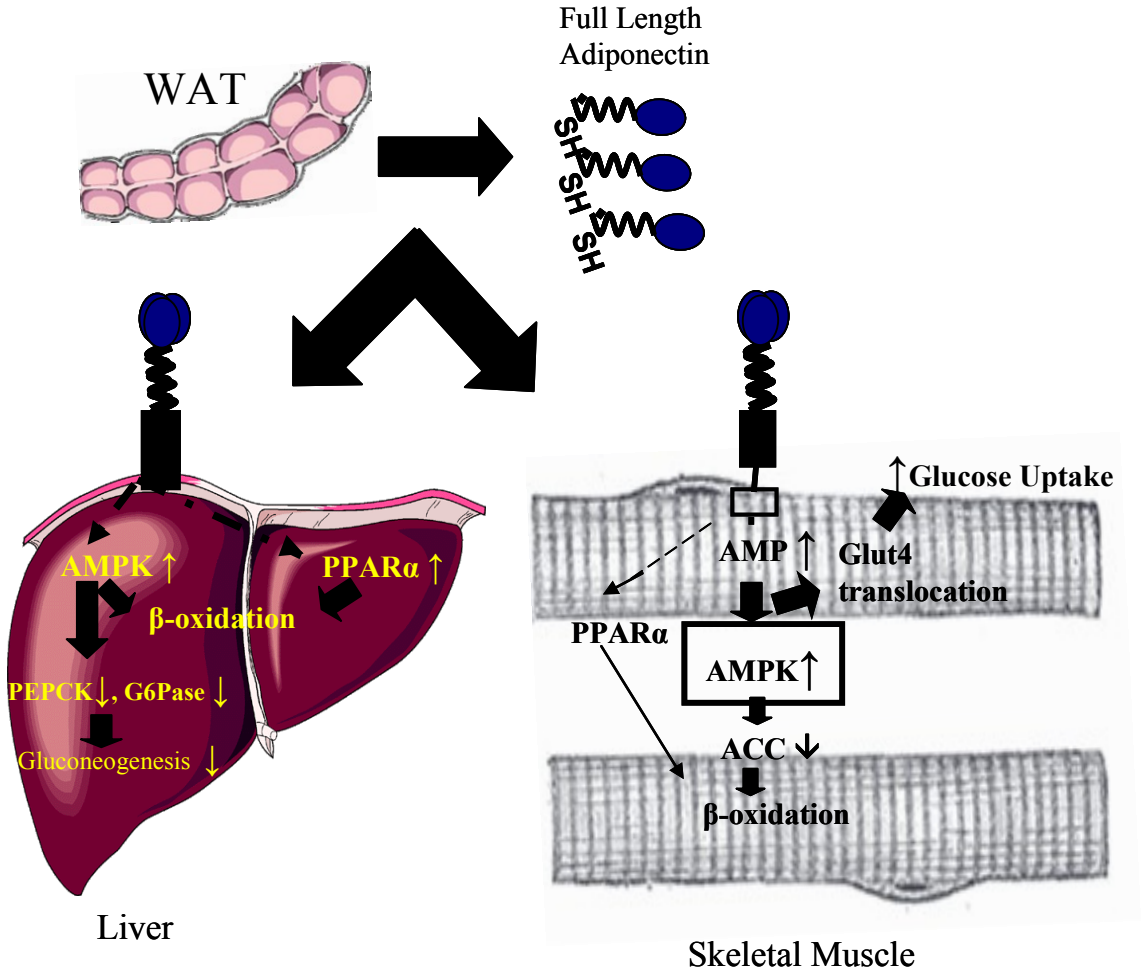


Figure 4. Adiponectin signalling in liver and muscle.

Adiponectin can activate AMPK and PPARα in the liver and skeletal muscle. In skeletal muscle, adiponectin activates AMPK, thereby stimulating phosphorylation of ACC, fatty-acid oxidation, and glucose uptake. Adiponectin activates PPARα, thereby also stimulating fatty-acid oxidation and decreasing tissue TG content in muscle. In the liver, adiponectin activates AMPK, thereby reducing enzymes involved in gluconeogenesis and increasing phosphorylation of ACC and fatty-acid oxidation. Adiponectin activates PPARα, thereby stimulating fatty-acid oxidation and decreasing tissue TG content in the liver. These alterations all increase insulin sensitivity *in vivo*. WAT, White adipose tissue; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose-6-phosphatase.

correlated such that those with an abundance of circulating adiponectin were less likely to develop type 2 diabetes (risk ratio 0.63 P=0.02). An increased adiponectin concentration provided more protection than age, fasting glucose, fasting insulin or waist circumference (68). A similar association was observed in a Japanese cohort that was followed for 5 years. Individuals with adiponectin levels in the lowest tertile of the study subjects were 9.3 times more likely to develop diabetes than those in the highest tertile (P=0.046)(69).

As stated earlier, biomarkers can be involved in the progression of the disease but more often are merely associated with the disease state. Which category adiponectin falls into remains equivocal. As evidence mounts from animal studies and gene mutant associations, it is becoming clearer that adiponectin is required for normal insulin sensitivity and in its absence the metabolic syndrome can develop (70). But there is also evidence that adiponectin may correlate with other markers of atherosclerosis, possibly giving an indication as to the severity of the disease. Since atherosclerosis is partially caused by inflammation (13), individuals at high risk have elevated levels of inflammatory markers such as C-reactive protein (CRP). In a study of male patients, adiponectin and CRP were negatively correlated ($r = –0.29$, $P < 0.01$) (71). Similar associations have been shown with other markers such as endothelial function and intima-media thickness (70).

In addition to being a marker of susceptibility for insulin resistance and diabetes, several animal studies have demonstrated the molecular association of adiponectin with impaired glucose metabolism. Adiponectin appears to modulate insulin sensitivity by stimulating glucose utilization and fatty acid oxidation via the phosphorylation and activation of AMPK in both muscle and liver cells (Figure 4) (49;62;70). During consumption of a high-fat and sugar diet, but not with a regular diet, adiponectin knockout

mice exhibit delayed clearance of free fatty acids from plasma, high levels of TNF-α mRNA in adipose tissue, and severe diet-induced insulin resistance with reduced insulin-receptor substrate 1-associated phosphatidylinositol 3-kinase (PI3-kinase) activity in muscle (66). However, with replenishment of adiponectin, these changes were reversed, decreasing the extent of insulin resistance (66). A similar result was observed in mice who received CLA, known to decrease adiponectin levels, for 4 weeks followed by 4 weeks without CLA. At week 8 the adiponcectin had returned to 50% of the original circulating levels (72). These findings suggest that restoration of adiponectin levels may provide a novel treatment for the metabolic syndrome.

1.3.3. Insulin Resistance

Of the risk factors for metabolic syndrome, insulin resistance is one of the leading factors for the development of further pathologies such as cardiovascular disease, stroke, non-alcoholic fatty liver disease, polycystic ovary syndrome, asthma and some cancers although the mechanisms have not yet been clearly elucidated (73-75).

Insulin binds to and activates the plasma membrane insulin receptor. Once activated the insulin receptor propagates its message by promoting the phosphorylation of selected tyrosines on the receptor and on post-receptor partners, insulin receptor substrate (IRS) proteins. From this point a number of signalling cascades are initiated (Figure 5). The major metabolic cascade, phosphatidylinositol 3-kinase (PI3-kinase) is responsible for the effects of insulin on glucose uptake, TG synthesis and storage. PI3-kinase subsequently activates both Akt and the atypical protein kinase Cs (ζ,λ). Akt is responsible for phosphorylation of FoxO1 on residues Thr-24, Ser-256 and Ser-319 (76) resulting in suppression of *trans*-activation, and exclusion from the nucleus and

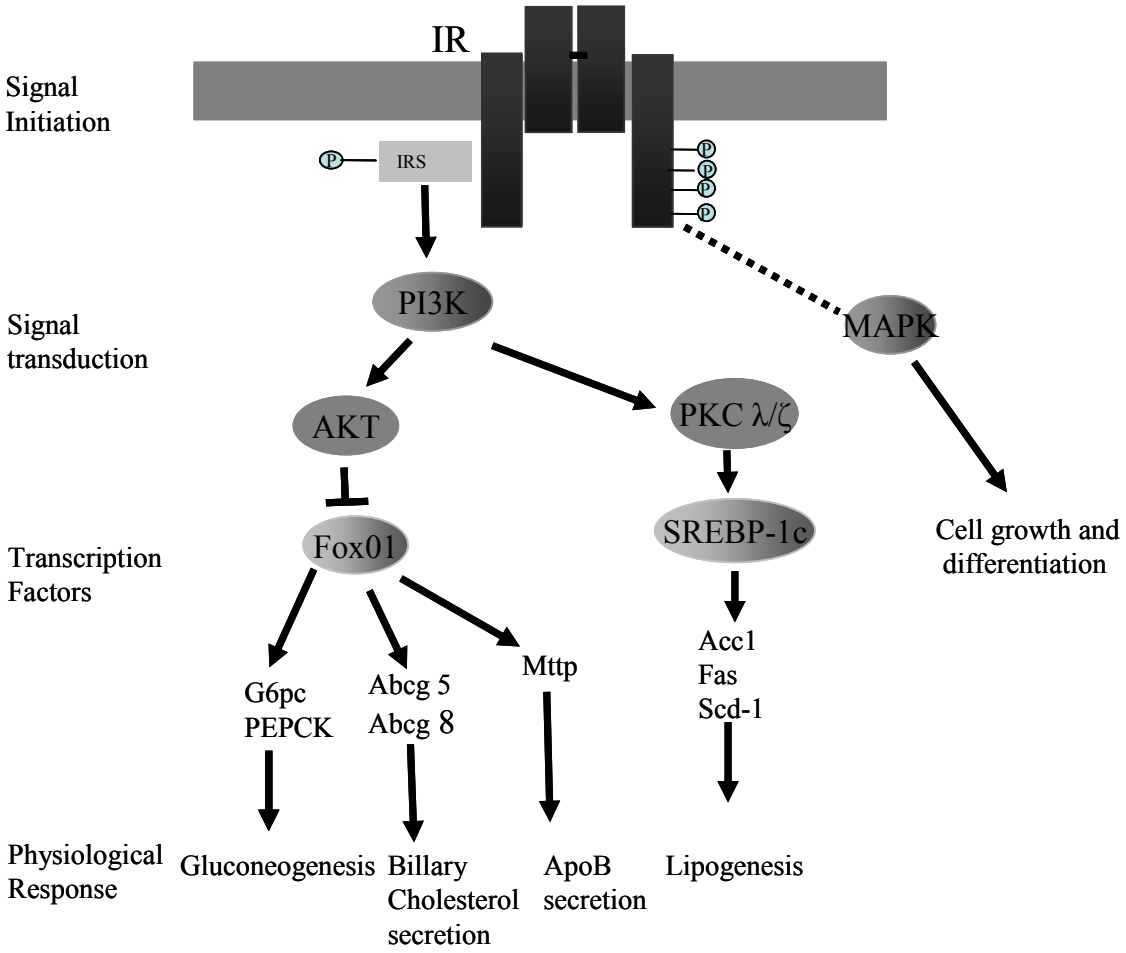


Figure 5. Insulin signalling.

Insulin initiates a complex network of signalling cascades. Shown here is a summary of the major pathways of these cascades, two important transcription factors, FoxO1 and SREBP-1c, and their downstream targets.

subsequent degradation (77). In the absence of insulin signalling, FoxO1 can bind directly to the insulin–response elements in the promoters of target genes or by co-activating or suppressing co-activators of other transcription factors. Protein kinase C propagates the signal to SREBP1c which initiates activation of numerous lipogenic genes. The third signalling pathway, mitogen-activated protein kinase, is predominately associated with cell growth and differentiation (78).

Insulin is responsible for regulating the expression of hundreds of genes at the transcriptional level either directly or through one of numerous signalling cascades (78). The Fox01 activates the expression of genes involved in gluconeogenesis, VLDL assembly and cholesterol efflux transporters. Gluconeogenesis is regulated through Fox01 activation of glucose-6-phosphatase (G6P)(79) and phosphoenolpyruvate carboxykinase (PEPCK) (80). Transgenic mice expressing a liver-specific, constitutively active FoxO1 had moderately elevated levels of fasting blood glucose and a 2.5-fold increase in insulin levels compared to wild-type mice (81). These observations suggest that the inability of insulin to regulate fasting glucose was due to the lack of signalling through FoxO1 (81). In the liver, lipogenesis and TG synthesis are regulated by insulin with hypertriglyceridemia present in the insulin resistant state. Surprisingly the transgenic FoxO1 mice had lower fasting plasma TG levels when compared to control, despite elevated levels of insulin. When circulating insulin is low, such as during fasting, endogenous FoxO1 proteins are active (located in the nucleus) and are able to promote the expression of genes that regulate gluconeogenesis and suppress the expression of genes involved in glycolysis, (including the pentose phosphate shunt and lipogenesis). Upon re-feeding, as insulin increases FoxO1 is inactivated through phosphorylation and as such gluconeogenesis would be inhibited and genes involved in glycolysis and *de novo*

lipid synthesis would be up-regulated (82). FoxO1 also targets microsomal triglyceride transfer protein (*Mttp*) which is involved in the lipidation of apoB a rate limiting-step in VLDL assembly (83). Comparing gain of function to loss of function studies in HepG2 cells the role of FoxO1 in the regulation of MTP was shown to be stimulatory while insulin inhibited MTP function (83).

While many of the effects of insulin on the expression of genes in the glucose metabolic pathway are regulated by FOXO1, many of its effects on lipogenesis appear to be mediated via the transcription factor sterol regulatory element–binding protein (SREBP)-1c. The SREBPs are a family of three nuclear transcription factors encoded by two genes (84). SREBP-1c, the dominant isoform in liver and adipose tissue, is capable of activating the entire program of monounsaturated fatty acid synthesis; it also appears to be involved in the regulation of gluconeogenesis genes. The SREBPs are subject to complex regulation at the transcriptional and posttranslational levels (84). Their transcripts encode membrane-bound inactive SREBP proteins, which are retained in the endoplasmic reticulum (ER). Two essential proteins regulate the cleavage process: SREBP cleavage-activating protein (SCAP) and insulin-induced gene (Insig). SCAP interacts with both the inactive precursor SREBP and Insig to act as an ER anchor (85;86). A specific signal, such as sterol depletion or insulin, causes SCAP to dissociate from Insig and assists in the transport of SREBP to the Golgi apparatus, where the proteolytical processing occurs to produce the mature active SREBPs. Once the N-terminal fragment of SREBP is released from the membrane, it can translocate into the nucleus to activate transcription. Several lines of evidence suggest that insulin directly regulates SREBP-1c. First, SREBP-1c transcript and nuclear protein are increased by insulin treatment in hepatocytes (87). Second, streptozotocin treatment, which renders

mice insulin deficient, results in a decrease in SREBP-1c (87). Similarly, fasting, which also lowers insulin levels, decreases SREBP-1c (88). Conversely, refeeding induces an exaggerated insulin response that is accompanied by an increase in SREBP-1c (88). Third, transgenic over-expression of constitutively active SREBP-1c induces the entire complement of genes necessary for fatty acid synthesis and obviates the need for insulin, whereas dominant-negative forms of SREBP-1c prevent lipogenic gene expression even in the presence of insulin. Insulin appears to increase SREBP-1c transcription, maturation, and activity. Insulin induces SREBP-1c transcription by activating the transcription factor, liver X receptor (LXR), which is known to bind the SREBP-1c promoter and activate its transcription in an insulin-dependent manner (30). Induction of SREBP-1c transcript appears dependent on PI3-kinase, through activation of PKCλ. Insulin also suppresses expression of Insig2a, which is the predominant Insig transcript in the livers of fasted animals. Suppression of Insig2a allows SREBP precursors to be escorted to the Golgi apparatus and undergo activation (89).

Insulin resistance results when normal plasma concentrations of insulin no longer efficiently activate the insulin receptor and downstream targets. This lack of proper signalling results in abnormal lipid metabolism that favours a pro-atherogenic milieu (90). Insulin signalling is often thought to be synonymous with glucose homeostasis but this simplistic definition fails to encompass all of the processes regulated by insulin such as the synthesis and storage of fat, protein synthesis, and non-metabolic processes such as cell growth and differentiation. As well, it implies that insulin resistance is an all or none phenomenon, in which the entire organism becomes insulin resistant at once. Ultimately, the phenotype of insulin resistance will depend on the exact components affected and the exact tissues in which they are affected. For example, insulin resistance caused by down-

regulation of the insulin receptor itself will decrease insulin signalling at all steps within the cell, whereas alterations in a signalling protein will alter only those pathways downstream of that specific protein (Figure 5). Hyperinsulinemia resultant from insulin resistance may actually up-regulate actions of insulin in tissues that are still insulin sensitive; for example, hyperinsulinemia acting on the liver, kidney, and ovary leads to hypertriglyceridemia, increased sodium retention and hypertension, and hyperandrogenism, respectively (91). An interesting observation was made in the livers of leptin deficient *ob/ob* mouse which supports the hypothesis that insulin resistance may occur at any one of the myriad of points along the signalling cascade. Lipogenesis, which is highly dependent on insulin for activation, is highly active in the livers of ob/ob mice which have a phenotype of severe hepatic insulin resistance and which also show large accumulations of nuclear SREBP 1-c (92). The failure to suppress gluconeogenesis while lipogenesis is up-regulated could reflect a down-stream interruption in the signalling as opposed to an effect on the receptor itself.

Complete hepatic insulin resistance, as studied using the liver insulin receptor knock out mouse (LIRKO), has been shown to be adequate to over-stimulate VLDL production and increase the risk of developing atherosclerotic lesions in the absence of overt plasma insulin changes (93). LIRKO mice display normal levels of TC and a 50% reduction in plasma TG, but the lipoprotein distribution, unlike the typical rodent profile with elevated HDL, is pro-atherogenic with elevated VLDL-C (93). Under insulin sensitive conditions insulin signalling would inhibit apoB secretion by increasing its degradation and preventing FoxO1-mediated transcription of MTP (83). Therefore, in the absence of insulin signalling, the liver is presented with an increased lipid load from circulating FFA, *de novo* lipogenesis and uptake of TG-rich particles from the plasma as

well as decreased degradation of apoB leading to both hypertriglyceridemia and hepatic steatosis.

Applying what has been gleaned from rodent knock-out models and dietary manipulations to humans requires caution. Similar to rodent models, various insulin pathways can be differentially affected. Insulin resistance and obesity often exist concomitantly in many individuals, presenting a horse and cart situation. Obesity has been shown to reduce insulin receptor binding, diminish phosphorylation of the insulin receptor as well as IRS1 in muscle tissue (94). The typical phenotype shows a substantial decrease in insulin activated IRS-1 signalling through PI-3 kinase leading to decreased glucose uptake (94-96), yet MAPK and Akt signalling are unaffected (94;95). This same expression pattern is observed in type 2 diabetics but with even greater decreases in phosphorylation of the IRS-1 protein and normal functioning of Akt and MAPK (94;95). Continuous insulin stimulation can lead to a decrease in the number of insulin receptors on the cell surface through internalization and degradation (97). There have been reports of obese individuals having fewer insulin receptors in adipose tissue although this is not consistently found (74). As already alluded to, the insulin resistance phenotype is tissue specific as the liver in obese subjects appears not to have a decrease in insulin receptor number or any decrease in receptor kinase activity and glycogenolysis under normal regulation (98).

An alternate view is that instead of insulin resistance resulting in elevated circulating levels of insulin, basal hyperinsulinemia initiates the insulin resistance (75). Marban and Roth (75) present evidence that in mice chronically over-expressing insulin, generalized insulin resistance results. The mice were transfected with varying copy numbers of the human insulin gene which produced basal plasma insulin levels 2-4-fold

higher than controls. The mice had elevated glucose and an exaggerated insulin response to glucose after feeding. The authors concluded that the hyperinsulinemia–induced insulin resistance was associated with reduced insulin receptor binding as well as hypertriglyceridemia that significantly correlated to the degree of hyperinsulinemia. It is also important to note that the mice over-expressing insulin were not obese, had normal fasting glucose and did not develop antibodies to insulin. In these mice it appears that the basal insulin levels were substantial enough to result in systemic insulin resistance.

Whether insulin resistance or hyperinsulinemia are the root cause of the metabolic disturbances the outcome is similar: elevated free fatty flux from adipose tissue to the liver, increased VLDL secretion and risk of atherosclerosis. Further studies are necessary to elucidate the exact molecular mechanisms of insulin signalling that are affected.

1.4. Endoplasmic Reticulum and Cellular Homeostasis

The ER is a crucial and central organelle for cellular homeostasis involving the synthesis, and posttranslational modification of transmembrane and nascent secretory proteins (99;100) as well as the synthesis of neutral and phospholipids (101). The classic function of the ER stress response or the unfolded protein response (UPR) was delineated through the observation that toxin-mediated impairment of ER protein folding resulted in transcriptional alterations (102;103). This initial finding led to the elucidation of the ER signalling pathways which have become known as the UPR (104). The aim of the UPR is to re-establish cellular homeostasis by down-regulating protein synthesis to decrease the folding load; increase folding capacity by increasing the amount of chaperones;

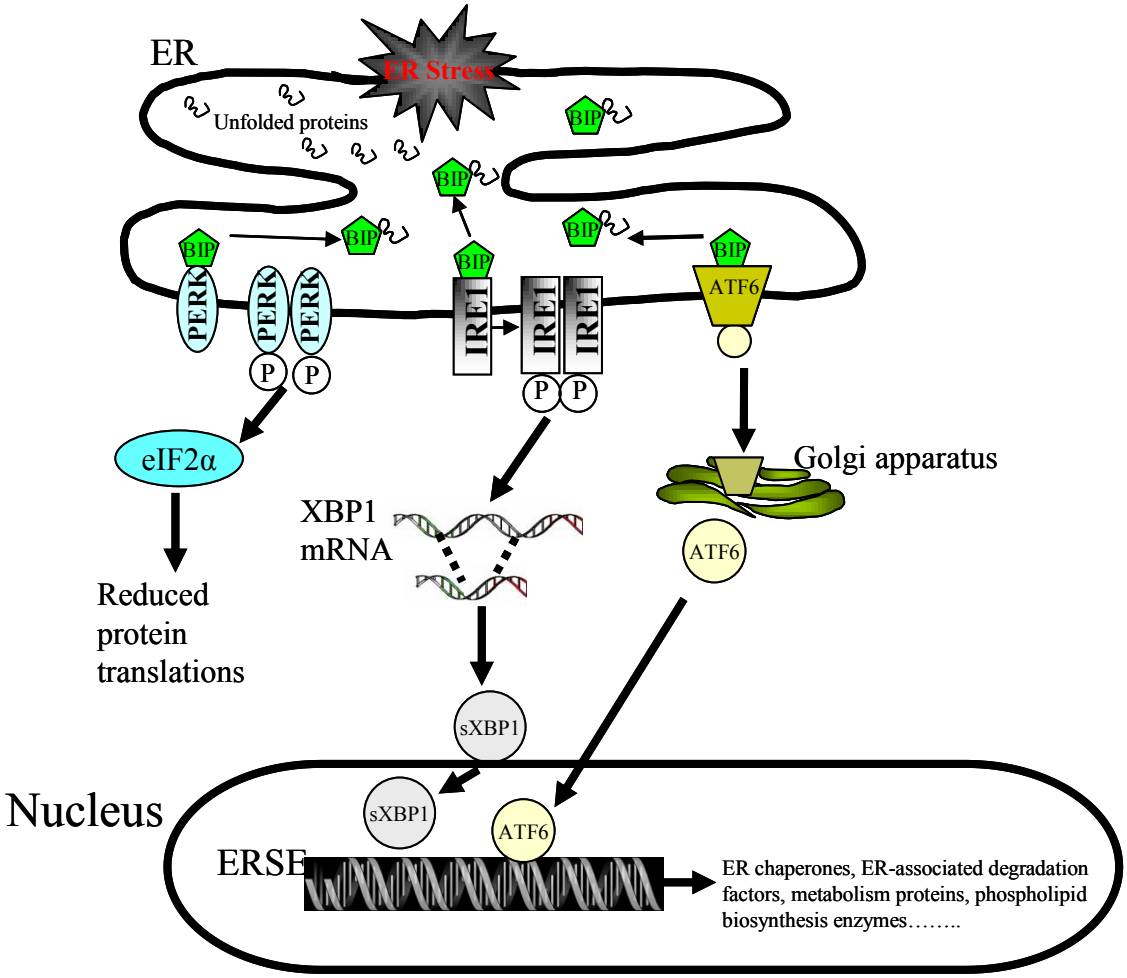


Figure 6. Endoplasmic reticulum stress and the unfolded protein response.

Endoplasmic reticulum stress leads to activation of the unfolded protein response in an attempt to restore cellular homeostasis. Upon ER stress, the chaperone BiP/GRP78 (Binding immunoglobulin protein/glucose regulated protein 78) dissociates from the luminal domain of three transmembrane stress receptors for UPR initiation. Protein kinase RNA (PKR)-like ER kinase (PERK) by dimerization and autophosphorylation becomes active and phosphorylates eukaryotic translation initiation factor 2α (eIF2α). This initiates a universal reduction in protein translation. Inositol requiring enzyme 1 (IRE1), after dimerization and autophosphorylation catalyzes the splicing of the X box binding protein1 (XBP1) mRNA. Activating transcription factor 6 (ATF6) is trafficked to the Golgi where it undergoes sequential proteolytic cleavage. XBP1 and ATF6 translocate to the nucleus and activate the transcription of UPR genes containing an endoplasmic reticulum stress response element (ERSE) in their promoter.

degradation of misfolded proteins and as a last resort apoptosis when homeostasis is unachievable. The UPR has evolved from a single linear pathway in yeast, to three distinct but complementary signalling pathways in the mammalian cell, all of which can regulate transcription (Figure 6). Expansion of the signalling repertoire is thought to have occurred in order to accommodate a broader range of physiological perturbations and allow for more sensitive responses specific to cellular needs (105). Evolution has diversified the UPR to include multiple stress sensors, responses that are regulated by many mechanisms, both transcriptional and non-transcriptional and by cellular pathways not typically considered part of the UPR (105).

During times of cellular homeostasis the transmembrane stress sensors, protein kinase RNA (PKR)-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) are anchored by the resident ER chaperone binding immunoglobulin protein/glucose regulated protein 78 (Bip/GRP78)(Figure 6). Upon sensing a "stress" the BiP/GRP78 dissociates to interact with the unfolded protein leading to the activation of the three sensors (104). The first response may be to reduce the protein load entering the ER through the PERK pathway. The cytoplasmic portion of PERK undergoes trans-autophosphorylation by oligomerization and then phosphorylation of the α-subunit of eukaryotic translation initiation factor-2 (eIF2α). Lower levels of the active form of eIF2α lead to a global reduction in translation of nascent proteins except for some proteins directly involved in the UPR. eIF2α, through the activating transcription factor 4 pathway (ATF4), up-regulates genes that encode amino acid transporters, genes that protect against oxidative stress, chaperones for the UPR protein XBP1 and the transcription factor C/EBP-homologous protein (CHOP), all in an attempt to reduce ER stress (106). Similar to PERK, upon release from BiP/GRP78, IRE1

undergoes homodimerization and *trans*-autophosphorylation activating its endoribonuclease activity (Figure 6). The activation of IRE1 results in the cleavage of an intron from the mRNA of XBP1 which encodes a transcriptional regulator, XBP1s, of UPR target genes (107). The unspliced mRNA encodes a more transient protein that represses UPR target genes. The final pathway involves the activated transcription factor 6 (ATF6) being trafficked from the ER to the Golgi upon induction of ER stress. The cytosolic DNA binding region is released through proteolytic cleavage (Figure 6) and the cleaved ATF6 fragment translocates to the nucleus to activate gene expression of ER chaperones and XBP1 (108).

Although the primary inducer of the UPR is thought to be the ER chaperone BiP/GRP78, this maybe a narrow view in light of recent studies showing that the UPR has a significant role in regulating physiological processes such as activation of the lipogenic pathway, mitochondrial fatty oxidation and VLDL synthesis and secretion (109). It has been suggested that if the UPR is unable to maintain cellular homeostasis in these processes then pathophysiological outcomes such as insulin resistance, type 2 diabetes, hepatic steatosis and even atherosclerosis may result.

A crucial function of the liver is to integrate whole body energy homeostasis by regulating carbohydrate and lipid metabolism. Hepatic lipogenesis is activated upon the intake of excess carbohydrates which are converted to TG (110). Hepatic lipogenesis is controlled by transcription factors like LXR, SREBP and cholesterol response element binding protein (ChREBP) which in turn are influenced by nutritional and hormonal conditions (84;111). SREBP-1c is strongly activated by insulin and increases gene expression related to fatty acid synthase (87;112). Given the complexity of regulation and the fine distinction between metabolic homeostasis and overload, it is probably not

surprising that the proteins originally identified as ER stress sensors are now found to have a role in metabolic pathways under non-stress conditions. The connection between ER stress and hepatic steatosis was suggested after the observation that a high fat diet induced markers of ER stress in the livers of *ob/ob* mice (113). In mice with an inducible disruption of the Xbp1 gene (Xbp1Δ) in the liver, plasma TG, cholesterol and free fatty acids were significantly reduced but surprisingly, were not accompanied by an increase in hepatic lipids (114). These findings were complemented by the significant down-regulation of key lipogenic genes such as those encoding stearoyl coenzyme A (CoA) desaturase 1 (Scd1), diacylglycerol acyltransferase 2 (Dgat2), and acetyl CoA carboxylase 2 (Acc2)(114). These lipogenic genes were induced upon high carbohydrate diet feeding in WT but not XBP1-deficient mouse liver. *De novo* synthesis of fatty acids, but not the central protein component (apoB) of lipoprotein assembly, was reduced in XBP1-deficient hepatocytes. XBP1 is not the only ER stress related protein to be connected to lipogenesis. Sustained dephosphorylation of eIF2α has been associated with a decrease in hepatic lipogenesis and steatosis in high-fat diet fed mice (115).

As discussed previously (Section 1.3.3) a paradoxical situation arises in the livers of *ob/ob* mice where in the presence of insulin resistance, SREBP-1c accumulates in the nucleus and lipogenesis is up-regulated. Similar findings have been reported in other rodent models of diabetes mellitus and lipodystrophy (92;116) as well as in HepG2 cells. Increased free fatty acid loads induced markers of ER stress and SREBP-1c along with downstream genes responsible for TG synthesis (117). These findings are supported by the observation that ATF6 and SREBP-1c share common mechanisms for proteolytic cleavage (118). Recently, it has also been shown that ER stress activation, independent of

insulin, can initiate and induce the cleavage of SREBP-1c leading to up-regulation of numerous lipogenic genes (119). This induction of SREBP-1c could be attenuated by the over-expression of the ER chaperone GRP78 which also led to a reduction of hepatic steatosis in mice (119).

Membrane expansion in the ER is a mechanism to accommodate an increase in cellular protein load. It has been shown that an increase in ER size is sufficient to reduce stress (120) and works co-ordinately with the ER stress response proteins to return homeostasis to the cell. Many of the proteins initially up-regulated by ER stress are key regulators of lipid biosynthetic pathways (104) and this role of the UPR has been highly conserved from yeast to higher organisms (121). Maintaining a functional membrane is of utmost importance and it has been shown that alterations in the phospholipid component either by depletion of phospholipid (122;123) or altering the unsaturated to saturated ratio of fatty acids of phospholipids (124), will activate the UPR. It remains to be determined if this is an intrinsic role of the ER sensors (PERK, ATF6 or IRE1) to monitor the lipid composition or whether it is a disruption in the protein-folding milieu. The main pathway in animals for sensing lipid quantity is through the SREBPs which have already been shown to interact with GRP78 (119) although there is little supporting evidence of overlap between the two (104). It has been suggested that these two pathways (SREBP and UPR) may oppose each other in that membrane cholesterol excess can induce ER stress (125). Additionally, experiments using small molecule inhibitors to block cholesterol biosynthesis up-regulated the eIF2α-phosphorylation pathway of the UPR and inhibited SREBP action (126).

Further work will be needed to delineate the relationship between lipid biosynthesis and the UPR in maintaining homeostasis and responding to ER stress. How

physiological ER stress and the UPR fit into the development of metabolic syndrome remains to be determined. Numerous metabolic perturbations such as obesity, high fat diet and insulin resistance have been shown to induce ER stress (113) Recent experiments showed that in *ob/ob* mice the use of chemical chaperones, which assist in protein folding, could lessen ER stress by rescuing insulin action and returning glucose to normal levels (127).

1.5. Hepatic Lipid Metabolism

The polypeptide backbone for the assembly of the triglyceride-rich lipoproteins is the 4536 residue hydrophobic apolipoprotein B (apoB). ApoB has two natural forms in humans; the full length hepatic apoB100 and the intestinal apoB48 which is the N-terminal 48% of the full length protein. The shorter apoB48 results from an mRNA editing process by a cytidine deaminase which converts a CAA (glutamine) to a UAA (stop codon) (128). In humans this mRNA editing occurs only in the intestine while in some rodents, mice and rats, it occurs in both the intestine and liver. Due to its size and hydrophobicity the crystal structure of apoB100 has yet to be determined but much has been learned about the lipid binding properties and binding partners from computer modeling (129) and research on similar structures such as the lamprey lipovitellin (130) leading to a clearer understanding of the structure and function of the N terminal region. The N terminal region contains numerous binding sites for MTP (131;132), which is required for the addition of lipid to apoB, as well as binding sites for scavenger receptors in macrophages (133) and for lipoprotein lipase (134).

Assembly of VLDL appears to occur in at least two steps. Initially, a small primordial particle, with a density similar to HDL, is formed co-translationally as apoB is

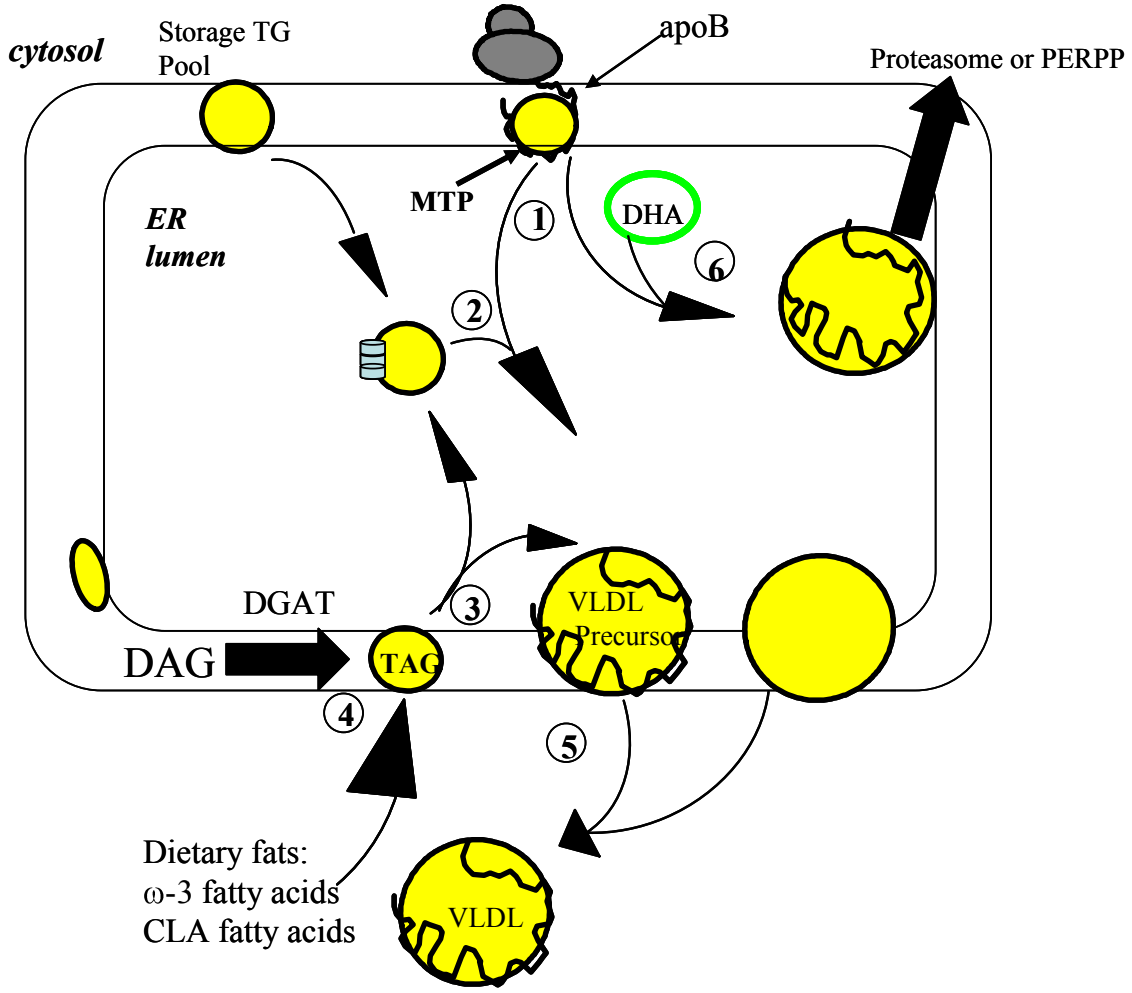


Figure 7. Assembly and secretion of VLDL.

The role of ApoB in the assembly of TG-rich particles begins with its association with the ribosome. ApoB is translated across the membrane and becomes enriched with lipids co-translationally **(1)** resulting in the formation of a nascent particle with a density similar to HDL. MTP activity may be required for this step. The nascent apoB particle acquires additional TAG from luminal lipid particles **(2).** Lumen TAG substrate exists as an apoB free particle but may associate with other lipoproteins such as apoCIII. MTP activity is required for the movement of lipid into the luminal droplet or into membranes of ER microsomes for VLDL assembly **(3).** The TAG supply is maintained through *de novo* synthesis, esterification of fatty acids from exogenous sources or previously stored TAG **(4).** The VLDL precursor particle transits from the ER to the Golgi where full lipidation occurs and secretion from the cell **(5).** Misfolded or insufficiently lipidated particles are marked for removal by either the ubiquitin/proteasome pathway or Post –ER- Pre-secretory Pathway (PERPP). The method of removal from the secretory pathway is also known to be influenced by lipid species **(6).**

translocated across the ER membrane. Subsequently, additional lipid is acquired by the particle to form a TG rich VLDL (135-137). Under conditions of poor lipid supply the assembly of VLDL is impaired and possibly terminated by ubiquitination/proteosomal degradation (138) or autophagy. The role of apoB in the assembly of TG rich particles begins during protein translation. ApoB is translated on the endoplasmic reticulum (Figure 7(1)) by membrane associated ribosomes. The newly translated polypeptide chain is translocated through the translocon into the ER lumen. ApoB becomes enriched with lipids co-translationally by the activity of MTP which assists in lipid transfer onto apoB (Figure 7 (2)). MTP has 2 subunits, one of which has lipid binding and transfer functions (139). It has been shown that the absence of a functional lipid transfer subunit results in abetalipoproteinemia, which is characterized by extremely reduced levels of apoB lipoproteins (140). Numerous studies have shown that non-lipoprotein expressing cells can be induced to secrete lipoproteins by the exogenous expression of MTP and apoB (141-143). Furthermore, small molecule inhibitors of MTP can decrease VLDL secretion from liver cells in culture (144). To ensure an adequate lipid supply in the vicinity of VLDL assembly, it appears that MTP is fundamental for the maintenance of a microsomal TG pool that is intimately involved in the bulk addition of lipids during the second step of VLDL assembly (Figure 7 (3))(144). *In vitro* studies, utilizing MTP inhibitors (145), and *in vivo* studies, using the liver specific *Mttp* knockout mouse (146), have enhanced our understanding of the interaction of MTP and cellular TG pools. From lipid pulse-chase analysis in primary hepatocytes inhibiting MTP activity resulted in a decreased output of VLDL TG and delayed removal of TAG from the cytosol and ER and Golgi membranes (145). When ultrastructures in the liver tissue from liver specific Mttp

knockout mice were examined there was a noticeable absence of lipid particles in the ER/Golgi lumen while lipid particles were clearly visible in wild-type controls (146). These studies suggest that MTP is responsible for the recruitment of TG to the microsomal lumen, to be used in the assembly of VLDL. Given the intimate relationship between VLDL and MTP activity, any alterations in MTP activity will have a direct effect on VLDL assembly therefore any regulation of MTP indirectly regulates VLDL assembly and secretion.

Feed–forward or substrate driven transcriptional regulation is a common mechanism in metabolic pathways to allow changes in gene expression to appear seamless (147;148). Coordinated regulation of Liver Fatty Acid Binding Protein (L-FABP) and MTP is possible through a conserved element in their promoters (149). It has been shown that PPARα dependent gene transcription sufficiently induces the expression of both L-FABP and MTP through their DR1 element, such that an efficient delivery of fatty acids will be secured for proper VLDL assembly. This PPARα activation is dependent upon the co-activator PPARγ coactivator β (PGC-1β) which in of itself can indirectly up regulate MTP through increased substrate delivery. PGC-1β is known to interact with the SREBP transcriptional regulators to increase lipogenic genes resulting in an increase in MTP expression (150). FoxO1, as previously discussed (Section 1.3.3) is also a regulator of MTP by binding directly to the MTP promoter (83) insulin opposes this action through the AKT signalling pathway (Figure 5).

The size and lipid composition of the secreted VLDL particle varies according to the nutritional status of the animal (151;152). Kinetic evidence has shown that the majority (~70%) of the TG that is secreted in VLDL must first enter cytosolic stores in the hepatocyte from which it is subsequently hydrolysed and re-esterified before

incorporation into secretory VLDL-TG (153-156). Thus, hepatic VLDL contains esters (predominantly TG) of the fatty acids consumed in the diet and, at least temporarily, stored in the liver. Since fatty acids consumed in the diet can have a direct impact on VLDL synthesis, it is important to understand how common classes of dietary fats; trans fats, polyunsaturated, monounsaturated and saturated fatty acids can improve or worsen the lipoprotein profile. Chicken hepatocytes were used to measure the secretion of TG and apoB in response to various medium-chain fatty acids (MCFA). All of the MCFAs (8:0, 10:0 and 12:0) decreased VLDL secretion compared to palmitate (16:0)(157). The extent of unsaturation of the acyl chain has also been shown to alter the secretion of apoB containing VLDL.

McA-RH7777 rat hepatoma cells, have been utilized extensively for studying the influence of fatty acids on apoB synthesis and secretion as they are one of the few cell lines that secrete authentic VLDL in the appropriate density range. When McA-RH7777 cells are exposed to exogenous oleate (18:1) there is a significant increase in TG rich VLDL compared to no supplement (137;158;159). In a comparison between oleate and eicosapentanoic acid (EPA) in McA-RH7777 cells, EPA significantly reduced TG secretion without affecting synthesis, resulting in a smaller dense apoB100 particle in the secretory pathway (160). It has also been recently shown that although cellular TG synthesis is similar in the presence of myristic acid (C14:0) and DHA (C22:6) there is a significant reduction in VLDL secretion with DHA supplementation suggesting that a regulatory process removes the fully lipidated particle from the secretory pathway (161). This process has been further characterized as the Post-ER-Pre-Secretory Pathway (PERPP) which is stimulated by oxidative modifications and subsequent aggregations of apoB particles (162). PUFAs were found to initiate oxidative modifications to apoB after

its exit from the ER but before leaving the Golgi. It has been suggested that this is both a regulatory step, to reduce the amount of apoB secreted, as well as quality control mechanism carried out by autophagosome-lysosomal degradation (162).

Trans fatty acids have been thought to increase plasma LDL levels, by increasing hepatic secretion of VLDL or by decreasing hepatic LDL uptake, either of which may be potentially atherogenic. When chicken hepatocytes were cultured with trans vaccenic acid (18:1) there was no significant effect on apoB production compared to BSA control (163). Conjugated linoleic acid (18:2) treatment in HepG2 cells resulted in significantly less apoB-100 synthesis and secretion as compared to treatment with SFAs or PUFAs (164). The chemical and structural characteristics of fatty acids have differential effects on VLDL synthesis and secretion. To simulate the dietary delivery of fatty acids, the concentration and length of exposure to various fatty acids has to be considered. Short term exposure to trans fatty acid (10trans, 12cis, 18:2) in $LDLr^{-/-}$ mice resulted in increased TG associated with elevated plasma VLDL. Paradoxically, longer duration of feeding decreased plasma VLDL-TG which the authors attributed to increased VLDL receptor up-regulation (165). ER stress has also been associated with a reduction in apoB secretion through an increase in ER-stress dependent degradation. When McA-RH7777 cells were treated with free fatty acids (FFA) with increasing fatty acid concentration, cell TG and ER stress markers increased. ApoB secretion was parabolic, in that low levels of FFA resulted in increased apoB secretion but higher levels decreased apoB secretion (166).

An apoB free lipid particle is synthesized and becomes post-translationally associated with the primordial sized apoB particle to form the secretion competent VLDL. This bulk addition of lipid occurs after the primordial apoB particle transits from

the ER to the Golgi. Lipid droplet proteins have been identified and it is known that they influence VLDL assembly, although the mechanisms have not been elucidated (167). The final structure of the VLDL is a neutral core of TG/CE surrounded by a surface monolayer of phospholipids, unesterified cholesterol and apolipoproteins.

Regulatory processes ensure that only properly folded and lipidated apoB particles continue through the secretory pathway. Polypeptides that fail to assemble a particle are degraded within the secretory pathway. How these species are identified is not yet clear. The cytosolic ubiquitin-proteasome pathway is the primary mechanism of intracellular degradation of apoB in HepG2 cells and plays a role in other systems as well. Newly synthesized apoB is ubiquitinated and degraded in a co-translational manner (168). Mechanisms which target apoB to the proteasome are incompletely understood and although numerous chaperones have been identified, their specific roles are yet to be confirmed. GRP78, an ER protein has been shown to be involved in increased degradation of apoB. Binding and retention of apoB by GRP78 may play a critical role in proteasomal targeting (169). Non-proteasomal pathways have also been reported for apoB degradation. Omega-3 fatty acids are thought to induce lipid peroxidation and oxidative stress that triggers the removal of VLDL particles via PERPP, as described above. Anti-oxidants can counter this degradation (162).

1.6. Bioactive Fatty Acids

There is increasing awareness that components of our food can provide more than simple nutrition. One of these nutrients, fatty acids, is often vilified as the root cause of obesity, insulin resistance and cardiovascular disease. Yet there appears to be certain types of fats which, when present in minor amounts, influence cellular activity and may

modify an individual's risk of developing disease. It is these bioactive fatty acids that were investigated for potential benefits in the treatment and prevention of metabolic syndrome.

1.6.1. Conjugated Linoleic Acid Structure

Conjugated linoleic acid (CLA) is a term used to describe a group of positional and geometric isomers of the essential dienoic fatty acid linoleic acid (LA, C18:2 *c*-9, *c*-12) (Fig. 8). The CLA isomers differ from LA and one another in the location and orientation of the double bonds in the acyl chain. To date, 28 isomers of CLA have been identified with varying geometry (*cis-cis, trans-trans, cis-trans, and trans-cis*) and double bond positions (which can be found on carbons 6 to 15) [reviewed in (170-173)].

In the human diet, CLA is found predominantly in the fat component of meat and dairy products from ruminant animals such as cows, deer, sheep and goats. More than 90% of CLA from these natural sources is the *cis*-9, *trans*-11 isomer (*c*-9, *t*-11). *c*-9, *t*-11 CLA is produced in the rumen of the intestinal tract as the first stable intermediate in the biohydrogenation of forage-derived unsaturated fatty acids such as oleic acid or LA to stearic acid. CLA can be directly absorbed at this point or further hydrogenated by the rumen bacteria to vaccenic acid (*trans*-11, octadecenoic acid) which upon absorption by the mammalian cell can be converted back to *c*-9, *t*-11 CLA by Δ-9 desaturase (174;175). The endogenous synthesis of *c*-9, *t*-11 CLA from the precursor *trans* vaccenic acid (18:1) has also been shown to occur in humans (176).

Recently, chemically synthesized preparations of CLA have become widely available as dietary supplements. The commercially available supplements are produced

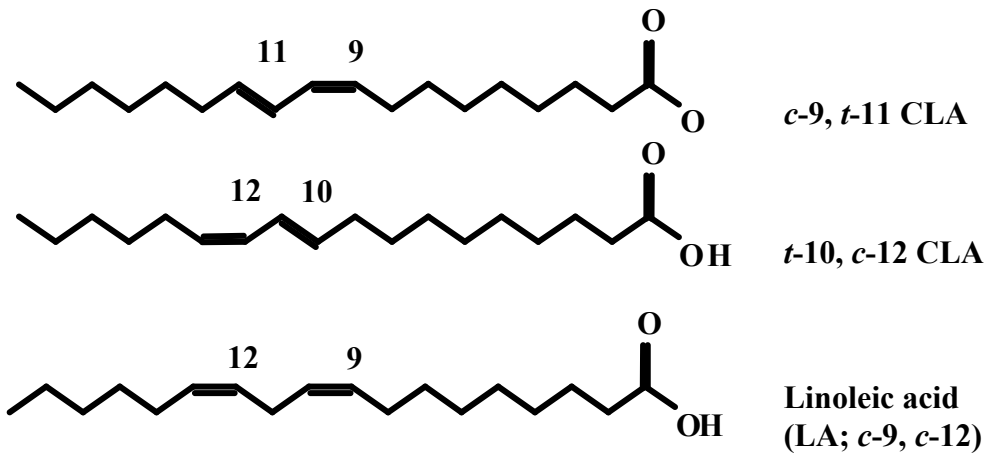


Figure 8. Structures of linoleic acid and conjugated linoleic acid.

The essential fatty acid linoleic acid is shown along with the two most common conjugated isomers; *t*-10, *c*-12 CLA and *c*-9, *t*-11 CLA.

by alkaline isomerization of oils rich in linoleic acid. The products of the chemical process are mixtures containing approximately 40% *c*-9, *t*-11 CLA, 40% *trans*-10, *cis*-12 (*t*-10, *c*-12) CLA and 20% other isomers (177;178). At the present time, manufacturers are able to purify the *c*-9, *t*-11 and *t*-10, *c*-12 CLA isomer preparations at commercial scale, although these are not generally available as supplements. However, the availability of single isomer supplements has allowed researchers to investigate the specific biological activities of each isomer.

Given the differences in structure and bond position of each isomer, there are differences in how *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA are metabolized once they are taken up by the tissue. A complicating factor to establishing the metabolic effects of each isomer is that both c-9, *t*-11 CLA and *t*-10, *c*-12 CLA can be further subjected to desaturation and elongation by Δ5 and Δ6 desaturases and elongases to form conjugated octadecetrienoic acid (18:3), conjugated eicosatrienoic acid (20:3) or conjugated eicosatetraenoic acid (20:4) all of which may have metabolic activities (179). *c*-9, *t*-11 CLA is more readily converted to C20:4 while *t*-10, *c*-12 CLA appears to be less readily elongated (179). It has also been shown that *t*-10, *c*-12 CLA is more readily oxidized because of the double bond positions as compared to *c*-9, *t*-11 CLA (180). Once taken up by the cell, fatty acids can be re-esterified into TG or incorporated into PL. Double bonds in the *cis* configuration promote the incorporation into PL, hence both the *c*-9, *t*-11 and *t*-10, *c*-12 isomers are more readily incorporated into neutral lipids than LA which is preferentially esterified into PL(181).

Although CLA is currently available as a dietary supplement and the food industry is developing methods to increase the content of CLA in some dairy products,

the true efficacy and safety of CLA has not yet been unequivocally established. It is not yet known if ruminant *trans* fatty acids have negative health implications for humans similar to those seen with *trans* fatty acids generated chemically by partial hydrogenation of polyunsaturated fats. Therefore, understanding the biological implications of differences in bond geometry and position of CLA isomers will have major implications for dietary recommendations, formulation of functional foods, and recommendations for dietary supplements for human consumption.

1.6.2. Biological Activities of CLA

The health benefits ascribed to CLA are wide ranging and are mainly based upon cell culture and animal studies with less supporting evidence from human trials. It is not clear in many of the studies which isomer was responsible for which biological activity or whether there is an interactive effect of the major isomers. Animal and human studies strongly indicate that the two major isomeric forms of CLA can have highly divergent physiological effects when administered individually (177;181). It has been suggested that CLA may have biological activities resulting in decreased development of atherosclerotic plaque, anti-carcinogenic properties, modulation of the immune response, altered plasma lipid metabolism, decreased body fat accumulation and decreased insulin resistance (170;181-183).

The anti-carcinogenic properties were the first to be identified for CLA (184) after a component of grilled beef, that appeared to inhibit mutagenesis, was isolated and determined to contain isomers of LA (185). A variety of cell culture studies have examined the ability of CLA to inhibit tumour growth in human cancer cells such as malignant melanoma, hepatoma, colorectal, prostate and breast cancer as well as in lung

adenocarcinoma cell lines (186-190) with breast cancer being the most thoroughly studied. Although both *c*-9, *t*-11 and *t*-10, *c*-12 isomers are able to reduce the proliferation of breast cancer cells their effectiveness is not equal (186). When breast cancer cells were incubated with a CLA mix or either of the individual isomers, the mixed isomer preparation and *c*-9, *t*-11 CLA reduced the number of viable cells by 60% while the *t*-10, *c*-12 CLA only reduced the cell number by 15% (186). Even epidemiological studies suggest that CLA supplementation may be effective in reducing breast cancer. Although CLA was not specifically quantified, milk consumption was found to be inversely related to breast cancer risk in a study involving nearly 4700 women over 25 years. Even when the risk was adjusted for other confounding factors like smoking the significant relationship remained (191). In postmenopausal Finnish women it was reported that CLA intake and serum levels where significantly lower in cancer patients compared to healthy controls (192). Further research is needed to fully understand the mechanisms by which milk or CLA may have beneficial effects in cancer.

Body fat loss was the next physiological parameter found to be influenced by CLA. After reports that mice had a 60% reduction in body fat and a 14% increase in lean body mass upon consuming a 0.5% CLA Mixture (50:50 *c*-9, *t*-11 and *t*-10, *c*-12) (193), research into CLA as a weight loss supplement increased dramatically. Unfortunately, results from human trials have been far less dramatic or conclusive. A recent meta-analysis of CLA as a fat loss agent in normal weight, overweight and obese individuals attempted to shed light on the discrepancies between animal and human weight loss (194). Plourde *et al* (194) reported that no study using normal weight subjects reported a significant body weight loss (n=9 studies) and the significant fat loss reported in two studies was confounded by physical activity training. In one study that followed

individuals for one year (195), there was no significant weight loss with 6 g/day CLA mix supplementation but there were improvements in glucose and insulin measures. Another one year study examined if the form of CLA impacted weight loss (196). When given as either a free fatty acid or as oil (TG), CLA mix resulted in a significant decrease in body fat mass compared to an olive oil placebo. The group receiving the free fatty acid mixture reported an increase in LDL-C. Some human trials have reported increases in markers of inflammation. Purified *t*-10, *c*-12 CLA but not the 50:50 mix supplemented at 3.4 g/day for 3 months resulted in increased oxidative stress, CRP and proinsulin and decreased insulin sensitivity in non-diabetic obese males (197). In healthy postmenopausal women CLA mix (50:50) increased markers of inflammation such as C-reactive protein, fibrinogen, and plasminogen activator inhibitor-1, as well as lipid peroxidation, a marker of in vivo oxidative stress measured as urinary free 8-iso-prostaglandin F_2 after 7 months of supplementation as compared to women receiving only *c*-9, *t*-11 CLA. This suggests that the *t*-10, *c*-12 CLA may be responsible for the adverse inflammatory response (198). Even though human trials have been less convincing than animal studies, it is universally accepted that the *t*-10, *c*-12 CLA isomer is biological active in the adipocyte (199;200). In both human primary adipocytes and an adipocyte cell line (3T3-L1) the *t*-10, *c*-12 isomer reduced TG accumulation and attenuated differentiation while the *c*-9, *t*-11 isomer had the opposite effect, increasing TG accumulation (199;201). One of the enzymes associated with the decreased TG accumulation is SCD-1, which has been consistently shown to be down-regulated by CLA (199). Kuhnt *et al* (176) reported that higher body fat accumulation was significantly associated with higher SCD-1 expression in women. After a 42 day CLA supplementation, the women had a 10-fold decrease in both SCD-1

and Glut 4 expression. CLA has been identified as a PPARγ ligand (202-204) with effects similar to TZDs such as troglitazone (202). In Zucker rats, CLA increased adiponectin levels, whose gene expression is under PPARγ regulation, which is thought to have alleviated hyperinsulinemia and obesity related hypertension (205). Other PPARγ targets such as TNFα, MCP-1 and NFκB (204;206) may be suppressed by CLA resulting in an anti-inflammatory response.

1.6.3. Omega-3 Fatty Acid Structure

Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentanoic acid (EPA, C20:5n-3) (Figure 9) are members of the very long chain omega (ω) -3 (or n-3) class of fatty acids. ω-3 PUFAs are considered "essential" long chain fatty acids as they must be consumed from animal or plant sources, since humans lack the Δ12-desaturase necessary to insert a double bond at the n-3 position of the fatty acid carbon chain. The precursor FA of the long chain n-3 PUFAs is alpha linolenic acid (ALA 18:3 n-3) (Figure 9). Dietary sources of ALA are the seeds of flax, canola, walnuts and chia and the chloroplast of green leafy vegetables (207). Once ingested, ALA undergoes Δ6 desaturation, elongation and further Δ5 desaturation to yield EPA. EPA undergoes further elongation and Δ6 desaturation before β-oxidation in the peroxisomes produces DHA. Estimated conversion rate is approximately 5–10% for ALA to EPA and only 2–5% for the subsequent conversion to DHA (208;209). The International Society for the Study of Fatty Acids and Lipids (ISSFAL) recently released an official statement on the conversion efficiency of ALA to DHA. They concluded that the conversion of ALA to DHA is on the order of 1% in infants, and considerably lower in adults (210). Because the conversion rate of ALA to

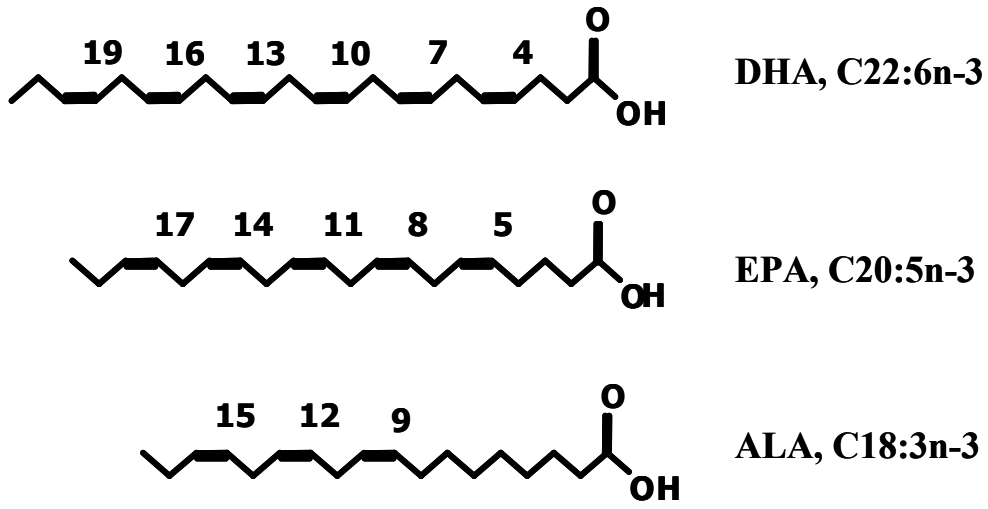


Figure 9 Structure of ω-3 fatty acids

DHA and EPA two common omega-3 fatty acids are found in the oil of fatty fish while ALA is a primary plant source of omega-3 fatty acids.

EPA/DHA is extremely low in humans it is necessary to consume DHA and EPA directly to obtain the health benefits. In the human diet, these fatty acids are consumed in fish oils from the flesh of fatty fish (salmon and mackerel) and sea mammals (whales). Enzymes in phytoplankton consumed by these fish produce the EPA and DHA from linolenic acid (18:3). Dietary supplementation of ω-3 fatty acids can be achieved by ingestion of capsules containing purified fish oils. Intake of PUFAs will increase the concentration of ω-3 fatty acids in tissue and plasma in a dose dependent manner. Numerous dietary intake studies have shown that the consumption of either DHA ethyl esters or DHA TG increased plasma red blood cell DHA concentration in adult humans (211-213). Supplementation with EPA ethyl esters increased plasma EPA concentrations but the conversion to DHA is negligible (214;215)

1.6.4. Biological Activities of Omega-3 Fatty Acids

The ability of ω-3 fatty acids to reduce cardiovascular disease was first identified from observational studies of the diet of Greenland Eskimos. It was observed that high fat intake was not equated with increased cardiovascular disease and that this increased fat intake was composed of a high percentage of ω-3 fatty acids (216). Since the 1970's numerous intervention studies have reported that EPA and DHA supplementation leads to a reduction in cardiovascular mortality and sudden death in both clinical and animal studies. A number of cardioprotective mechanisms have been suggested, from anti-arrhythmic effects, reduction in blood pressure, improved endothelial function, retarded growth of atherosclerotic plaque to hypotriglyceridemic effects in hypertriglyceridemic individuals (217-219). A meta-analysis of 72 placebo controlled trials with an omega

fatty acid dose of ≤7 g/day showed a clear dose response in the reduction of plasma TG, if the minimal dose was 3-4 g/day (220). The lowered plasma TG has been attributed to increased lipolysis and decreased hepatic lipogenesis (221). From meta-analysis the average reduction in TG is approximately 15% with a 1.6 mg/dL increase in HDL-C (222). Not all studies have found positive benefits as the effect of ω-3 PUFAs on improving the diabetic profile has been controversial (223-225). As well, potentially adverse increases in LDL-C have been reported in conjunction with a potent concentrated ω-3 prescription preparation (219). In these trials the positive effects on TG and HDL-C must be tempered with the observation that LDL-C increased by 45% and apoB kinetics revealed that the percent conversion of VLDL to LDL increased without an increase in LDL apoB levels (219).

From the intervention studies and epidemiological data it is clear DHA and EPA have been ascribed a wide array of potential benefits. The cardioprotective benefits can be categorized as anti-inflammatory, inflammation resolving, TG lowering, regulation of transcription factors/gene expression, membrane fluidity, anti-arrhythmic and antithrombotic (207;216;221). In terms of the present study, the TG lowering and transcription factor regulation are of most interest, although the development of atherosclerosis and metabolic syndrome, as pointed out previously, are also diseases of inflammation. Inflammation of the endothelial cells in the artery walls is a key component to atherosclerosis (12;13) while inflammation of the adipocyte has been suggested as the underlying cause of obesity (226;227) and subsequent development of insulin resistance and metabolic syndrome. ω-3 PUFAs can respond to inflammation either directly through transcription factors (NFκB and PPARs) or indirectly via the production of eicosanoids or

through the activation of inflammatory resolving lipid mediators (207). Both of these mechanisms can diminish the inflammatory response and prevent chronic inflammation. There have been mixed results in the role of ω-3 PUFAs in reducing the concentration of circulating cytokines which are thought to be related to the differences in study design, specifically type of diet, amount and duration of ω-3 PUFA supplementation and the initial health status of the subjects (207).

Adiponectin, an adipocytokine thought to indirectly or directly alter the risk of cardiovascular disease is also affected by DHA and EPA (228). In sucrose fed rats the addition of fish oil for 2 months decreased plasma free fatty acids and TG levels and increased plasma adiponectin without modulating gene expression. The resulting improvement in dyslipidemia was concomitant with improvement in whole body insulin resistance (229). In C57Bl/6J mice fed a high fat diet (35% wt/wt) for 5 weeks with 15% of the fat exchanged for EPA/DHA, significant reductions in plasma TG, NEFA and insulin were reported (230). In this study, caloric restriction only significantly reduced plasma glucose and TG. EPA/DHA supplementation was also able to up-regulate *Adipoq* mRNA in isolated adipocytes which produced a significant increase in circulating adiponectin. The up-regulation of *Adipoq* may be a result of EPA/DHA binding to PPARγ (231). The same up-regulation of adiponectin mRNA did not accompany a significant increase in plasma adiponectin in genetically (*ob/ob*) or high fat diet induced obese mice given dietary EPA for 2 or 4 weeks (232). An ω–3 PUFA diet also increased plasma adiponectin in *ob/ob* mice after 5 weeks of feeding (233)**.** Many of the results obtained in murine models of obesity have not been replicated in human studies. Itoh et al (232) were able to translate their findings in genetically and high fat diet induced obese mouse

models to humans. Obese subjects were given EPA (1.8g/day) for 3 months after which there was a significant increase in plasma adiponectin as compared to the control group. This improvement in adiponectin was independent of adiposity changes.

Alterations in plasma TG are probably one of the most consistent findings in both animal and human studies of EPA/DHA supplementation. As previously discussed, both EPA and ω-3 PUFA supplementation can decrease plasma TG in rodent models of obesity in as little as 4 weeks (229;230;232;233). In hypertriglyceridemic men, 3g /day of DHA over 45 days resulted in a significant reduction (25%) in fasting and post-prandial plasma TG. This improvement in lipid profile was accompanied by a reduction in small dense LDL particles, a reduction in the total number of LDL particles as well as a 13% decrease in plasma concentration of apolipoprotein CIII (234). This reduction in apolipoprotein CIII would increase the activity of lipoprotein lipase increasing TG clearance from the plasma. Although not specifically investigated in this study, it was shown previously that PPARα associated changes in LPL can occur as a result of down-regulation apoCIII expression either directly (235) or through its inhibitory affect on NFκB which up-regulates apoCIII (236). Thus, DHA supplementation could result in an increase in lipoprotein lipase and ultimately increase TG clearance from the plasma. This may be one of the mechanisms by which ω-3 PUFA reduce plasma TG levels.

The second mechanism that could explain how ω-3 PUFA lowers plasma TG is through modulation of VLDL assembly and secretion secondary to a decrease in TG synthesis. An adequate lipid supply is mandatory for the formation of a mature VLDL particle. Anything that disrupts the lipid supply would subsequently alter the final particle assembly. Both EPA and DHA have been shown to be potent inhibitors of SREBP-1c

(237). SREBP-1c suppression results from the PUFA interaction with the lipid binding domain (LBD) of LXR, inhibiting the binding of LXR/RXR complex to the LXR responsive elements (LXREs) in the SREBP-1c promoter. EPA was found to be a stronger inhibitor than either DHA or LA, and all were substantially stronger inhibitors than OA; SFA had no effect (237). Suppression of SREBP-1c would decrease the expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) with an overall reduction in available fatty acids to be incorporated into TG for VLDL. It has been suggested that simultaneous to the inhibition of SREBP-1c, ω-3 PUFA could be activating β-oxidation through PPARα-induced gene expression (237) to efficiently and reciprocally regulate the two pathways and prevent futile cycling. Another possible TG lowering mechanism is through decreased activity of the TG biosynthetic enzymes phosphatidic acid phosphohydrolase (PAP) or acyl-CoA:diacylglycerol acyltransferase (DGAT) that catalyze the conversion of phosphatidate to diacylglycerol (DG) and DG to TG respectively (167;207). Contradictory results have been obtained when trying to use EPA and DHA to inhibit these enzyme activities. Studies in rat liver microsomes showed that EPA and DHA could inhibit both PAP and DGAT activity, while other studies have shown no effect on enzyme activity (221). An important caveat to these studies is that most used supraphysiological concentrations of the ω-3 fatty acid or employed extremely short experimental periods (1 dose, 7 hr). Thus, given the current data it appears that a direct inhibition of enzyme activity is unlikely to play a role in reducing VLDL-TG secretion.

1.7. Animal Models of Metabolic Dysfunction

The importance of lipid and lipoprotein metabolism in the development of atherosclerosis has been demonstrated by the observation in rodents that perturbations to normal metabolism are necessary for the development of atherosclerotic lesions (238). This is true in the C57BL/6 mouse on a high fat high cholesterol diet, in gene targeted mice, including LDL receptor deficient and apoE deficient, and in transgenic mice, such as those expressing a receptor-defective form of human apoE, apoE*3-Leiden (239). Less commonly, this is also true in the hamster fed a high fat high cholesterol diet (240). All of these perturbations cause marked changes in lipoprotein profile and the development of vascular disease. We presume that the same is true for the human disease and in large part this is supported by population studies. As a result, plasma lipid and lipoprotein levels are often the major markers for atherosclerotic risk. Nevertheless, the use of the appropriate model to test the anti-atherosclerotic effects of pharmacologic and dietary agents is constantly in debate (241;242) and this is certainly true for studies of bioactive fatty acids (243;244). Part of this uncertainty may result from the fact that no clear animal model has yet emerged in which the beneficial effects of bioactive fatty acids have been established unequivocally. Furthermore, there is no standard methodology which has been employed universally for the assessment of atherosclerotic lesions. Furthermore, a consensus is building that it is lesion composition, rather than size, that determines the acute complications of atherosclerosis in humans. Unfortunately, analysis of lesion composition is not standardized or commonly carried out in rodent studies of atherosclerosis. As well, mechanisms of lesion initiation and early progression are the objectives of most interventions rather than modulating the advanced disease state that is most often treated in humans. These discrepancies may play a major role in the difficulties of translating

findings from rodent studies to human therapies (242). Most animal studies of the effect of bioactive fatty acids on atherosclerosis have, by necessity, used high fat, cholesterol-containing atherogenic diets to induce lesions in the rodent models. However, as described below, the levels of dietary fat, cholesterol and bioactive fatty acid have varied markedly between studies. In addition, the base diet, chow or semi-purified, has also varied and this can lead to considerable difficulty in the interpretation and comparison of results (245).

1.7.1. Assessment of Atherosclerosis

Two common methods of evaluating the severity of atherosclerotic lesion development are measurements of the cross-sectional area at the aortic root and measurement of the total plaque in the aorta using an *en face* preparation. It has been established that in the mouse models of atherosclerosis, lesions develop initially and most extensively in the aortic root (246;247). Evidence from Tangirala (248) suggests that the severity and extent of atherosclerosis can be evaluated by either measurement. However, this may not be the case in other rodents. Even though each method individually provides evidence of vascular disease, a combination of the two measurements may be more informative at the early stages in some models. Since the aortic origin is a predominant site of lesion development, it may be a more appropriate site to assess the extent of lesions when there is less severe hyperlipidemia, whereas the *en face* preparation may be more appropriate for regression and other studies where extensive atherosclerosis and advanced lesions are observed.

1.7.2. The Hamster as a Model of Metabolic Dysfunction

The hamster has proven to be a useful model for studies of dyslipidemia, in part due to the similarity of the lipoprotein profile to that of humans when fed a high fat diet containing cholesterol (249). The Syrian Golden hamster and the F_1B hamster, an inbred strain which may be more susceptible to atherosclerosis, have been used for studies of bioactive fatty acid metabolism. Unlike other rodents, when challenged with a high fat high cholesterol diet (western diet), the hamster carries the additional plasma cholesterol in the very low density lipoprotein (VLDL), rather than the high density lipoprotein (HDL) (240;249). In contrast to rabbit studies, which have uniformly used semi-purified diets, the majority of hamster studies have used CLA supplementation of a chow-based diet, as it has been reported that atherogenic changes in the hamster are greater on chow-based than on semi-purified diets (250). Even on chow-based diets, however, the lesions in the hamster are generally much less severe than other models, and most often described as early fatty streak lesions, representative of the early stages of atherogenesis.

1.7.2.1. CLA and Atherosclerosis in the Hamster

Early studies using mixed isomer CLA in the hamster showed that atherosclerotic lesions that developed in animals fed an atherogenic high fat diet with cholesterol (Table 2) were not reduced significantly by mixed isomer CLA, when compared to LA (251). There was, however, a decrease in lesion development with CLA compared to LA when the fat content of the diet and the CLA were both increased (252). Each isomer has been shown to have distinct biological activities and this has been explored in the hamster as well. When supplemented to a chow-based high fat diet with cholesterol, both the *c*-9, *t*-11 and the *t*-10, *c*-12 CLA isomers (1%, w/w) showed a

tendency to reduce the number of atherosclerotic lesions, but these changes were not statistically significant (253). Cholesterol accumulation in the aorta was used to assess atherosclerotic lesion development in the F_1B strain (254). When the CLA isomers were supplemented at 0.5% (w/w), neither *c*-9, *t*-11 CLA nor *t*-10, *c*-12 CLA significantly reduced cholesterol accumulation when compared to the LA supplement. These two studies suggest that if CLA has an effect on atherosclerosis in the hamster, the effect is small.

On the other hand, Valeille (255) reported that in the Syrian Golden hamster the *c*-9, *t*-11 CLA isomer reduced aortic lipid accumulation by 30-35%, more effectively than fish oil, when supplemented to a chow-based atherogenic diet. These changes were associated with beneficial effects on the expression of a number of genes in the aorta, including reduced inflammatory markers and increased activation of the PPARα/LXRα signalling cascade. Subsequently, the authors reported that aortic lipids were reduced when the *c*-9, *t*-11 CLA isomer was provided in combination with a diet low in overall and saturated fat (cheese oil) compared to cheese oil alone (256). These studies reinforce the suggestion that CLA supplementation may be more atheroprotective when used in combination with an overall reduction in dietary fat and cholesterol. More recently, when Syrian Golden hamsters were provided a semi-purified diet supplemented with 1% *c*-9, *t*-11 or *t*-10, *c*-12 CLA isomer, no fatty streaks were observed (257).

The reduction in lesion development or reduced aortic cholesterol accumulation in the hamster has not been clearly associated with a concomitant reduction in plasma TG or cholesterol levels (Table 2). In most studies of the Syrian Golden hamster there appears to be no beneficial effect of CLA supplementation with individual isomers to a chow-based diet. Plasma cholesterol levels showed no significant change in four studies

(253-256). However, when *c*-9, *t*-11 CLA was supplemented to a semi-purified diet, significant improvements in plasma cholesterol were noted in comparison to oleic acid or mixed isomer CLA supplemented diet (257). In this study, the *t*-10, *c*-12 CLA did not have a significant effect on plasma cholesterol. The lack of effect of the *t*-10, *c*-12 isomer on plasma lipids was also observed by Navarro (258).

Therefore, a consensus regarding the effect of CLA supplementation on atherosclerosis or on plasma lipid risk markers remains elusive. The picture has not been clarified by use of individual isomers or by use of semi-purified diets. Although there is some indication that CLA in conjunction with a lower fat diet may reduce lesions in the hamster, the roles of dietary fat and cholesterol content, and potential interactions with CLA, on hamster atherosclerosis has not been explored systematically. These studies deserve some attention in order to fully explore the potential of the hamster as a model.

Table 2. Summary of CLA and atherosclerosis in the hamster.

	Hamster Strain Duration	Diet	Control Group	Plasma Lipids	Atherosclerosis
Nicolosi *et al*, 1997	F_1B 11 weeks	Chow-based - 10% coconut oil, 1% safflower oil, 0.12% cholesterol 0.025%, 0.05% or 0.5% mixed isomer CLA	0.5% LA	TC ↓ TG ↔ nonHDL-C ↓	Lesion area ↔
Wilson *et al*, 2000	F_1B 12 weeks	Chow-based- 20% coconut oil, 2% safflower oil,0.12% cholesterol 1% mixed isomer CLA	1% LA	TC ↔ TG ↑ nonHDL-C ↔	Lesion area ↓
itchell *et al*, 2005	Syrian Golden 12 weeks	Chow-based - 20% coconut oil, 2% safflower oil, 0.12% cholesterol 1% CLA pure isomer	1% LA	TC ↔ TG ↔ HDL-C ↑ (*t*-10, *c*-12)	Lesion area ↔
Valeille *et al*, 2005	Syrian Golden 12 weeks	Chow-based- 20% butter oil, 0.12% cholesterol 1% CLA (90% c-9, t-11) or fish oil	20% butter oil	TC ↔ TG ↑	Lesion area not assessed
Valeille *et al*, 2006	Syrian Golden 12 weeks	Chow-based - 20% cheese oil 0.12% cholesterol 0.9% *c*-9, *t*-11 CLA	20% cheese oil	TC ↔ TG ↓ HDL-C ↔ nonHDL:HDL-C ↔	Lesion area not assessed
Wilson *et al*, 2006	F_1B 12 weeks	Chow-based - 10% coconut oil 0.1% cholesterol 0.5% individual isomer	0.5% LA	TC ↔ TG ↓ (*c*-9, *t*-11) HDL-C ↔	Lesion area not assessed
Navarro *et al*, 2007	Syrian Golden 6 weeks	Semi-purified - 10% palm oil 0.1% cholesterol 0.5% or 1% *t*-10, *c*-12 CLA	0.5% LA	TC ↔ TG N/A VLDL-C ↓	Lesion area not assessed
LeDoux *et al*, 2007	Syrian Golden 12 weeks	Semi-purified -10% coconut oil, 1% safflower oil 0.12% cholesterol 1% individual or mixed isomers	1% OA	TC ↓ (*c*-9, *t*-11) TG ↑ (mix) HDL-C ↓, LDL-C↓ (*c*-9, *t*-11)	Lesions not found

1.7.3. The Mouse as a Model of Metabolic Dysfunction

Although the lipid metabolism is more similar to human metabolism in the hamster, the lack of more advanced atherosclerotic plaque development in a reasonable length of time has led to the use of genetically modified mice. Mice are normally resistant to the development of atherosclerotic lesions, even when fed a high fat diet, because excess cholesterol is not carried in the pro-atherogenic low density lipoprotein but rather in the anti-atherogenic high density lipoprotein. However, several gene manipulations can cause the development of atherosclerosis in the mouse, especially when coupled with dietary manipulations [reviewed in (259)]. Since lesion development in several mouse models appears to be similar to atherogenesis in humans, the use of genetically modified mice has allowed for the study of the mechanisms of action of various pharmaceutical and nutraceutical products (242). Four strains of mice have been used to study the effect of CLA on atherogenesis: C57BL/6, the apoE deficient mouse, APOE3*Leiden transgenic mouse and, most recently, the $ApoE^{-/-}/LDLr^{-/-}$ double knockout mouse.

The C57BL/6 mouse is an inbred mouse model of diet-induced atherosclerosis, originally characterized by Paigen and colleagues (247;260). Although this model has been extremely useful for the further development of mouse models of atherogenesis, the distribution and progression of atherosclerotic lesions in this model is substantially different from the human disease (238). In addition, the levels of dietary cholesterol required to produce lesions are much higher than found in the human diet.

The $ApoE^{-/-}$ mouse has elevated levels of atherogenic lipoproteins due to a reduced ability to clear these particles from the plasma. These mice develop spontaneous atherosclerotic lesions even on a low fat diet without cholesterol (261;262). The progression of the lesion from fatty streak to complex lesion has been shown to resemble

that of human lesions [reviewed in (238;263)] and is exacerbated by the addition of saturated fats and cholesterol. This trait has made the ApoE$^{-/-}$ mouse a valuable model for investigating how multiple factors, such as dietary fat content, can influence the progression of arterial lesions. The ApoE$^{-/-}$ mouse has high plasma cholesterol levels, which are increased further by high fat feeding. However, the extreme changes in lipoprotein metabolism also make the model somewhat less relevant to human disease, and this is not the only drawback to the ApoE$^{-/-}$ model. The apoE protein may play a substantial role in modulating the inflammatory response that is important in the progression and resolution of atherosclerotic lesions, and the absence of this protein may alter atherosclerotic plaque development independent of the abnormalities in lipoprotein metabolism (263). This issue may be clarified experimentally using the apoE*Leiden mouse, which maintains some functions of apoE with a less pronounced lipoprotein abnormality. Most studies have not reported obesity in the ApoE$^{-/-}$ or insulin resistance even on a high fat diet (264;265) two major components of the metabolic syndrome. Although not completely understood this protection against obesity and insulin resistance may be related to apoE having a role in adipocyte TG storage (266). It has been suggested that under the right dietary conditions, the ApoE$^{-/-}$ mouse can display traits of metabolic syndrome. King et al. (267) reported that when ApoE$^{-/-}$ mice were placed on a 60% (kcal) fat diet for 17 weeks they had a marked increase in body weight and atherosclerotic lesion development compared to low fat (10% kcal) fed animals. The high fat fed animals were also glucose intolerant and had increased serum amyloid A concentrations indicating systemic inflammation making all factors in the metabolic syndrome therefore making this animal model a useful tool for delineating mechanisms connecting hyperlipidemia and the metabolic syndrome (267).

The LDL receptor knockout mouse ($LDLr^{-/-}$), mimics, when fed a high fat high cholesterol diet, the elevated plasma cholesterol levels observed in human familial hypercholesterolemia. Unlike the $ApoE^{-/-}$ mouse, which carries excess cholesterol primarily in the VLDL and remnants, the $LDLr^{-/-}$ mouse carries excess cholesterol in the LDL fraction, which is more similar to humans. Lesions in the $LDLr^{-/-}$ mouse also progress from fatty streaks to complex lesions over time. Although both the $LDLr^{-/-}$ and the $ApoE^{-/-}$ mouse models can be used to study atherogenesis, the results obtained from each model may not always be comparable because of differences in pathogenic mechanisms. It appears that these two murine models of atherogenesis respond differently to high dietary cholesterol, resulting in defect-specific lipid and lipoprotein disturbances.

1.7.3.1. CLA and Atherosclerosis in the Mouse

Only two reports have appeared examining atherosclerosis in the C57BL/6 mouse. In the first study, Munday and colleagues (268) observed that there was an increase in the size of aortic root atherosclerosis when CLA was added to a semi-pure atherogenic diet, despite improvements in plasma lipid risk factors (Table 3). In the second study, Lee and colleagues (269) used a chow diet supplemented with 0.6% (w/w) mixed isomer CLA, in which the CLA was enzymatically incorporated into olive oil. Supplementation with this oil showed improvements in plasma atherosclerosis risk factors and reduced aortic fatty streak lesions. These markedly disparate observations were despite the use of diets with a similar high fat and cholesterol content. The reason for the marked discrepancy between the two studies may be related to the differences in base diet. Although fat and cholesterol content were similar, semi-purified diets and chow

based diets may not be comparable, especially since the sourcing of components of chow diets may be subject to tremendous variability (245).

The most promising report of CLA suppression, and indeed regression, of atherosclerosis is that of Toomey and colleagues (270). These authors reported that CLA prevented the progression and induced regression of atherosclerotic lesions in the ApoE$^{-/-}$ mouse (Table 3). In this experiment, mixed isomer CLA markedly reduced the lesion area (5.5 ± 5.5%) compared to mice fed a saturated fat diet (37 ± 8.4%). The authors observed decreases in macrophage accumulation in lesion areas and down-regulation of proinflammatory genes, suggesting a role for CLA in modification of the immune response.

The CLA mixture used in this investigation, with 80% *c*-9, *t*-11 CLA, is different from the 50:50 mix used in a recent study in the same model (271). In the latter study, (Table 3) there was no significant improvement in atherosclerotic lesion area after 12 weeks on CLA supplement, assessed in either *en face* preparations or in cross sections of the aortic root. The 50:50 isomer mixture did not change plasma TC compared to the diet without supplementation or when supplemented with LA or *c*-9, *t*-11 CLA. However, supplementation with the *t*-10, *c*-12 CLA caused a significant increase in both plasma TC and TG. The explanation for the profound differences between the two studies is not clear, although dietary fat type, cholesterol content or isomer ratios in the CLA preparations may all play roles.

There is some evidence to suggest opposing effects of the two isomers on atherosclerosis in the ApoE$^{-/-}$ mouse. The individual CLA isomers have been supplemented to a semi-purified diet (272). The *c*-9, *t*-11 isomer significantly reduced the cross sectional lesion area of the aortic root, whereas the *t*-10, *c*-12 isomer significantly increased lesion area compared to control.

Table 3. CLA and atherosclerosis in the mouse.

Reference	Mouse Model Duration	Diet	Control Group	Plasma Lipids	Atherosclerosis
Munday *et al*, 1999	C57BL/6 15 weeks	Semi-purified -14.5% TG from corn, olive oil and milk fat, 0.5% cholate 1% cholesterol 0.25% or 0.5% mixed isomer CLA	0.5% LA	TC ⇔ TG ↓ HDL:TC ↑	Lesion area ↑
Lee *et al*, 2005	C57BL/6J 4 weeks	Chow-based - 7.5% cocoa butter, 7.5% lard, 0.5% cholate 1.25% cholesterol 5% lard, olive oil or CLA-structured lipid	5% olive oil	TC ↓ TG ⇔ HDL-C ↑ HDL:TC ↑	Lesions not found in the treatment group
Arbones-Mainar *et al*, 2006	ApoE$^{-/-}$ mouse 12 weeks	Semi-purified -12.3% fat 0.15% cholesterol 1% CLA isomer	1% LA	TC ↓ (*c*-9, *t*-11) ↑ (*t*-10, *c*-12) TG ↑ (*t*-10, *c*-12) HDL-C ↑ (*t*-10, *c*-12)	Lesion area ↓ with *c*-9, *t*-11 Lesion area ↑ with *t*-10, *c*-12
Nestel *et al*, 2006	Insulin-deficient apoE$^{-/-}$ mouse 20 weeks	Chow-based - 4.5% fat No added cholesterol 0.9 % synthetic *c*-9, *t*-11 CLA	No CLA	TC ⇔ TG ↓ HDL-C ↑	Lesion area ⇔
Toomey *et al*, 2006	ApoE$^{-/-}$ mouse 16 weeks	Chow-based -15.8% fat 1% cholesterol 1% saturated fat (SF) or 1% CLA mix (80:20, *c*-9, *t*-11:*t*-10, *c*-12)	1% SF	TC ⇔ TG ↑	Lesion area ↓
Franczyk-Zarow *et al*, 2007	ApoE/LDLr $^{-/-}$ 8 weeks	Semi-purified -No fat 0.1% CLA supplemented eggs or 0.1% CLA enriched eggs	CLA-free eggs	TC ⇔ TG ⇔	Lesion area ⇔
Cooper *et al*, 2008	ApoE$^{-/-}$ mouse 12 weeks	Semi-purified - 16% fat 1.25% cholesterol 0.5% CLA isomers or mix	0.5% LA	TC ↑ (*t*-10, *c*-12) TG ↑ (*t*-10, *c*-12)	Lesion area ⇔

En face examination of the vessel revealed that the *t*-10, *c*-12 CLA supplementation increased lesion area in specific regions of the vessel and suggested that the *t*-10, *c*-12 isomer induced a pro-oxidative state. Since supplementation may be necessary to achieve the increases in CLA intake that may be required for biological effectiveness, further exploration of pure and enriched *c*-9, *t*-11 CLA preparations is warranted.

Study of apoE deficient mice with additional physiologic abnormalities has also begun. When $ApoE^{-/-}$ were rendered diabetic by streptozotocin injection, the *c*-9, *t*-11 CLA isomer (0.9%, w/w) supplementation of a low fat chow diet did not significantly affect the aortic arch lesion area when compared to the animals fed a low fat chow diet without CLA (273). Although there was no improvement in aortic lesions, there was significant reduction in plasma TG levels with the CLA diet in the diabetic animals, reducing the mean plasma TG levels similar to those found in the non-diabetic animals. CLA supplementation had no effect on the plasma cholesterol levels in either the diabetic or non-diabetic animals.

1.8. Health Significance

Although the mortality rate from cardiovascular disease has declined by 15% over the past twenty-five years (StatsCan 2004) the economic toll from treatment and disability of survivors is still an issue for an overburdened health care system. Developing non-medical interventions to prevent or reduce this burden are paramount. One of the main modifiable risk factors for the development of heart disease is the amount and quantity of dietary fat consumed. It is becoming clear that the type of fat in the diet is more closely associated with benefits than the reduction in overall consumption. Therefore diet modification may alter liver lipid metabolism resulting in decreased atherosclerotic risk.

Functional foods and nutraceuticals have the potential to become the treatment of the future if we can understand the benefits and risks of components such as fatty acids. Because functional foods are similar to conventional foods and are consumed as part of a regular diet, they provide the opportunity for introducing elements such as ω-3 fatty acids or conjugated linoleic acids into readily consumed foods to provide physiological benefits which may lead to the prevention of atherosclerosis. It is my intention to help to elucidate the benefits or risks of consuming these fats on liver lipid metabolism.

1.9. Hypothesis

I hypothesize that dietary supplementation with bioactive fatty acids will improve central obesity, hepatic lipid and lipoprotein metabolism, plasma lipoprotein profiles and insulin sensitivity thereby reducing the risk of developing metabolic syndrome. By reducing the risk factors for metabolic syndrome it may be possible to reduce the development of atherosclerosis in susceptible rodent models. Furthermore, physiological ER stress responses, induced by a high fat or high fructose diet may be further exacerbated (*t*-10, *c*-12 CLA) or resolved (*c*-9, *t*-11 CLA or DHA) by dietary supplementation with bioactive fatty acids leading to differential regulation of lipogenic genes.

CHAPTER TWO - MATERIALS AND METHODS

2.1. Materials

Bovine serum albumin (BSA; essentially fatty acid free), linoleic acid, oleic acid, triolein, cholesterol oleate and egg yolk phosphatidylcholine were purchased from Sigma Aldrich Co. (Oakville ON). Purified conjugated linoleic acid (CLA) isomers *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA (>95% verified by gas chromatography; Matreya, Inc., Pleasant Gap, PA) were used in cell culture experiments. All tissue culture materials, including Dulbecco's modified Eagles medium (DMEM), trypsin, fetal bovine serum (FBS) were obtained from Invitrogen Corp. (Burlington, ON). Methanol, hexane, chloroform, diethyl ether, acetic acid, sodium sulfate and Silica Gel G TLC plates were purchased from Fisher Scientific International, Inc. (Nepean, ON). All chemicals were of the highest purity available. Radiochemicals, [9,10-^{3}H] oleic, palmitic and myristic acid and [^{35}S] Promix, a mixture of [^{35}S] methionine and [^{35}S] cysteine, were purchased from Amersham Biotechnologies (Baie d'Urfe, QC).

2.2. *In Vivo* Methods

2.2.1. Animals

All procedures involving animals were approved by the Dalhousie University Committee on Laboratory Animals in accordance with the guidelines of the Canadian Council on Animal Care.

Outbred male Syrian Golden hamsters (Mesocricetus auratus, CRL:LVG(SYR), 80-120 g) were obtained from Charles River Laboratories (Montreal, QC) and housed in pairs on an alternating 12 h light/dark cycle.

Homozygous ApoE$^{-/-}$ or LDLr $^{-/-}$ male mice (8-10 weeks old), on a C57BL/6 background (Jackson Laboratories, Bar Harbour ME) were housed in groups of 4-5 animals on an alternating 12 h light/dark cycle.

2.2.2. Dietary Treatments

2.2.2.1. Mice: High Fat Diet Supplemented with CLA Isomers, DHA or EPA

Mice were assigned to five groups of 10-15 animals each matched for average weight and were fed, *ad libitum*, a semi-purified western diet (WD) containing 16% (w/w) fat and 0.1% (w/w) cholesterol (Bio-Serv AIN-93G, Frenchtown, NJ) for 11 weeks. Control animals received the western diet, while the diets of treatment groups were supplemented to 0.5% (w/w) with LA (Sigma-Aldrich, Oakville, ON), *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA or a 1:1 mixture of the two CLA isomers (Tonalin® FFA 80; 39% *c*-9, *t*-11 CLA/ 38% *t*-10, *c*-12 CLA). CLA was provided by Cognis Nutrition and Health and produced by Natural Lipids (Hovdebygda, Norway). Fatty acids were added to the base diet as an ethanol solution and the ethanol was then removed by lyophilization. All diets were stored as -20ºC until use, and were used within 2-3 weeks of preparation.

Fatty acid composition of each diet has been previously determined (Table 4) (274). Briefly, fatty acid composition was determined in total lipid extracts, prepared using a ratio of 18 parts 2:1 chloroform-methanol (v/v) to one part diet. Phases were separated by centrifugation following the addition of six parts 0.9% NaCl. Lipids were

Table 4. Fatty acid composition of experimental mouse diets
(%wt mean ± S.D.)

Fatty Acid	Diet Supplement				
	None	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
C16:0	22.5 ± 0.2	21.0 ± 0.8	21.1 ± 0.7	20.6 ± 2.7	20.5 ± 1.1
C18:0	30.6 ± 1.1	27.9 ± 1.5	25.6 ± 2.9	27.4 ± 3.4	26.1 ± 4.7
C18:1 (*n*-9)	35.8 ± 7.0	35.3 ± 7.4	37.4 ± 9.8	32.8 ± 6.4	36.7 ± 10.25
C18:2 (*n*-6)	5.9 ± 0.7	9.5 ± 2.5	6.2 ± 1.2	5.3 ± 0.6	6.0 ± 1.2
C18:3 (*n*-3)	0.2 ± 0.3	0.4 ± 0.4	0.2 ± 0.3	1.2 ± 1.6	0.5 ± 0.1
c-9, *t*-11 CLA	ND	ND	4.0 ± 1.5	ND	1.0 ± 0.2
t-10, *c*-12 CLA	ND	ND	ND	2.6 ± 0.9	1.0 ± 0.2
C20	2.2 ± 2.8	2.3 ± 3.0	2.1 ± 3.7	2.0 ± 3.5	3.9 ± 3.7
C20:1 (*n*-9)	1.4 ± 2.4	1.9 ± 2.4	1.8 ± 3.1	2.5 ± 2.4	2.7 ± 3.8

dried from the chloroform phase and hydrolyzed using 1 N KOH in ethanol to release free fatty acids, which were then extracted using diethyl ether/hexane. Methylation of the free fatty acids was carried out according to Yurawecz *et al* (275).

2.2.2.2. Hamster: High Fat Diet Supplemented with CLA Isomers, DHA or EPA

After 1-2 weeks of acclimation to the animal unit on chow diet, animals were separated into five groups. Hamsters were housed in individual cages and monitored for food intake and changes in body weight. The high-fat-high-cholesterol, (HFHC, Table 5) diet contained 20% (w/w) hydrogenated coconut oil, 2% (w/w) safflower oil and 0.12% cholesterol and was prepared by Research Diets, Inc. (New Brunswick, NJ) on a base of standard chow (Purina 5001). Linoleic acid, purified CLA isomer (Nu-Chek Prep, Elysian, MN, USA; >90% pure as assessed by gas chromatography), DHA or EPA was added to the experimental diet to 1% (w/w) as an ethanol solution and the ethanol was then removed by lyophilization. All diets were stored at -20°C until use and pellets were replenished twice weekly. Food and water were available *ad libitum* and the hamsters were weighed each week.

2.2.2.3. Hamster: Fructose Diet Supplemented with *c*-9, *t*-11 CLA, DHA or EPA

The base diet (160005 modified Dyets NRC Hamster diet, Table 6) contained 6% (w/w) corn oil, 22% (w/w) casein, 46% (w/w) corn starch and 16% Dyetrose and was prepared by Dyets Inc, (Bethlehem, PA). The base diet had a caloric density of 3642 kcal/Kg. The 60% fructose diet (161506 Casein Hamster diet) substituted 60% fructose for the dyetrose and cornstarch all other ingredients remained as per base diet giving the fructose diet a caloric density of 3655.4 kcal/Kg. Purified *c*-9, *t*-11 CLA isomer (Nu-Chek

Table 5. Fatty acid composition of HFHC hamster diets

Fatty Acid	Diet (mg/g)		
	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA
C8:0	12.4	8.7	11.5
C10:0	10.3	7.4	9.9
C12:0	83.4	62.2	82.2
C14:0	32.8	25.1	31.3
C16:0	25.4	17.3	20.4
C18:0	20.1	13.6	13.6
C18:1	10.7	7.0	7.0
C18:2 (LA)	27.8	14.2	15.9
c-9, *t*-11 CLA	ND	9.1	ND
t-10, *c*-12 CLA	ND	ND	6.8

Table 6. Composition of Base and 60% fructose hamster diets

Ingredient	Diet (g/Kg)	
	Base	60% Fructose
Casein	220.0	220.0
L-Arginine	1.0	1.0
L-Tryptophan	1.1	1.1
Fructose	0.0	600
Corn Starch	460.9	0
Dyetrose	160.0	0
Corn Oil	60.0	60
Cellulose Fiber	50.0	70.9
Salt Mix	35.0	35
Vitamin Mix	10.0	10
Choline Bitartrate	2.0	2

Prep, Elysian, MN, USA; >90% pure as assessed by gas chromatography), DHA or EPA was added to the experimental diet to 1% (w/w) as an ethanol solution and the ethanol was then removed by lyophilization. All diets were stored at -20°C until use and pellets were replenished twice weekly. Food and water were available *ad libitum* and the hamsters were weighed each week.

2.2.3. Quantification of Atherosclerosis

Mice were anaesthetized by inhalation of isoflurane. Once complete general anaesthesia was achieved, exsanguination by ventricular puncture was performed. The right atrium was then nicked and the vasculature was perfused with 10 mL PBS by injection into the puncture site at the apex of the left ventricle. The heart and aorta, from the aortic arch to the iliac bifurcation, were excised, and prepared for histological analysis as previously described (274). Briefly, aortas were placed in 5% formalin in PBS and stored at 4°C for approximately 1 week. Aortas were cleaned of surrounding fascia and fat deposits before their full length was bisected along the outer curvature of the arch, adjacent to the carotid arteries. Aortas were then pinned open *en face* and stained with 0.5% Sudan IV and images were collected using a PixeLink PL-A686, 6.6 mega-pixel microscope camera attached to a Leica MZ6 dissecting microscope. To control for bias, *en face* images were coded and randomized to "blind" each investigator to the dietary intake group and to prevent comparison between investigators. For each aorta, the percent plaque area (lesion area/total aorta area) was estimated using ImageJ software. The average of each of the three investigator's values was used to obtain a single value for each animal.

For aortic root cross-sections, the apex of the heart was removed and the upper portion of the heart was mounted in embedding medium (Tissue-Tek O.C.T., Sakura Finetech USA, Inc., Torrance, CA). Approximately 60 cross-sections of 10-14 μm were prepared from each aortic root using a MICROM HM500 0 cryostat (-20 to -25°C) and mounted on gelatin-coated microscope slides. The slides were dried at room temperature for 2-3 days, stained for lipid using Oil Red O and counterstained with hematoxylin. For each aortic root, digital images were captured and, as with the *en face* preparations, blinded to the investigators for quantification. The proportion of each cross-section occupied by lipophilic material was quantified and the mean of the three values for each section was determined.

2.2.4. Plasma Measurements

At the end of the feeding period anaesthesia was induced by inhalation of isoflurane. Exanguination by ventricular puncture was carried out and blood was collected into syringes containing 2 μL of 0.5 M EDTA solution, as anticoagulant. Plasma was isolated by centrifugation at 15,600 x g for 10 minutes at 4ºC to remove red blood cells and then refrigerated prior to density gradient ultracentrifugation (see below). For plasma lipid and protein analyses, aliquots of plasma were stored frozen -80ºC.

2.2.4.1. Density Gradient Ultracentrifugation

For plasma lipoprotein profile analysis, sucrose was dissolved in freshly isolated plasma (800 – 1000 μL) to a final concentration of 12.5% sucrose (w/v). The plasma was then diluted to 1.6 mL with 12.5% sucrose solution (in phosphate-buffered saline, PBS). A 4 mL polyallomer ultracentrifugation tube (Beckman-Coulter) was used to construct a

discontinuous sucrose gradient by underlayering 1.2 mL PBS with the 12.5% sucrose plasma sample and then underlayering the plasma sample with 670 μL of 25% sucrose and 670 μL of 47% sucrose solution (both in PBS). The plasma lipoproteins were separated by ultracentrifugation at 55,000 rpm for 20 h in a SW60Ti rotor. Upon completion, aliquots (200 μL for 20 fraction gradients) were collected from the top of the gradient. Cholesterol and triglyceride concentrations in plasma samples and gradient fractions were determined by enzymatic assays from Roche Diagnostic or Sigma-Aldrich, respectively, adapted to microtiter plate format. For plasma lipid analyses, 5-10 μL aliquots were assayed; for density gradient ultracentrifugation fractions, 10-25 μL aliquots were assayed. Lipoprotein (VLDL, LDL and HDL) cholesterol and triglyceride content was calculated as the area under the curve of the density gradient profile.

2.2.4.2. Plasma Lipids and Blood Glucose Concentrations

Cholesterol and triacylglycerol concentrations in plasma samples and gradient fractions were determined by enzymatic assays (Roche and Genzyme Diagnostics respectively) adapted for microtiter plate format. For cholesterol assays aliquots of 1.25 μL (plasma) and 2.5 μL (density fractions) were measured. For triacylglycerol analysis, aliquots of 5 μL (plasma) and 25 μL density fractions were measured. The cholesterol and triacylglycerol content of each class of lipoproteins were calculated as the sums of the fractions as defined by density; VLDL (1-4, ρ=1.02 -1.04 g/mL), LDL (5-10, ρ=1.04 - 1.07 g/mL), HDL (11-20, ρ=1.07-1.23 g/mL).

Blood glucose concentrations were measured immediately at collection with an Accu-chek® Advantage Meter (Roche Diagnostics, Quebec City, QB).

2.2.4.3. Measurement of Plasma Adiponectin and Insulin

Plasma adiponectin and insulin were measured by enzyme-linked immunosorbent assay kits (EZMADP-60K and EZRMI-13K, respectively, Millipore, Billerica, MA) according to manufacturer's instructions. Briefly, for adiponectin, diluted samples, standards and assay controls were incubated, in microtiter wells coated with monoclonal anti-adiponectin antibody, with a polyclonal biotinylated anti-adiponectin detection antibody. Following 2 h incubation at room temperature the wells were rinsed three times with Wash buffer and then incubated with streptavidin-conjugated horse radish peroxidase solution. Following 30 min incubation at room temperature, the unbound proteins were washed away and substrate (3,3',5,5'-tetramethylbenzidine) solution was added. The development of blue color was monitored at 370 nm (5-15 mins). Once the absorbance had reached 1.2-1.8 in the highest standard, stop solution (0.3 M HCl) was added to all wells and the absorbance was then read at 450 nm (measurement) and 590 nm (blank). Dose-response curves for standards were fit to a 4 or 5 parameter logistic equation and sample and control values were calculated by interpolation. A similar protocol was utilized for the insulin determination.

2.2.4.4. Measurement of Non-esterified Fatty Acids (NEFA)

Plasma NEFA were measured using an ACS-ACOD MEHA enzymatic assay formatted for microtiter plates. (WAKO Chemicals USA, Richmond, VA).

2.2.5. Measurement of Hepatic Lipids

Following exsanguination, the liver was excised, immediately weighed and frozen in liquid nitrogen. The tissue was stored at -80° C until analysis. Total lipid was extracted from a portion of each liver according to a method adapted from Folch *et al*. (276), using a ratio of 18 parts 2:1 chloroform-methanol (v/v) to one part sample. Phases were separated by centrifugation following the addition of 6 parts 0.9% NaCl. Total lipid in extract was determined by weight after drying under nitrogen. Internal standard, tricaparin, was added to aliquots of liver lipids and the lipid species were derivatized with bis(trimethylsilyl)triflouroacetamide (BSTFA) at room temperature for 1 h. Lipid classes in the sample were quantified using temperature-programmed gas-liquid chromatography on a Hewlett-Packard 6890 Capillary FID gas chromatograph fitted with a 30 m column (0.32 mm i.d.) coated with crosslinked 5% Ph Me silicone (0.25 μm film thickness; Hewlett-Packard HP-5; Palo alto, CA) attached to a 0.53 i.d retention gap (274).

2.2.5.1. Phospholipid Mass Assay

Phospholipid mass was determined utilizing an enzymatic method based on the addition of N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline to produce a blue pigment which is measured spectrophotometrically at 600 nm and 700 nm (Wako). Hepatic lipids samples (Section 2.2.3.6) were dissolved in chloroform to obtain 50 mg/mL with total lipid between 5-20 mg. A 100 μL lipid sample was dried under nitrogen and resuspended in an equal volume of 1% Triton X-100. The samples were then incubated in a shaking water bath for 15 min at 37 °C. Standards (Choline chloride, 0-300 mg/dL), quality control samples Precipath® and Precinorm® (Boehringer Mannheim,

Germany) and lipid samples were prepared according to manufacturer's instructions and the absorbance determined at 600 nm and 700 nm.

2.2.6. PCR Array Analysis

Frozen liver tissue was homogenized and total RNA was purified using RNeasy® Plus Mini kit (Qiagen, Valencia CA) according to the manufacturer's instructions. RNA concentration and purity were assessed in a Powerwave™ spectrophotometer (Biotek, Winooski, Vermont). First strand cDNA was synthesized from 2 μg total RNA using RT^2 First Strand Kit (SABiosciences, Frederick, MD) according to manufacturer's instructions. Customized PCR array plates were designed and produced by SABiosciences (SABiosciences, Frederick, MD) containing the primers for SREBP1c, SREBP2, SCD1, PPARα, PPARγ, PPARδ, LDLr, LXR, FoxO1 and housekeeping gene β-actin was used as an endogenous control. Primer amplification and specificity were previously verified by SABiosciences. The expression of the genes of interest was measured by real-time quantitative PCR with 2X SABiosciences RT^2 qPCR Master Mix containing SYBR green (SABiosciences, Frederick, MD) in a Mastercycler $RealPlex^2$ system (Eppendorf, Westbury NY). Relative quantitation of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

2.2.7. Protein Determination

Protein mass was quantified using the Bio-Rad Protein Assay based on the method of Lowry (277) and modified for use with the microtiter plate reader. Briefly, the assay is based on the reaction of protein with the alkaline copper tartrate solution and

Folin reagent. Color development is measured at 750 nm after 15 min incubation. BSA was utilized for the standard curve (0-15 mg/mL) and prepared in duplicate.

2.2.8. Trichloroacetic Acid Precipitation

In order to concentrate the protein in nuclear extracts, TCA precipitation was performed. From the Lowry assay the volume of each sample was calculated in order to obtain 75 μg of protein. To the original sample, TCA was added to 10% (w/v), mixed thoroughly and incubated on ice for 30 minutes. The sample was then subjected to centrifugation at 15,000 RPM for 10 min at 0°C to pellet the protein fraction. Two volumes of 10% TCA were added to one volume of the original sample and again subjected to centrifugation (15,000 RPM for 10 min at 4°C). Supernatant was removed and the pellet washed twice with 4 volumes of acetone, with sample centrifugation at 15,000 RPM for 10 min at room temperature between washes. Supernatant was aspirated and the pellet left to dry. The pellet was re-suspended in of β-mercaptoethanol-containing sample buffer for SDS-PAGE.

2.2.9. Immunoblot Analysis of Protein Mass

Frozen liver tissue (30-50 mg) was homogenized in 500 μL solubilizing buffer (10 mM Tris HCl pH 7.5, 150 mM NaCl. 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 1% NP-40, 10 mM Na pyrophosphate and protease inhibitor (Roche)) using a mechanical homogenizer. Homogenate was then subjected to centrifugation at 600 x g for 5 min at room temperature. Protein concentration of the supernatant was determined as previously described (Section 2.2.7). Equal amounts of protein from all samples were resolved by

SDS-PAGE and transferred to nitrocellulose membranes (Bio Rad). The membranes were incubated with the following antibodies: SREBP1c (Millipore), PPARγ (Santa Cruz), PPARα (Santa Cruz) and actin (Millipore). A goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated second antibody was used followed by ECL® (Roche) visualization and quantified using Scion imaging.

For nuclear fractionation, total lysates from fresh mouse livers were prepared as previously described (111). Briefly, the tissue was rinsed in ice-cold phosphate-buffered saline solution containing protease inhibitors (Roche). After mincing 20-50 mg of tissue in ice-cold homogenization buffer (2 M sucrose, 10 mM Hepes pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.5 mM DTT) the lysates were passed through a Dounce homogenizer until all tissue was disrupted. The homogenate was layered on 2 M sucrose cushion and subjected to centrifugation at 80,000 x g in a Beckman MLA 130 rotor for 35 min at 0°C. The nuclear pellets were then rinsed in buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and resuspended in 200 μL of buffer (20 mM Hepes pH7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and incubated on ice for 15 min. The lysates were cleared by centrifugation for 5 min at 10,000 rpm at 0°C and the supernatant was stored at -80°C. The protein content was determined as described previously.

2.2.10. Statistical Analysis

Analysis of variance (ANOVA) was used to determine differences between the WD treatment group and supplemented dietary treatment groups for phenotypic data, plasma measurements and gene expression data. Data are presented as mean ± S.E.M. and differences were considered significant at $p < 0.05$ as determined by the Games Howell

post hoc analysis (SPSS for Windows, Version 17.0). Games Howell *post hoc* analysis was selected as being the most powerful and accurate test when sample sizes are unequal and the homogeneity of variance assumption has been violated. Principal component analysis (278) was used to examine the relationship between variables thought to cluster in relationship to metabolic syndrome.

For repeated measures analysis a mixed design ANOVA was utilized (SPSS for Windows, Version 17.0). The F ratio for within-subject effects was calculated using the Greenhouse-Geiser method as the assumption of sphericity was violated. For main effects of diet supplementation Games Howell *post hoc* analysis was used as the homogeneity of variance assumption had been violated

2.2.11. Body Composition Analysis

At baseline, 2, 4 and 6 weeks anaesthesia was induced by inhalation of isoflurane and the percent body fat of each animal was determined using dual energy x-ray absorptiometry (DEXA) on a PIXIMUS™ densitometer and software (GE Lunar). A quality control measurement using a QC phantom, was performed daily prior to image acquisition. Percent body fat was determined from the predetermined region of interest (ROI) which excluded the head.

2.2.12. Intraperitoneal Glucose and Insulin Tolerance Tests

Baseline blood glucose was obtained after the hamsters were fasted overnight (14 h) as previously described (Section 2.2.4.2). Hamsters were then given an intraperitoneal injection of 1.5 g glucose/kg body wt, given as a 20% glucose solution in

0.9% NaCl. Glucose was measured from saphenous vein blood samples at subsequent intervals of 30, 60, 90 and 120 min. The same procedure was used for the insulin tolerance test with insulin (Humulin®), administered by intraperitoneal injection at a concentration of 1 U/kg body weight in a sterile 0.9% NaCl solution.

2.2.13. Culture of Primary Hepatocytes

Hepatocyte isolation was conducted under 4% isoflurane-induced general anaesthesia. Briefly, livers were isolated from the circulatory system by suturing the thoracic aorta, the abdominal aorta, and the portal artery. The portal vein was then severed, and livers were perfused via the inferior vena cava with 50 ml of liver perfusion medium containing 0.1% penicillin-streptomycin (Invitrogen) followed by 25 ml of liver digest medium (Invitrogen) at 42°C. Following perfusion, the liver was excised and minced in ice-cold hepatocyte wash medium. Digested liver tissue was filtered through a cell strainer (100 μm), and the released hepatocytes were collected by centrifugation (60 *g*, 3 min), washed 3 times in hepatocyte wash medium, re-pelleted by centrifugation, and then resuspended in attachment medium (Williams E containing 5% FBS, 1 μg/ml insulin, 0.1% penicillin-streptomycin). Cells were seeded on collagen coated Primaria cell culture plates (BD Biosciences, Bedford, MA) at a density of 1–1.5 million cells/35-mm dish and incubated for 3 h (37°C, 5% CO_2) to facilitate attachment. Viability of hepatocytes was measured by Trypan blue dye exclusion; hepatocytes were not used for experiments if the viability was less than 70%.

2.3. *In Vitro* Methods

2.3.1. Fatty Acid-BSA Emulsion

Fatty acids for cell culture supplementation were prepared in complex with BSA (279). With the exception of PA, the concentration of the emulsion was 10 mM sodium fatty acid, 1.45 mM BSA with a fatty acid: BSA molar ratio of 6.9:1. For OA, myristic acid (MA), LA, *c*-9, *t*-11 CLA or *t*-10, *c*-12 CLA, 100 μmol of free fatty acid was dissolved in 1 mL of absolute ethanol and neutralized with 50 μL of freshly prepared 5 N NaOH. The ethanol was then evaporated under nitrogen gas and the residual sodium salt was dissolved in 5 mL of 0.15 M NaCl at 60°C. Five millilitres of ice-cold 20% BSA was then added and the mixture was stirred at room temperature for 10 min and filtered through a 0.22 μm syringe filter. For PA, 50 μmol of free fatty acid was dissolved in 1 mL of absolute ethanol and the titration and emulsion preparation was performed as above, resulting in a 5 mM emulsion. Emulsions were stored in aliquots at -80°C until required.

2.3.2. Incorporation of [^{3}H]-Fatty Acid into Lipids

Radiolabelled fatty acid was prepared by drying 40 μL of [^{3}H] fatty acid (1 mCi/mL, 3.6 μM fatty acid in toluene) under nitrogen gas and resuspended in 100 μL of absolute ethanol. Two microlitres of 1 N NaOH was added and the fatty acid salt was dried under nitrogen. The residue was dissolved in a mixture of 20 μL DMEM and 20 μL of 20% BSA. For experiments with a single fatty acid, cells were treated with 0.4 mM test fatty acid (MA, PA or OA) containing [^{3}H] labelled tracer fatty acid at a final radiochemical concentration of 2 μCi/mL. After the indicated labelling periods, the

medium was collected and cells were harvested in 1 mL PBS. Lipids were extracted, separated by thin layer chromatography (TLC) and quantified as described below (section 2.3.3).

2.3.3. Lipid Extraction

Lipids were extracted from cell and medium samples by addition of 2 mL of methanol and 4 mL of chloroform. Samples were vortexed and centrifuged for 15 min in a Sorvall centrifuge at ~200 rpm to separate the phases. For cell and medium extracts the chloroform phase was passed through a 2 mL column of anhydrous sodium sulfate and then evaporated under nitrogen at 37°C. Samples were separated by thin layer chromatography (TLC) on heat-activated (50^{o}C, 2 h) silica gel TLC plates. The plates were developed according to established protocols (280) in two stages; first the phospholipid species were resolved in chloroform:methanol:acetic acid:H_2O (70:30:12:2) developed to 10 cm, and allowed to dry. Then the plate was developed in hexane:diethyl ether:acetic acid (70:30:1) to a height of 20 cm to separate the neutral lipid components. A lipid standard containing egg yolk phosphatidylcholine (EYPC, L-α-phosphatidylcholine), cholesterol oleate (CO) and triolein (TO) was used to establish the migration of the PC, CE and TG respectively. To visualize the lipid species, the TLC plates were stained with iodine vapour and the TG and PC were recovered from the plate into vials containing 0.5 mL of water. Radioactivity was quantified in 5 mL of Ecolite by liquid scintillation counting in a Beckman LS 6000 IC counter.

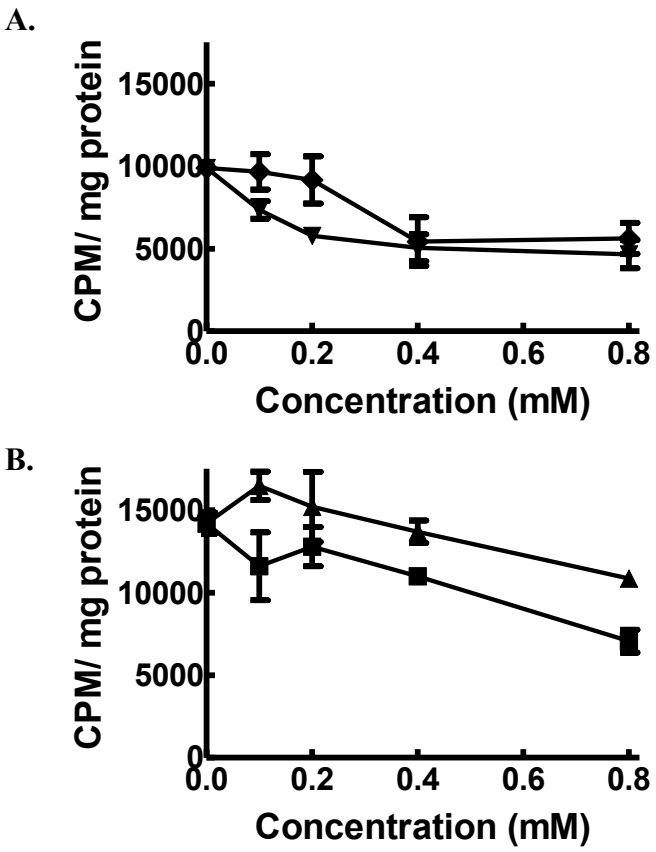


Figure 10. ApoB100 synthesis in response to varying concentrations of bioactive fatty acids.

Hamster hepatocytes were labelled for 1 h with medium containing [^{35}S] methionine/cysteine and 0.1 mM to 0.8 mM of the indicated fatty acid. ApoB100 was recovered from cell lysate by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity associated with apoB 100 was quantified by liquid scintillation counting. A.) ▼ *c*-9, *t*-11 CLA; ♦ *t*-10, *c*-12 CLA; B.) ▲ EPA; ■ DHA.

2.3.4. Analysis of ApoB Metabolism

2.3.4.1. Optimization of Fatty Acid Concentration

The effect of fatty acid concentration on apoB synthesis was evaluated by metabolic labelling in primary hamster hepatocytes. Cell monolayers were incubated in the absence of serum with 0.1 - 0.8 mM of the indicated fatty acid for 1 h. ApoB 100 was recovered from cell lysate by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity associated with apoB 100 was quantified by liquid scintillation counting. The resulting dose response curves for *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA and DHA and EPA (Figure 10) indicated that in the presence of serum, apoB 100 synthesis was reduced at fatty acid concentrations greater than 0.4 mM, and no differences were detected between a concentration of 0.2 mM and 0.4 mM.

2.3.4.2. Metabolic Labelling of Cellular ApoB 100

Hamster hepatocytes were incubated in methionine/cysteine-free DMEM for 1 h to deplete endogenous cysteine and methionine and then in methionine/cysteine-free DMEM containing 200 μCi/mL [^{35}S] methionine/cysteine for pulse-labellng. After the 1 h pulse, cell lysate was harvested by solubilization in 1% sodium dodecyl sulphate radioimmunoprecipitation assay (SDS-RIPA) [1% (w/v) SDS in 0% SDS-RIPA (50 M Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT) and 0.015% (w/v) phenylmethylsufonylfluoride (PMSF)] and heated to 75°C for 15 min and then placed on ice. After storing the samples at 4^{o}C overnight, the cell lysates were again heated to 75°C and diluted 10-fold with 0% SDS-RIPA containing 1 mM DTT and 0.015% (w/v) PMSF.

2.3.4.3. Pulse Chase Analysis

Cells were plated on 35 mm culture dishes (Falcon Primaria®) and cultured to 80% confluence. The medium was removed and replaced with 2 mL of methionine/cysteine-free DMEM containing 0.2 mM fatty acid and incubated for 1 h. After a 1 h pulse with DMEM containing 0.2 mM fatty acid and [^{35}S]ProMix® (200 μCi/mL), the labelling medium was removed and replaced with chase medium containing DMEM, fatty acid and unlabelled cysteine (0.6 mM) and methionine (2 mM). At each chase time, cells were harvested and the medium was collected into Eppendorf tubes containing 100 μL of 10 x 0.1% SDS-RIPA [0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl, 10 mM EDTA, 1% SDS (w/v), 10% (v/v) Triton X-100, 10 mM DTT, 0.15% PMSF]. ApoB was quantified following immunoprecipitation and SDS-PAGE as described above.

2.3.4.4. SDS Polyacrylamide Gel Electrophoresis

ApoB100 proteins were resolved on 5% polyacrylamide gels following established protocols (281). The 5X running buffer was comprised of 125 mM Tris base, 960 mM glycine and 0.5% SDS in 1 L water. The 2X sample buffer contained 20 mM Tris-HCL pH 6.8, 4% (w/v) SDS, 16% (v/v) glycerol, 0.004% (w/v) Bromophenol Blue and 10% (v/v) 2-mercaptoethanol.

CHAPTER THREE – STUDIES IN THE ApoE$^{-/-}$ MOUSE

3.1. ApoE$^{-/-}$ Mouse: A Model for Hyperlipidemia and Atherosclerosis

The ApoE deficient mouse (ApoE$^{-/-}$) is a model of hyperlipidemia (Section 1.7.3) rather than metabolic syndrome dyslipidemia, which is characterized by elevated TG levels and reduced HDL-C levels. However, the ApoE$^{-/-}$ mouse phenotype (elevated cholesterol, early lesion development) remains a highly useful background against which to investigate either detrimental or protective factors such as dietary fat. Previously we investigated the effect of CLA on apoE$^{-/-}$ mice fed a high-cholesterol diet (1.25% w/w) designed to rapidly induce severe atherosclerosis and dyslipidemia (274). From this study it was apparent that the severity of lesion development may have been so overwhelming that any potential beneficial reduction by CLA was masked. Therefore using a susceptible murine model (ApoE$^{-/-}$) we wished to investigate whether reducing the cholesterol content (0.1%) of a high fat diet would allow for the expression of any beneficial effects from supplemented bioactive fatty acids. By examining hepatic lipid metabolism, plasma lipoprotein profiles and atherosclerotic lesion development over an 11 week period it was hoped that possible mechanisms of action for CLA could be determined.

3.2. Western Diet with CLA Supplementation

3.2.1. Body and Liver Weights

Eight week old littermates were divided into diet supplement groups (n=10-13). Animals that were substantially different than the average were excluded from the study. Mice were housed 4-5 per cage and food consumption was measured every 3 days (Table 6) and averaged per animal. There were no significant differences in initial weights or

Table 7. Phenotypic characteristics of the ApoE$^{-/-}$ mouse.

ApoE$^{-/-}$ mice were fed a WD either supplemented or not with 0.5% bioactive fatty acid for 11 weeks. Food consumption was determined every 3 days. Liver weight was determined at sacrifice. †† $p < 0.001$ vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
Food Consumption (g/day/animal)	3.0 ± 0.2	2.8 ± 0.1	2.7 ± 0.1	3.0 ± 0.4	2.8 ± 0.3
Initial Weight (g)	23.9 ± 0.5	24.6 ± 0.4	23.1 ± 0.5	23.8 ± 0.8	22.1 ± 0.5
Weight Δ (g)	11.9 ± 0.8	9.3 ± 0.7	10.2 ± 0.9	4.2 ± 0.5 ††	9.0 ± 0.7
Liver wt (% body wt)	5.6 ± 0.3	4.9 ± 0.4	5.4 ± 0.3	12.1 ± 0.6 ††	8.2 ± 0.2 ††

food consumption regardless of diet supplement (Table 6). The *t*-10, *c*-12 CLA dietary group gained approximately half of the weight that all other groups gained ($p < 0.001$). Nevertheless, supplementation with either the *t*-10, *c*-12 CLA or CLA Mix resulted in significantly greater liver weight both on an absolute basis (data not shown) and as a percentage of body weight (Table 6; $p<0.001$) the size and appearance of the livers were strikingly different, as previously described (274).

Given the previously reported dramatic weight loss associated with the *t*-10, *c*-12 CLA isomer in other studies (193;282), it was somewhat surprising that none of the animals lost weight. The significantly enlarged livers in both the *t*-10, *c*-12 CLA and CLA Mix supplementation countered any potential weight loss.

3.2.2. Plasma Measurements

Diet supplementation did not substantially influence the plasma cholesterol concentrations in the $ApoE^{-/-}$ mice supplemented with LA, *c*-9, *t*-11 CLA or *t*-10, *c*-12 CLA compared to WD (Figure 11A). Interestingly, mice fed WD supplemented with CLA Mix had lower TC levels than either individual isomer, or this was significantly lower than *c*-9, *t*-11 CLA ($p<0.05$). Supplementation had a more pronounced effect on the plasma TG concentrations, *t*-10, *c*-12 CLA and CLA Mix significantly reduced TG by 25% and 50% respectively compared to WD ($p<0.05$ and $p<0.01$ respectively) (Figure 11B).

Examination of the lipoprotein profiles (Figure 12) revealed that the majority of plasma cholesterol is carried in the VLDL and LDL fractions. Consistent with a decrease

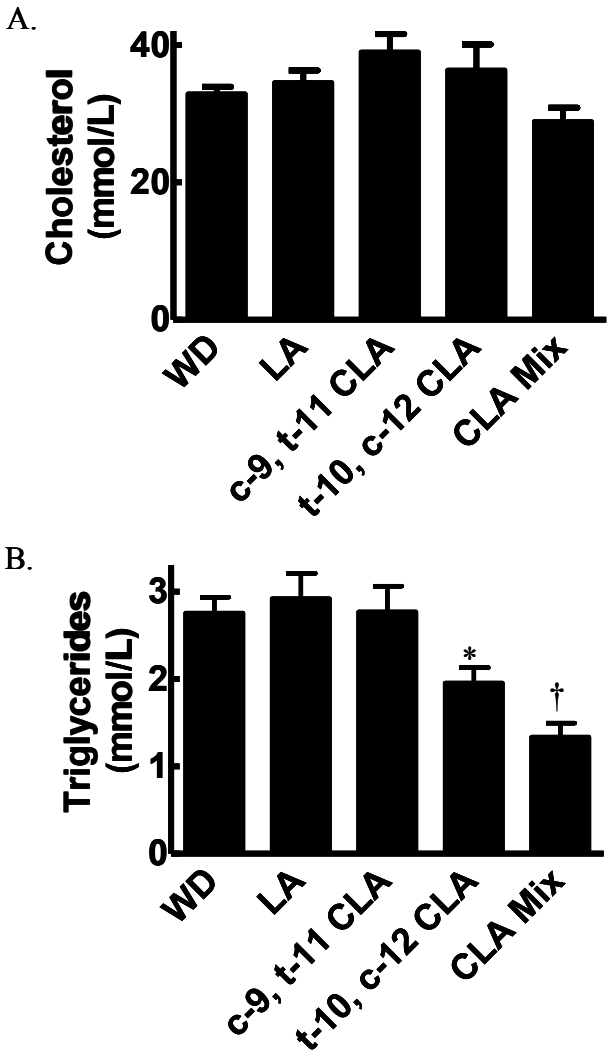


Figure 11. Plasma total cholesterol and triglycerides for ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ mice were fed a WD either supplemented or not with 0.5% bioactive fatty acid for 11 weeks. Blood was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and total cholesterol (A) and triglycerides (B) were measured (mean ± S.E.M., n=10-13). * $p<0.05$ vs. WD; † $p<0.01$ vs. WD.

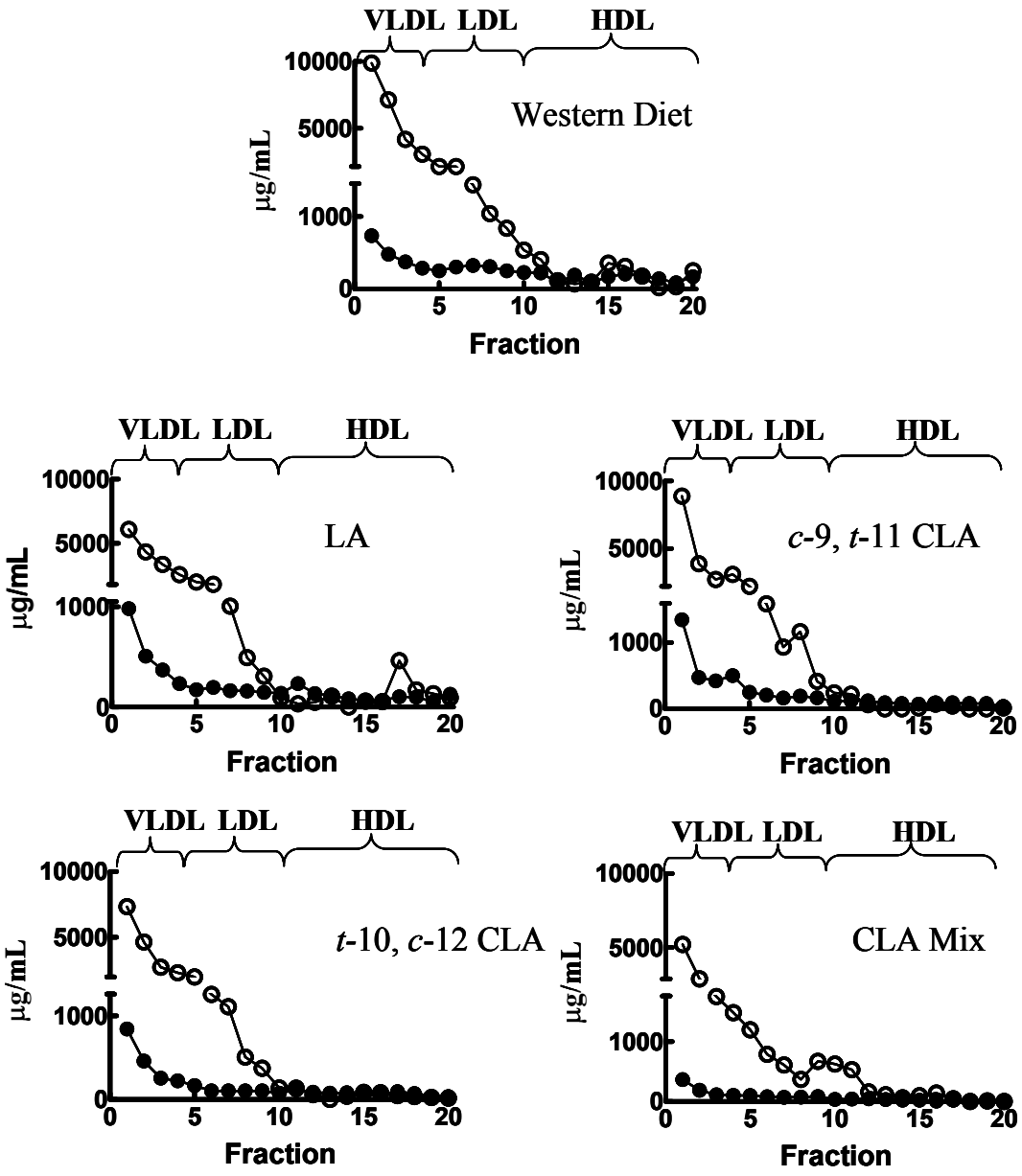


Figure 12. Plasma cholesterol and triglyceride lipoprotein profiles for ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ mice were fed a WD either supplemented or not with 0.5% bioactive fatty acid for 11 weeks. Blood was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and pooled plasma samples (n=4-5) were separated by density gradient ultracentrifugation. Cholesterol (○) and triglyceride (●) were measured in each fraction.

in plasma TC the profiles from the CLA Mix supplementation group showed a decrease in VLDL-C. TG profiles reveal that the majority of TG is carried in the VLDL fractions and minimal TG is distributed amongst all other fractions. Consistent with the reduction in plasma TG (Figure 11) VLDL-TG was substantially reduced in the CLA Mix supplemented animals (Figure 12).

Elevated blood glucose and plasma insulin are also indicators of the development of the metabolic syndrome. Using the guidelines from the National Institute of Health (NIH) Mouse Metabolic Phenotyping Center (MMPC), a 5 h fast was used to represent a more physiological state and reduce metabolic stress (283). In the ApoE$^{-/-}$ animals blood glucose was obtained at sacrifice and measured immediately using a hand held Glucometer. Fasting blood glucose was similar among all supplementation groups (Table 7). As in our previous study (274), where blood glucose was unaffected by CLA supplementation but insulin was markedly elevated by *t*-10, *c*-12 CLA, the insulin levels in this group in the current study were significantly elevated (16-fold) compared to WD, LA and *c*-9, *t*-11 CLA (Table 7). Hepatic insulin resistance is thought to be mediated by an increased flux of fatty acids to the liver. In the current study this is unlikely to be a contributing factor to the elevated insulin levels as NEFA levels for *t*-10, *c*-12 CLA supplemented group did not differ from the WD animals (Table 7). In contrast, the CLA Mix, which also had higher plasma insulin levels, had lower NEFA levels compared to WD (P=0.06)(Table 7). Nevertheless, neither NEFA nor insulin changes with the Mix diet was significant.

Adiponectin, normally an extremely abundant adipocytokine, was dramatically reduced with *t*-10, *c*-12 CLA supplementation in the ApoE$^{-/-}$ mice (Table 7). As adipose tissue decreases with weight loss in humans, adiponectin increases. Therefore it might be

Table 8. Plasma measurements for ApoE$^{-/-}$ mice.

After 11 weeks on a WD either supplemented or not with 0.5% bioactive fatty acid plasma from mice fasted for 5 h was obtained. Data are presented as mean ± S.E.M. (n=10-13). *p<0.05 vs. WD; †† p<0.001 vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
Glucose (mM)	16.3 ± 0.7	14.2 ± 0.9	14.5 ± 0.8	14.2 ± 0.6	16.8 ± 0.7
Insulin (ng/mL)	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	10.3 ± 3.0*	1.6 ± 0.8
Adiponectin (μg/mL)	11.2 ± 0.5	11.3 ± 0.8	9.3 ± 0.6	0.8 ± 0.1††	2.7 ± 0.2††
NEFA (mEq/L)	0.5 ± 0.03	0.5 ± 0.04	0.4 ± 0.02	0.6 ± 0.06	0.4 ± 0.09

expected to increase in the mouse as well. Since the *t*-10, *c*-12 CLA mice gained less weight this might suggest that an increase in adiponectin would be observed. The diminished adiponectin level may be indicative of a decrease in adipose tissue function (See Discussion Section 3.3).

3.2.3. Quantification of Atherosclerosis

Atherosclerotic lesions were assessed by quantifying lipid staining of the *en face* preparation consisting of the aorta from the aortic arch to the iliac bifurcation (Figure 13A) and in the lesions that developed in the aortic root (Figure 13B). After 11 weeks on a WD or a WD supplemented with LA or *c*-9, *t*-11 CLA ApoE$^{-/-}$ mice had oil red O staining lesions covering approximately 15% of their total aorta (Figure 13C). Supplementation with the *t*-10, *c*-12 CLA or CLA Mix significantly reduced lesion development ($p < 0.05$) compared to WD, with the CLA Mix having approximately 50% less oil red O staining. Lesions in the aortic root were similar between diet groups with LA and *c*-9, *t*-11 CLA supplementation and comparable to WD (Figure 13D). The CLA Mix group also had the least lesion area in the aortic root although this was not significantly different from the WD. The *en face* preparations were further divided into arch, thoracic and abdominal regions (Figure 13E) to examine whether any of the diets had localized effects on lesion development (272). The thoracic region had the least lesion involvement in all diet groups while the abdominal region contained the greatest area of involvement. However there were no significant differences between diet groups for any of the regions.

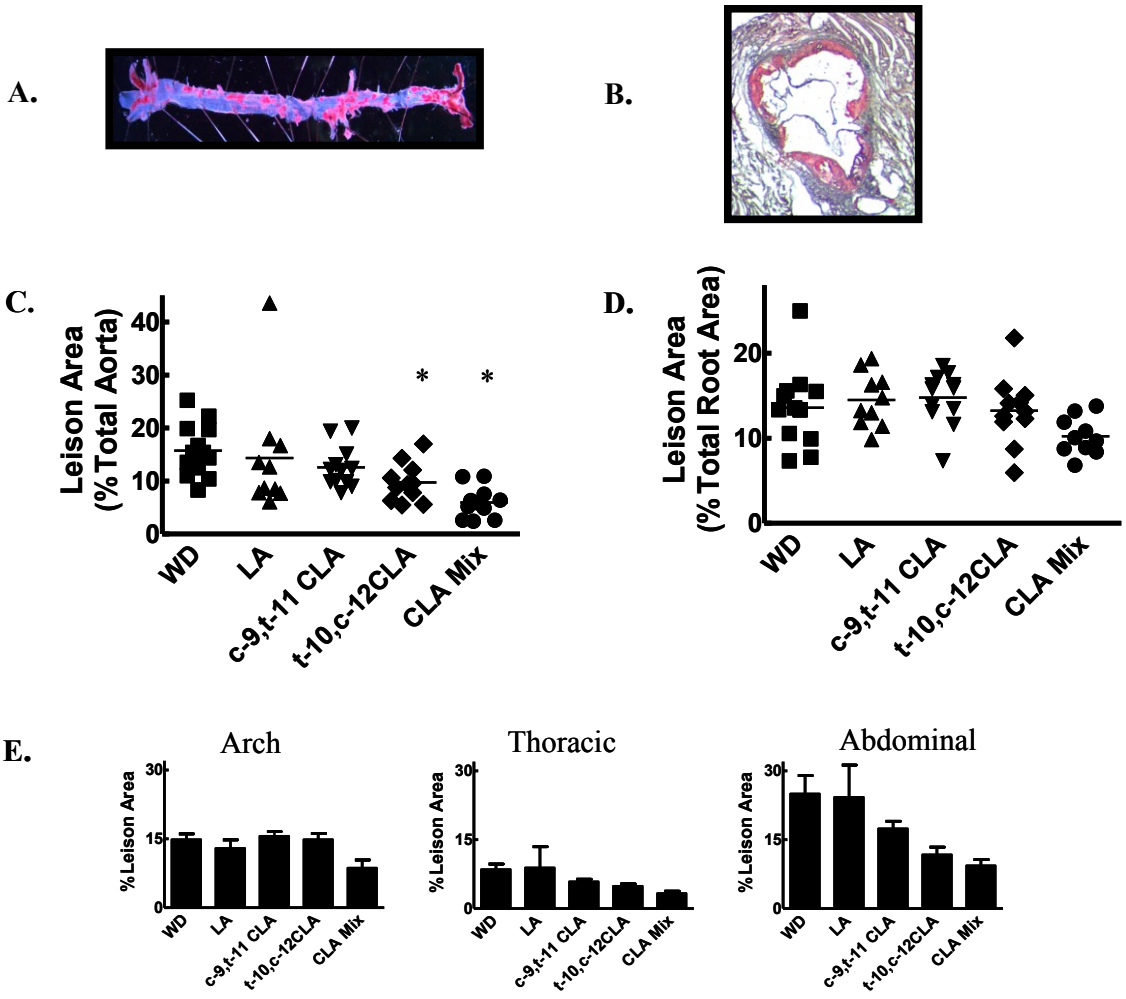


Figure 13. Atherosclerotic lesion measurements in aortic root cross-section and aortic *en face* preparations.

The images show a sample preparation for (A) *en face* and (B) aortic root from ApoE$^{-/-}$ mice. Mice were fed a WD with or without 0.5% supplementation for 11 weeks. (C) Quantification of lesion area relative to total aorta area determined as described in the Materials and Methods. (D) Quantification of cross-sectional area of the aortic root occupied by lesion relative to total cross-sectional area determined as described in the Materials and Methods. In (C) and (D), each data point represents the mean of two measurements by independent researchers. (E) Quantification of arch, thoracic and abdominal region of total aorta as previously described. Bars represent mean value for each group (n=10-13 animals per group). * $p < 0.05$ vs. WD.

3.2.4. Liver Lipid Mass

In our previous study (274) the increased liver weight in the *t*-10, *c*-12 CLA dietary group could be accounted for by a nearly three-fold increase in lipid mass, predominately TG. In the current study, although there was a significant increase in hepatic TG, this alone probably does not account for the 2-fold increase in liver weight. Hepatic TG with *t*-10, *c*-12 supplementation was approximately 30% higher than WD alone and 50% higher than with LA supplementation ($p<0.01$). LA, which has been shown previously to have anti-atherosclerotic and lipid lowering properties (284), significantly reduced hepatic TG compared to all other dietary groups except *c*-9, *t*-11 CLA supplementation (Table 8)($P=0.04$). As was observed in our previous study (274) CLA Mix supplementation significantly reduced hepatic CE compared to the WD dietary group (Table 8) ($p<0.01$). As well, in the current study, FC was significantly reduced by CLA Mix supplementation compared to WD (Table 8)($p<0.05$).

From these results it is clear that it is not simply an accumulation of hepatic lipids that resulted in the altered appearance of the livers with the *t*-10, *c*-12 CLA supplementation. Interestingly, the hepatic PL content was lower in the *t*-10, *c*-12 CLA group (Table 8) than any other diet group. We speculate that the hepatic lipid load may be disrupting the endoplasmic reticulum unfolded protein response (UPR) which is usually associated with an increase in PL to accommodate an expanded endoplasmic reticulum (123).

Table 9. Liver lipid mass for ApoE$^{-/-}$ mice.

ApoE$^{-/-}$ mice were fed a WD either supplemented or not with 0.5 % bioactive fatty acid for 11 weeks. Livers were obtained at sacrifice and lipids extracted in chloroform. TG, FC and CE were quantified by gas-liquid chromatography and PL by phospholipid C enzymatic assay. Data are presented as total mass per gram of liver weight (mean ± S.E.M.). (TG, triglycerides; FC, free cholesterol; CE cholesterol esters; PL phospholipids) * $p < 0.05$ vs. WD; † $p < 0.01$ vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
TG (mg/g liver)	194.0 ± 15.8	132.2 ± 11.7	148.8 ± 17.9	264.0 ± 14.8†	242.9 ± 12.7
FC (mg/g liver)	4.2 ± 0.2	4.7 ± 0.2	4.0 ± 0.2	3.8 ± 0.2	3.5 ± 0.1*
CE (mg/g liver)	22.6 ± 1.6	21.7 ± 1.8	17.2 ± 1.7	18.5 ± 2.2	9.5 ± 0.8†
PL (mg/g liver)	10.1 ± 0.4	10.9 ± 0.4	9.6 ± 0.4	8.3 ± 0.3†	9.2 ± 0.4

3.2.5. Hepatic Gene Expression

In order to examine the mechanisms of CLA action on liver lipid metabolism hepatic gene expression was analysed. Using liver tissue from 11 week WD fed animals, with or without fatty acid supplementation, mRNA abundance for a number of key lipogenic enzymes and metabolic transcriptional regulators were measured by real-time PCR using an expression array. Results for the $ApoE^{-/-}$ mice must be interpreted with caution as the number of samples containing "no calls" (C_t > 30, below the level of reliable detection) was high. In addition the variability between replicates was high. As a result, not every animal could be represented in the group data.

After 11 weeks of feeding, the hepatic mRNA for SREBP-1c, a key regulator of lipogenesis, was increased 2.5-fold in WD compared to chow (Figure 14). There were no significant differences in SREBP-1c gene expression between supplementation groups compared to WD, although there was a further 2.6-fold and 1.9-fold increase in the *c*-9, *t*-11 CLA and t-10, *c*-12 CLA supplemented groups, respectively.

Expression of SCD1 mRNA, another lipogenic gene, was reduced by all diets compared to chow and was significantly reduced by both LA and *c*-9, *t*-11 CLA supplementation compared to WD (Figure 14; $p<0.01$). Expression of LXR, which is an upstream regulator of SCD1, was also reduced by the WD which was further decreased by *c*-9, *t*-11 CLA supplementation (12.9-fold) (Figure 14) although this difference did not reach statistical significance. Expression of SREBP-2, considered a regulator of cellular cholesterol metabolism, was reduced by 29-fold with *c*-9, *t*-11 CLA supplementation ($p<0.05$) compared to WD.

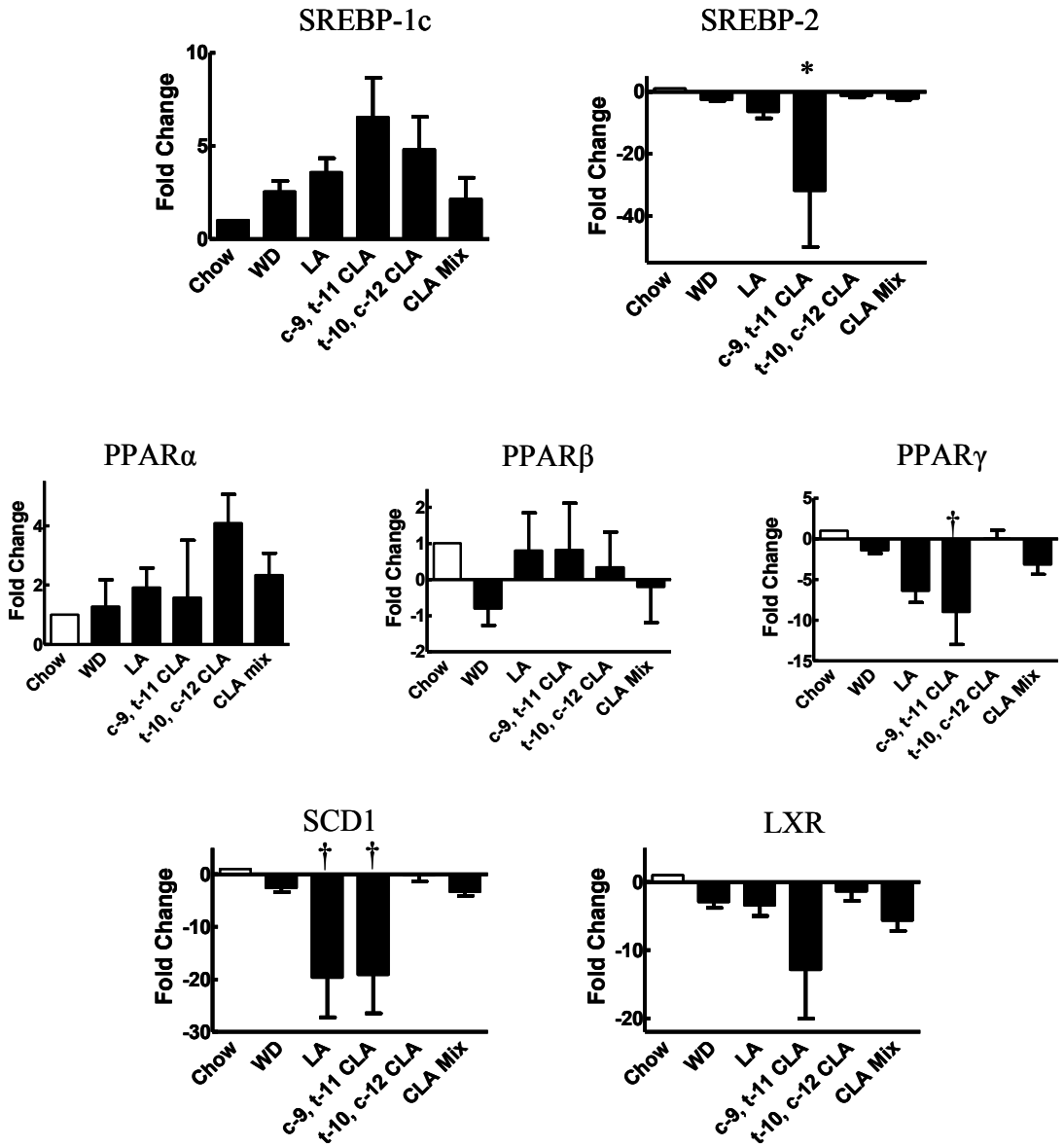


Figure 14. Hepatic gene expression in ApoE$^{-/-}$ mice.

Expression of hepatic genes was measured in the livers of mice fed a WD supplemented with fatty acids. mRNA levels were analyzed by quantitative real-time PCR using specific primers and SYBR green probe. mRNA abundance was calculated as the ratio to the actb mRNA level in each cDNA sample and expressed as a fold change, assigning the value in ApoE$^{-/-}$ mice fed a chow diet as 1. Values represent means ± S.E.M. (n = 5-10 mice). * $p<0.05$ vs. WD; † $p<0.01$ vs. WD.

Transcriptional regulators PPARα, PPARβ and PPARγ, which are known to regulate the expression of many genes involved in liver metabolism (285), were also measured (Figure 14). PPARα, which is highly expressed in the liver, was not altered by the WD, and although the *t*-10, *c*-12 CLA supplementation did result in a further increase in PPARα mRNA this was not statistically significant. PPARβ expression was highly variable within each diet group and supplementation did not alter mRNA expression compared to either the chow or WD fed animals. PPARγ, which is highly expressed in adipose tissue and to a lesser degree in hepatocytes, was reduced 1.4-fold by the WD compared to chow and was further reduced (7.5-fold) by the *c*-9, *t*-11 CLA supplementation ($p<0.01$).

3.3 Discussion

The current study was undertaken to examine the influence of dietary cholesterol on CLA supplementation of a high fat (16%) diet using the ApoE$^{-/-}$ mouse as a model of hyperlipidemia. Previously, we had examined a high cholesterol (1.25%) diet in the same mouse model (274) but, given the importance of ApoE in the cholesterol and lipoprotein transport system, felt that the cholesterol content may be masking any effects that CLA may have in terms of reducing atherosclerosis or improving the lipid and lipoprotein profiles. In the present study the cholesterol content was reduced to 0.1% with all other diet components remaining the same (Section 2.2.2.1). All animals gained weight over the course of the 11 weeks, with the *t*-10, *c*-12 CLA animals gaining 45% less weight than all other groups ($p<0.001$). This is in agreement with Liu *et al* (286) who reported that 1.5% (w/w) CLA 50:50 mix resulted in a 39% decrease in weight gain over 45 days. By

contrast, in the high fat high cholesterol (HFHC) study (274) we saw the *t*-10, *c*-12 CLA animals gain significantly more weight than all other groups in large part due to profound hepatomegaly. Many CLA studies have reported no changes in body weight with CLA supplementation yet the CLA animals had an appreciable decrease in body fat (193;287-290). Nevertheless, some have reported a decrease in both weight gain and fat pad mass (291;292). Although fat loss was not directly measured in this study the 2-fold increase in liver weight (Table 6) coupled with the lower total weight gain in this group suggested that adipose tissue was likely reduced. This was further supported by the significant decrease in adiponectin (Table 7) suggesting a loss of adipose tissue or a reduction in functional adipose tissue.

A number of plasma biomarkers of atherosclerosis were measured at sacrifice. As expected for the ApoE$^{-/-}$ mouse, which is a severely hyperlipidemic model, the mice had elevated TC (Figure 11) consistent with other mouse models fed a HF diet (293), with typical lipoprotein profiles (Figure 12) displaying elevated VLDL-C in all dietary groups. The influence of CLA on plasma TG and TC reported in the literature has been mixed, as a result of varying experimental protocols and mouse strains. In the ApoE$^{-/-}$ mouse no change in TC has been reported with a mixed isomer preparation (270;273;294); a decrease with the *c*-9, *t*-11 isomer (272), an increase with the *t*-10, *c*-12 isomer (272;274) and the current study a significant decrease with a 50:50 mixed CLA preparation. This variable response is evident even in the C57Bl/6J strain where similar trends were reported, with no change using 0.25% or 0.5% mixed CLA (268). With a higher dose (1%) of mixed CLA, Poirier *et al* (295) reported an increase in plasma TC or a decrease in TC with CLA structured lipid (269).

Comparing the effect of cholesterol content in the diet, the animals in the current study received 12.5-fold less cholesterol and yet plasma cholesterol was only minimally reduced (10%) for the WD, LA and *c*-9, *t*-10 CLA supplemented groups compared to similar groups in the HFHC study (274). The plasma TC levels for both the *t*-10, *c*-12 CLA and CLA Mix supplemented animals were substantially reduced compared to the 1.25% cholesterol groups (by 10 mmol/L and 5 mmol/L respectively) (274). This reduction in plasma TC with CLA Mix supplementation was significant compared to WD (Figure 11). *t*-10, *c*-12 CLA and Mix CLA supplementation also improved plasma TG compared to WD (Figure 11) which is in agreement with studies in the C57Bl/6J (286;296) but in contrast to previous *t*-10, *c*-12 CLA supplementation of semi-purified diets in $ApoE^{-/-}$ mice (272;274) and with a 80:20 mix of *c*-9, *t*-11 and *t*-10, *c*-12 CLA in this mouse model on a chow diet (270).

Hypoadiponectinemia and hyperinsulinemia are both known to be associated with a pro-atherogenic state. The previously reported disruption to plasma adiponectin levels by *t*-10, *c*-12 CLA (72;286;291) was profoundly evident in the current study (Table 7). Adiponectin is normally an abundant circulating adipocytokine but was nearly non-detectable in the *t*-10, *c*-12 CLA group and significantly reduced in the CLA Mix group (Table 7). Numerous studies have reported highly divergent actions for each CLA isomer and have concluded that the CLA Mix, depending on the proportion of each isomer, does not have intermediate actions (292;297;298). This is clearly evident in the current work. Individual isomers either elevated or had a similar effect on plasma TC as did WD, but the CLA Mix significantly reduced plasma TC. Similarly, plasma TG levels with the *c*-9, *t*-11 isomer were not affected compared to WD but *t*-10, *c*-12 isomer reduced TG and the CLA Mix reduced TG even further (Figure 11). Adiponectin levels in the animals fed the

CLA Mix diet were 70% lower than with the *c*-9, *t*-11 isomer (Table 7). Thus it appears that for some biological activities the CLA Mix has effects that more closely resemble dominant actions of the *t*-10, *c*-12 CLA isomer.

Since *t*-10, *c*-12 CLA supplementation did not reduce TC below levels observed in the WD and that this isomer severely reduced adiponectin and elevated insulin levels (Figure 11, Table 7) it is surprising that the aortic lesions, as measured in the *en face* preparations, are significantly reduced compared to WD (Figure 13). Mix CLA supplementation had a more favourable lipid profile (Figure 11) and less severe hypoadiponectinemia and hyperinsulinemia (Table 7) and also had the most profound reduction in aortic lesion development (Figure 13). Both CLA supplement groups had reduced *en face* lesions but not those in the aortic root, which may be an indication that the root lesions develop first and are the most severe in this model. The gene-targeted disruption of the apoE, coupled with the dietary cholesterol content, may be too overwhelming for the CLA supplementation to counter. This improvement in atherosclerosis was not observed in our previous HFHC diet where CLA supplementation did not alter lesions compared to WD (274). The 12.5-fold decrease in dietary cholesterol which resulted in minimal plasma TC changes was accompanied by a 50% reduction in lesions with *c*-9, *t*-11 and *t*-10, *c*-12 supplementation. In the earlier work HFHC groups with or without supplementation had approximately 20-25% of the total aorta (*en face* preparation) covered with atherosclerotic lesions (274). In the current study, with 0.15% cholesterol, *en face* lesion staining occupied between 6-15% of the total aorta. Similarly less cross-sectional area was affected in the aortic root measurements. On a diet containing 1.25% cholesterol approximately 40% of the aortic root was involved, while on a diet with 0.15% cholesterol only 15% of the aortic root contained lesions. This

profound effect of dietary cholesterol in lesion development is evidence for an interaction of diet and mouse genetics. The ApoE mouse develops lesions even on a chow diet (241) and dietary cholesterol content only influences lesion development in the early stages. After lesions are established, they are indistinguishable between groups receiving different dietary cholesterol content (241).

There are many discrepancies in the literature regarding the effectiveness of CLA at reducing atherosclerosis (Table 3). The *c*-9, *t*-11 CLA isomer was the more effective agent in reducing atherosclerosis biomarkers in some studies (254;272;273;299;300). However, in many of these studies lesion development was not measured directly. Risk reduction is not always reflected by lesion reduction (254;273). Even within the same strain ($ApoE^{-/-}$) there has not been agreement as to the effects of each isomer of CLA. One reason for differences within the same strain may be differences in the base diet used in the individual studies, which can play a significant role in assessment of lesion development. It has been previously reported that a semi-purified diet will induce lesion development under less severe dietary manipulations of cholesterol and fat content (301;302). Using a semi-purified diet supplemented with 1% CLA isomer and 0.15% cholesterol, Arbones-Mainar *et al* (272) showed that the affected lesion area decreased with *c*-9, *t*-11 isomer supplementation while the *t*-10, *c*-12 isomer increased lesion area. In contrast, when a higher cholesterol level and lower CLA concentration was used we saw no reduction in lesion area by either isomer (274).

The underlying mechanisms involved in the anti-atherosclerotic effects of mixed CLA or individual isomers are not completely understood and results from *in vivo* and *in vitro* studies are often contradictory. The role of CLA in decreasing atherosclerosis may in part be mediated through PPARα or γ. Both PPARs are expressed in endothelial cells,

macrophages and smooth muscle cells (303). Ringseis *et al* (204) utilized human smooth muscle cells to show that each of the individual CLA isomers, *c*-9, *t*-11 and *t*-10, *c*-12, could inhibit the formation of the eicosanoid precursor arachidonic acid and decrease NFκB binding activity. This resulted in reduced eicosanoids and prostaglandins and a muted inflammatory response. The effectiveness of the CLA isomers was equivalent to the PPARγ agonist troglitazone and was partially abolished by a PPARγ antagonist, suggesting that the actions of CLA on lesion development are, at least in part, mediated through PPARγ (204).

Previous studies have demonstrated that despite circulating hyperinsulinemia and insulin resistance, insulin signalling was intact in the arterial wall of obese C57BL/6 mice fed a high-fat diet (304). Insulin-sensitizing medications, such as thiazolidinediones, which are ligands for peroxisomal proliferator activated receptor, reduce atherosclerosis in male $LDLr^{-/-}$ mice fed a high-fat diet without improvements in plasma lipids. These agents may have direct effects on arterial wall cells and macrophage biology (304). In the current study, either CLA isomer may have acted as the PPARγ ligand and thereby reduced atherosclerotic lesion development, without concurrent improvements in lipid profile.

The modest improvements in plasma lipid and lipoprotein profiles observed with *t*-10, *c*-12 CLA supplementation are overshadowed by significant negative changes. Our observations support previous reports that this isomer is responsible for decreased weight gain, increased liver weight, diminished adiponectin levels, hyperinsulinemia and the development of hepatic steatosis (72;286;289;290;292;295;305-308). These characteristics are nearly ameliorated by the addition of an equimolar amount of *c*-9, *t*-11

CLA, as observed in the hepatic and plasma profiles of the CLA Mix supplemented mice (Tables 7 and 8). Given the significant increase in liver weight, it was hypothesized that hepatic lipid analysis would reveal that accretion of hepatic TG accounted for this increased weight as previously reported (274;286;289;290;304;309). Hepatic TG accumulation was also predicted based on the observation that *t*-10, *c*-12 supplementation resulted in hyperinsulinemia with moderately elevated NEFA levels (Table 7) which also associate with increased hepatic TG. Although the increase in hepatic TG was not as profound as with HFHC diet (3-fold increase)(274) it was still significantly increased compared to WD, LA and *c*-9, *t*-11 CLA groups (Table 8). Hepatic CE in CLA Mix supplementation group were reduced almost 50% compared to all other diets groups (Table 8).

Changes in adipose tissue, which have been suggested as the first tissue to be affected by the *t*-10, *c*-12 isomer (308), could be the reason for decreased adiponectin resulting in hyperinsulinemia. The loss of functional adipose tissue may result in a reduction in PPARγ as well as its down-stream targets (310;311). Purushotham *et al* (72) were able to show that when CLA was removed from the diet adiponectin levels could be restored to 50% of the original level. This level of recovery was sufficient to increase body weight and fat stores, as well as reduce liver weight. Similar results were obtained when a physiological dose of leptin and adiponectin were administered to lipoatrophic mice, also improving hyperinsulinemia and hepatic steatosis (312). Since adiponectin is a target gene of PPARγ (313), the CLA-mediated down-regulation of this nuclear receptor (306;314) may contribute to decreased adiponectin levels. The specificity of *t*-10, *c*-12 CLA supplementation for decreasing adiponectin levels has been consistently shown in numerous studies (72;274;291;295). The most common mouse model used to investigate

the effect of *t*-10, *c*-12 CLA supplementation has been the C57Bl/6J mouse which appears to be very sensitive to adiponectin alterations as in most studies authors used a CLA mix preparation, yet have observed similar decreases in adiponectin as in single isomer studies (72;291;295).

The level of plasma TG, representing primarily VLDL-TG, is reflective of a dynamic balance between lipoprotein lipase (LPL) mediated clearance of VLDL in peripheral tissues and the secretion of hepatic VLDL-TG. If the balance moves toward increased clearance or decreased synthesis, a reduction in plasma TG will be observed. The liver constitutively synthesizes VLDL to meet energy requirements of peripheral tissues. A number of transcription factors regulate this process in the liver including PPARα for which *t*-10, *c*-12 CLA has been identified as a potent ligand (315). PPARα may influence the LPL-mediated clearance of lipoproteins by directly stimulating hepatic LPL expression (316) or by altering inhibitors or activators of LPL activity such as apoCII or apoCIII (235). Degrace *et al* reported that it was not a decrease in lipoprotein secretion that resulted in hepatic steatosis but rather an induction of LDL receptor, leading to an increase in hepatic lipoprotein clearance (317). Increased lipoprotein clearance was also reported in C57Bl/6J mice that had been fed 1% (w/w) *t*-10, *c*-12 CLA (318). After 12 days of CLA supplementation CD36 and VLDL receptor gene expression were increased suggesting that *t*-10, *c*-12 CLA could stimulate increased liver lipid uptake, possibly through PPARγ induction of CD36. The lipids accumulated as result of inhibition of β-oxidation at carnitine palmitoyltransferase-1 (CPT-1) by the CLA (318). Recent findings in $ApoE^{-/-}$ mouse showed the nuclear receptor NR4A and its down-stream target CD36 were both upregulated by *t*-10, *c*-12 supplementation (319). In contrast, however, Liu *et al* (286) observed significant hepatic accretion without altering

expression of genes involved in β-oxidation (PPARα, CPT-1). Even though VLDL-TG production may not be diminished in all models, hepatic steatosis may still occur because there is reduced fatty acid removal by the dysfunctional adipose tissue causing increased liver fatty acid flux.

Liver gene expression was unaffected by *t*-10, *c*-12 supplementation compared to WD and did not provide insight as to the mechanism of increased liver weight. Interestingly, microarray analysis of hepatic gene expression in 3 month old C57Bl/6J mice showed no association between lipid metabolism genes, PPARα, SREBP-1, SREBP-2, PGC1α or PGC1β, with liver lipid content (309). Supplementation with *c*-9, *t*-11 CLA has been studied to a lesser degree, as it has not been shown to induce profound hepatic steatosis accompanied by hyperinsulinemia. By examining the gene expression data we may be able to infer some of the unique actions of the *t*-10, *c*-12 isomer by the differential expression observed with *c*-9, *t*-11 CLA supplementation. *c*-9, *t*-11 CLA supplementation resulted in elevated plasma TG and TC levels, a 40% reduction in hepatic TG accretion and similar hepatic levels of FC and CE compared to *t*-10, *c*-12 CLA supplemented animals. From the gene expression analysis SREBP-2, PPARγ and SCD1 were all down-regulated, although not significantly, compared to *t*-10, *c*-12 CLA supplementation. SREBP-2 preferentially activates genes involved in cholesterol synthesis (320). Since WD feeding, with or without supplementation, induced extreme hypercholesterolemia in the $ApoE^{-/-}$ mouse it is not surprising that all diet groups had low expression of SREBP-2 mRNA and the further suppression by *c*-9, *t*-11 may not play a physiological role at the protein level. Under normal conditions, PPARγ is not abundantly expressed in the liver, therefore similar to SREBP-2 down-regulation may not translate into a change in protein

level. Further investigation is warranted into the differential gene expression and transcriptional regulation between *c*-9, *t*-11 and *t*-10, *c*-12 isomers to delineate the mechanisms of the hepatic steatosis.

By reducing the cholesterol content of the diet (274) the *t*-10, *c*-12 isomer was able to exert a stronger positive influence on reducing plasma TG and atherosclerotic lesion development but with the same deleterious hyperinsulinemia and hypoadiponectinemia. The conundrums remain – weight reduction, improved plasma TG and reduced atherosclerosis lesion development with *t*-10, *c*-12, no improvement in plasma lipids, lesion development, or hepatic lipids with *c*-9, *t*-11 supplementation, yet it is the former that has elevated insulin, diminished adiponectin levels and livers that are nearly twice as large. Hepatic gene expression was unremarkable and did not provide a clear explanation as to the underlying cause of the increased liver size. The inconsistent findings are consistent with the literature. Clearly the two isomers have divergent mechanisms of action as been previously stated (272;292;319) as well they affect a myriad of tissues and metabolic processes making for complex regulation of numerous biological functions.

CHAPTER FOUR – STUDIES IN THE LDLr$^{-/-}$ MOUSE

4.1. LDLr$^{-/-}$ mouse: A Model for the Metabolic Syndrome

Human familial hypercholesterolemia is one of the most severe forms of dyslipidemia and occurs due to a mutation in the LDL receptor which decreases the clearance of atherogenic LDL from the plasma (Section 1.7.3). Having a more "human" lipoprotein profile (i.e. elevated LDL), the LDLr$^{-/-}$ mouse (321) develops a moderate hypercholesterolemia on a chow diet. When given a western diet (21% fat and 0.15% cholesterol) the LDLr$^{-/-}$ mice have been reported to develop more severe hypercholesterolemia and extensive atherosclerosis (321). Obesity and insulin resistance have also been diet-induced in the LDLr$^{-/-}$ mouse with a diet containing 20% fat (304). This phenotype made the LDLr$^{-/-}$ mouse a more suitable model for expanding our studies beyond simply trying to reduce atherosclerosis with bioactive fatty acids supplementation to examining the effects of bioactive fatty acids on risk factors for the metabolic syndrome.

4.2. Western Diet with CLA Supplementation

4.2.1. Body and Liver Weight

The LDLr$^{-/-}$ mice were of similar initial weights and consumed equal amounts of food regardless of diet supplement (Table 9). Over the 11 week supplementation period all mice gained weight with the WD and *c*-9, *t*-11 CLA groups gaining a similar amount (Table 9). The LA and CLA Mix gained approximately 40% less weight ($P = 0.08$ and $P=0.06$ respectively) while the *t*-10, *c*-12 CLA group gained 50% less than the WD group

Table 10. Phenotypic characteristics of the LDLr$^{-/-}$ mice.

LDLr $^{-/-}$ mice were fed a WD either supplemented or not with 0.5% bioactive fatty acid for 11 weeks. Liver weight was determined at sacrifice. Values represent mean ± S.E.M.(n = 10-11 animals). * $p < 0.05$ vs. WD; † $p < 0.01$ vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
Food Consumption (g/day/animal)	2.4 ± 0.03	2.0 ± 0.03	1.8 ± 0.05	1.9 ± 0.03	2.0 ± 0.04
Initial Weight (g)	18.7 ± 0.6	18.2 ± 0.3	17.2 ± 0.6	16.9 ± 0.5	17.6 ± 0.6
Weight Δ (g)	9.5 ± 1.2	6.0 ± 0.5	7.6 ± 0.7	4.7 ± 0.5 †	5.7 ± 0.6
Liver wt (% body wt)	3.7 ± 0.2	3.5 ± 0.1	3.8 ± 0.1	8.7 ± 0.5*	4.6 ± 0.1*

($p< 0.01$). In contrast to other studies utilizing the $LDLr^{-/-}$ mouse as an atherogenic model (304) we did not see a large enough weight gain to consider the mice obese. Previous studies used slightly higher fat (20% vs 16% wt/wt) and cholesterol content (0.5% vs. 0.1 % wt/wt) which may have resulted in the excessive weight gain (301;302;304). Liver weight was found to be similar between the WD, LA and *c*-9, *t*-11 CLA supplement groups (Table 9) while both the *t*-10, *c*-12 and CLA Mix livers were significantly larger ($p < 0.05$).

4.2.2. Principal Component Analysis

Atherosclerosis and its risk factor the metabolic syndrome are known to be a result of perturbations in a number of physiological parameters. To capture the interrelationship between plasma and hepatic parameters, principal component analysis was performed *in lieu* of standard ANOVA comparisons. In multifactorial diseases such as atherosclerosis and metabolic syndrome many variables may have an underlying relationship, for example blood glucose and plasma insulin could be measuring facets of the same physiological parameter. The goal of principal component analysis is to compress the original data set into a set of linear components and determine which variables might contribute to each component (278). The extraction of factors (components) must be performed critically such that the resulting linear components adequately reflect the original data but in a manageable form. In the current study, principal component analysis was primarily designed to generate hypotheses as to which factors had the greatest influence on separating the animals based on their response to

diet. All variables were transformed prior to statistical analysis because of a non-normal distribution.

Examination of the TC, TG, (Figures 15A and B) and lipoprotein profiles (Figure 16) revealed an increase in the atherogenic lipoproteins with the *t*-10, *c*-12 CLA supplemented diet and a decrease in these particles and the associated lipids with the CLA Mix in the $LDLr^{-/-}$ mice. *t*-10, *c*-12 CLA supplementation resulted in a significant increase in plasma TC ($p<0.01$). Consistent with an increase in plasma TC the VLDL-C fraction in the *t*-10, *c*-12 CLA supplement group was substantially increased compared to all other diet groups (Figure 16, open circles). Interestingly the plasma concentrations of TG and TC observed in the mice supplemented with CLA Mix were not intermediate to the concentrations observed in the mice supplemented with either individual isomer, both of which increased TG and TC. Since the *t*-10, *c*-12 CLA supplementation decreased lesion area (Figure 18); it was surprising that the atherogenic fractions (VLDL-LDL) were the ones carrying the excess TC and TG in this group.

The *t*-10, *c*-12 CLA had increased blood glucose compared to the WD treatment group ($P=0.06$) (Table 10). As in our previous studies with $ApoE^{-/-}$ mice (274), where blood glucose was not significantly affected by CLA supplementation, insulin was markedly elevated with *t*-10, *c*-12 CLA but due to large variations in variance did not reach statistical significance. Fasting insulin is often considered a surrogate marker for insulin resistance. Therefore with a 12-fold increase in fasting plasma insulin the *t*-10, *c*-12 CLA mice (Table 10) appear to be showing signs of decreased insulin sensitivity.

Adiponectin was reduced by almost 90% in *t*-10, *c*-12 CLA ($p<0.01$) and by 50% in the CLA Mix ($P=0.06$) groups compared to WD. This isomer specific reduction

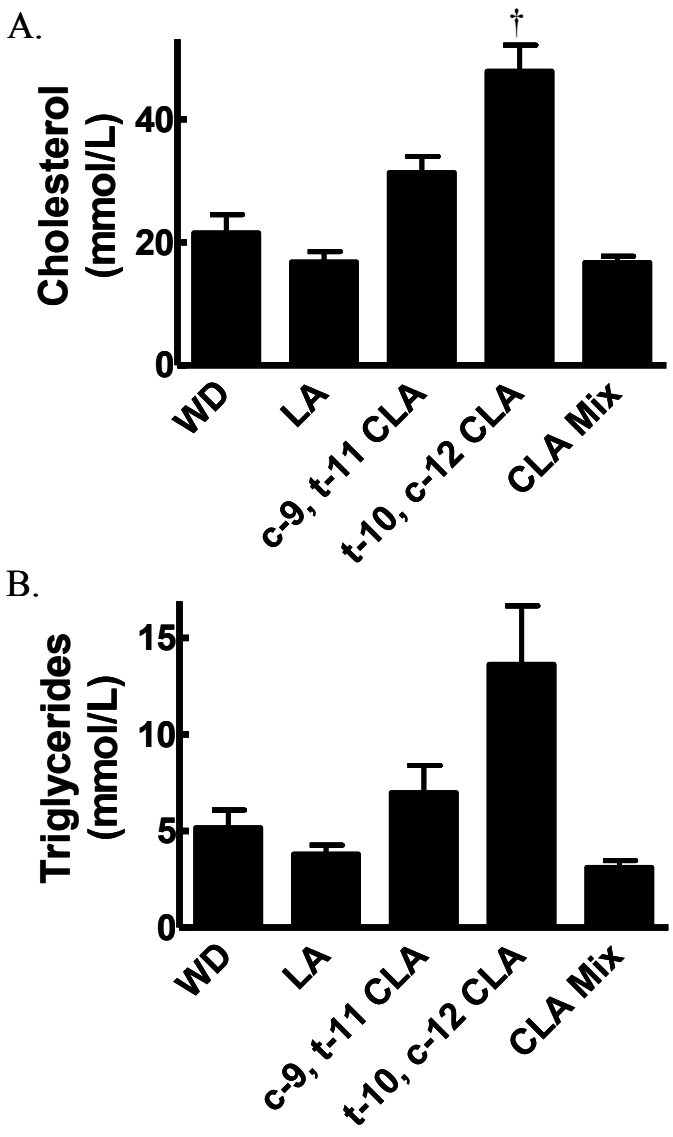


Figure 15. Plasma total cholesterol and triglycerides for LDLr$^{-/-}$ mice.

LDLr$^{-/-}$ mice were fed a WD either supplemented or not with 0.5% bioactive fatty acid for 11 weeks. Blood was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and total cholesterol (A) and triglycerides (B) were measured (mean ± S.E.M., n=10-11animals). † $p < 0.01$ vs. WD.

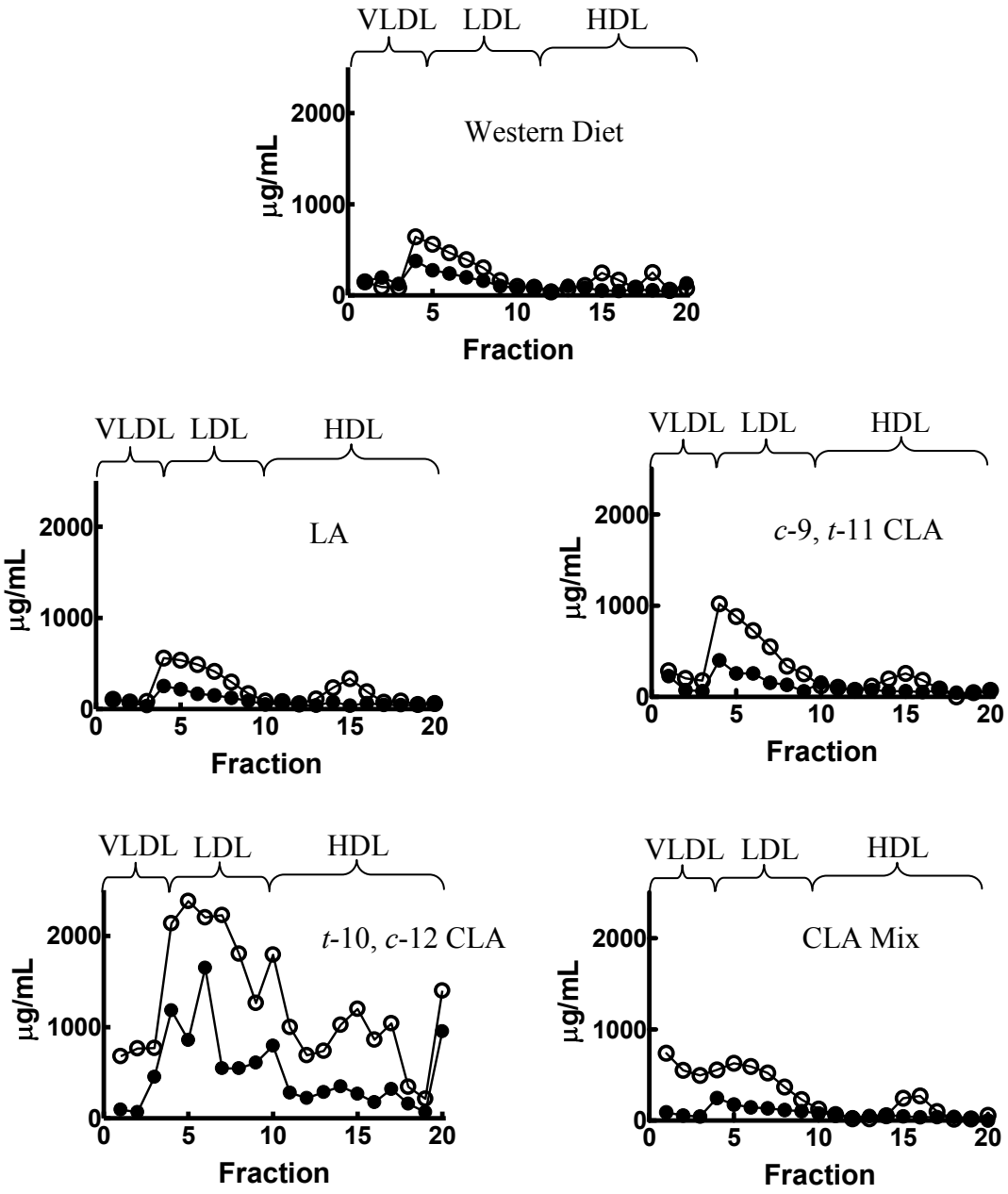


Figure 16. Plasma cholesterol and triglyceride lipoprotein profiles for LDLr$^{-/-}$ mice.

After 11 weeks on a WD either supplemented or not with 0.5% bioactive fatty acid serum was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and pooled plasma samples (n=4-5) were separated by density gradient ultracentrifugation. Cholesterol (○) and triglyceride (●) were measured in each fraction.

Table 11. Plasma measurements for LDLr$^{-/-}$ mice.

Plasma from LDLr$^{-/-}$ mice fasted for 5 h was obtained after 11 weeks on a WD either supplemented or not with 0.5 % bioactive fatty acids. Data are presented as mean ± S.E.M.(n=10-11). † $p < 0.01$ vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
Glucose (mM)	10.5 ± 0.6	9.5 ± 0.5	10.6 ± 0.7	13.6 ± 0.9	12.6 ± 0.9
Insulin (ng/mL)	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	7.1 ± 3.2	0.8 ± 0.1
Adiponectin (μg/mL)	13.8 ± 2.4	13.1 ± 1.1	13.4 ± 0.7	1.6 ± 0.2†	5.9 ± 1.4
NEFA (mEq/L)	1.0 ± 0.1	1.2 ± 0.1	0.3 ± 0.1†	0.8 ± 0.1	0.5 ± 0.1†

has been reported previously in the literature (274;286;291) and has been reported to precede any decrease in body fat (295). In addition, the influence of a WD on increasing adiposity was observed, as adiponectin was reduced compared to chow fed animals (13.8 ± 2.4 vs. 27.9 ± 0.9μg/mL respectively).

Elevated FFA flux from adipose tissue to the liver, as found in the insulin resistant state, often promotes TG accretion in the liver. Surprisingly, the *t*-10, *c*-12 CLA group did not have elevated NEFA compared to WD (Table 10). Both the *c*-9, *t*-11 CLA and CLA Mix supplementation resulted in a significant reduction in NEFA compared to WD ($p<0.01$) (Table 10).

To capture the interrelationship between phenotypic, plasma and lesion parameters, principal component analysis was performed in addition to standard ANOVA comparisons. PCA score plot revealed a distinct group clustering trend according to dietary manipulation along PC3, which accounts for 11.5% of the total variance (Figure 17, top panel) in the data matrix. Along PC3 the animals were separated primarily according to adiponectin levels regardless of dietary intervention. Moving from negative to positive values reflects a progressive reduction in plasma adiponectin. Those mice fed the WD characteristically had negative values of PC3 and had normal levels of adiponectin. In contrast, mice fed the *t*-10, *c*-12 CLA and CLA Mix supplemented diets had positive PC3 values and below normal adiponectin levels. The second variable that separated the mice by diet was liver weight, expressed as a percentage of body weight (% body wt, Figure 17 bottom panel). Mice fed the *t*-10, *c*-12 CLA and CLA Mix supplemented diets typically had positive scores on PC3 and WD mice had negative values which correspond to enlarged livers and normal weight livers respectively. Four of the *t*-10, *c*-12 CLA fed animals had negative values for PC3. Upon closer examination of

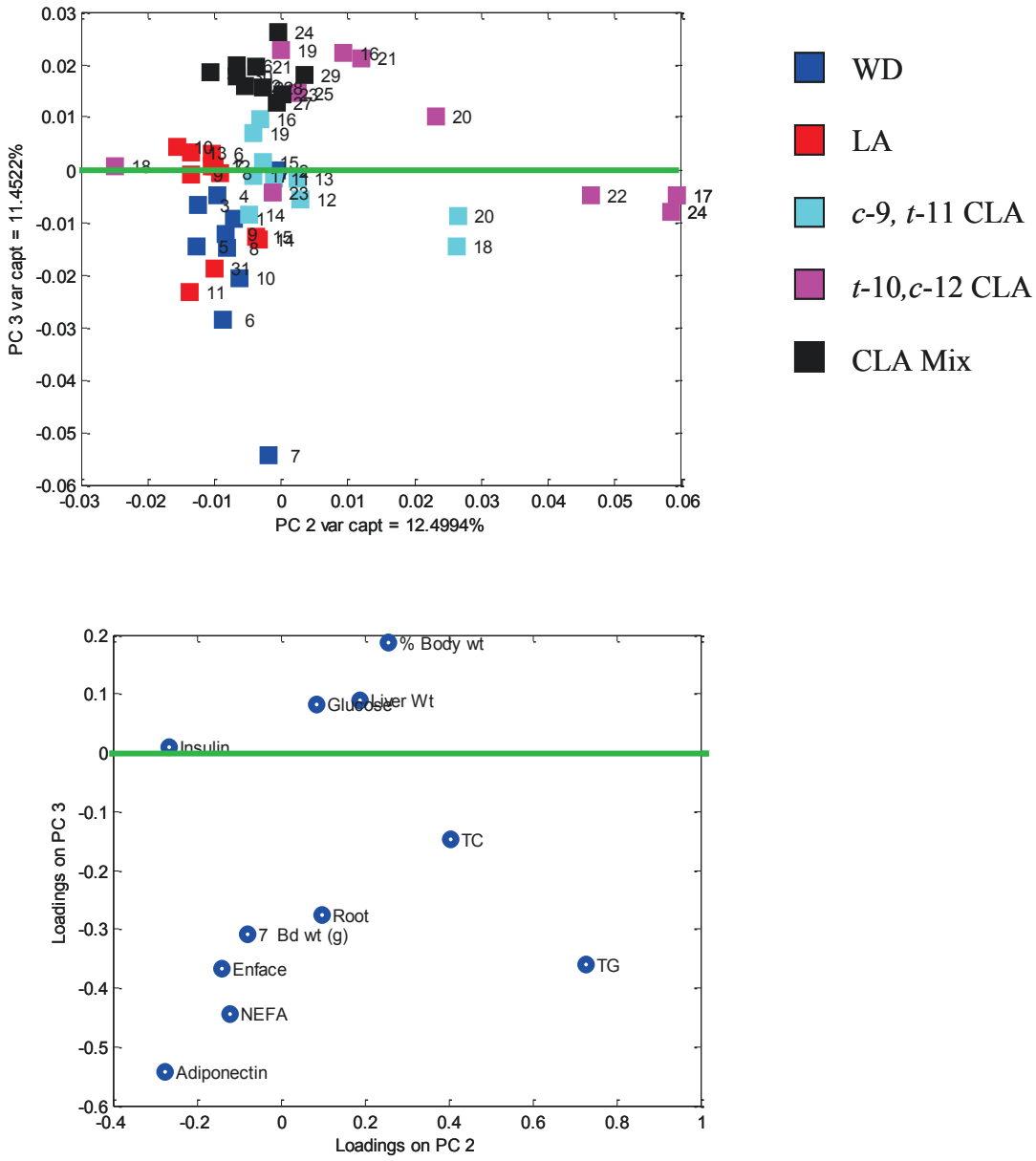


Figure 17. Principal component analysis for LDLr$^{-/-}$ mouse plasma and lipid data.

Score plots were derived from PCA. The two component model explained 12.5% (PC#2) and 11.5% (PC#3) of the variation of the data.

these animals it appears that other variables, such as plasma insulin, TC or TG are having a greater influence than liver weight. PC2, which accounts for 12.5% of the total variance in the data matrix, did not separate the mice by distinct diet groups. Animals that had elevated plasma TG and TC levels, regardless of diet group, had positive values while animals with lower plasma TG and TC had negative values (Figure 17 bottom panel). PCA results support our previous data that has shown that the biological activities of the CLA Mix more closely resemble those of *t*-10, *c*-12 CLA than *c*-9, *t*-11 CLA. The size of each diet group (n=10-11) limits more extensive interpretation of the data but relationships regarding the influence of various parameters in determining metabolic perturbations was revealed which may not have been evident by conventional ANOVA analysis.

4.2.3. Quantification of Atherosclerosis

En face lipid staining revealed that approximately 16% of the aorta in $LDLr^{-/-}$ mice fed a WD contained atherosclerotic lesions (Figure 18C). Lesion development was lower, by almost 50% in all fatty acid supplemented groups with a significant reduction observed in the LA ($p < 0.05$), *t*-10, *c*-12 CLA and CLA Mix ($p < 0.01$) groups (Figure 18). Further scrutiny of the regions of the total aorta did not reveal any localized influence of the diet supplementation as all groups had less lesion development in all regions when compared to the WD (Figure 18E). Consistent with previous reports that early lesion development begins in the aortic root, the cross-sections revealed extensive lesion development in all diet groups. Mice fed a WD developed extensive and severe lesions with lipid-staining material covering on average 25% of the total root area (Figure 18D). While supplementation reduced this to approximately 20% in the *c*-9, *t*-11 CLA and *t*-10,

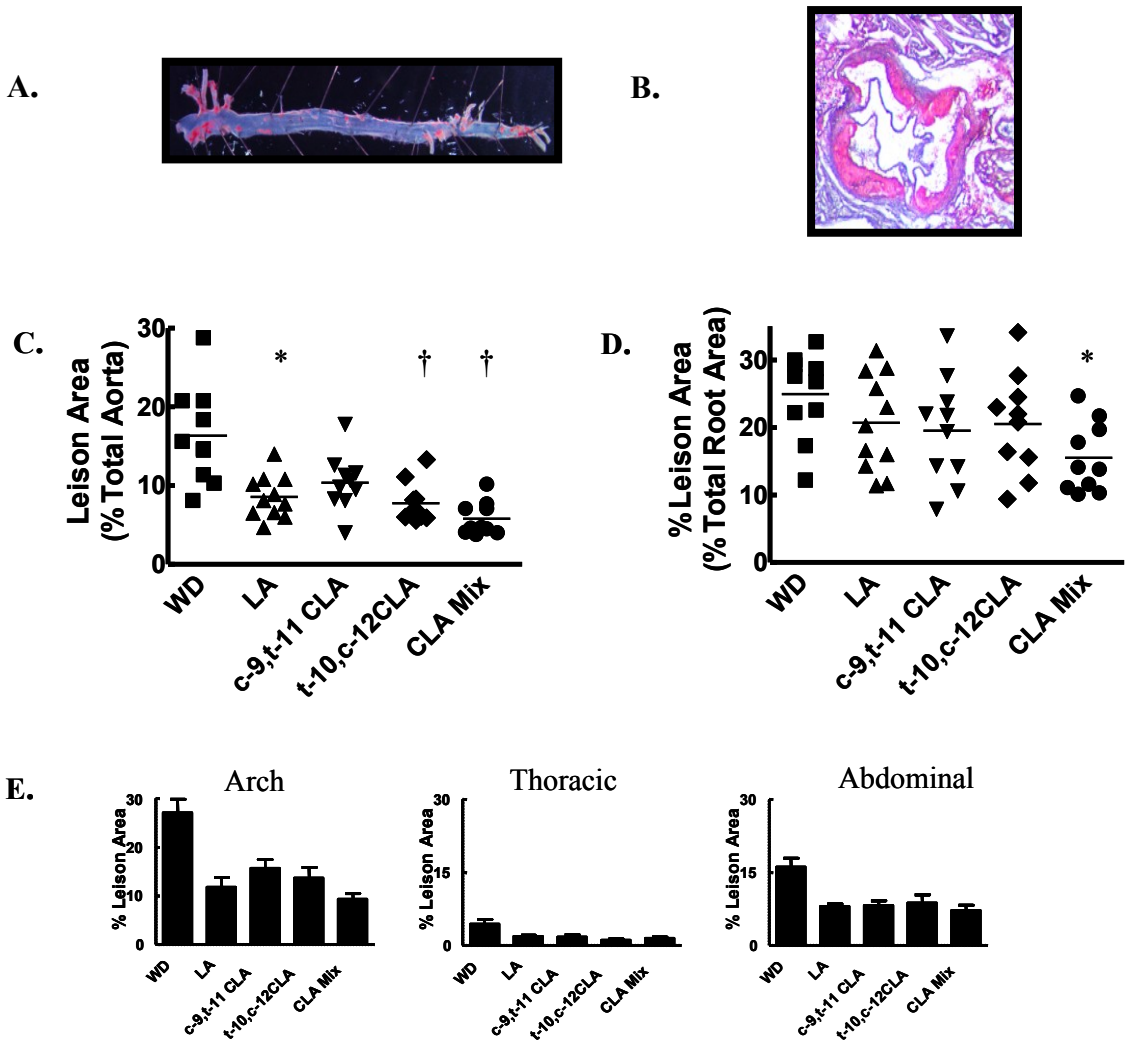


Figure 18. Atherosclerotic lesion measurement in aortic root cross-section and aortic *en face* preparations.

The images show sample preparations for (A) *en face* and (B) aortic roots from LDLr$^{-/-}$ mice. Mice were fed a WD with or without supplementation as previously described. (C) Quantification of lesion area relative to total aorta area determined as described in the Materials and Methods. (D) Quantification of cross-sectional area of n=10-13 animals per group). (E) Quantification of arch, thoracic and abdominal region of total aorta as previously described. * $p < 0.05$ vs. WD; † $p < 0.01$ vs. WD.

c-12 CLA groups, this difference was not statistically significant. However, CLA Mix supplementation significantly reduced lesion development ($p< 0 .05$) compared to WD.

4.2.4. Liver Lipid Mass, Hepatic Gene Expression and Protein Mass

Given that liver weight and insulin along with hepatic TG mass were significantly increased and plasma adiponectin significantly decreased by the *t*-10, *c*-12 CLA isomer, it was anticipated that possible mechanisms for this would be suggested from gene expression analysis in the $LDLr^{-/-}$ mice. In addition to mRNA levels, protein levels of key transcriptional regulators were measured by immunoblotting with densitometric analysis for quantitation.

In the $LDLr^{-/-}$ animals *t*-10, *c*-12 CLA supplementation significantly increased hepatic TG stores compared to WD (Table 11; $p<0.01$). FC was not different between groups with or without supplementation. In addition, the *t*-10*, c*-12 CLA supplementation resulted in significantly increased CE accumulation compared to WD (Table 11; $p < 0.01$).

The transcriptional regulator LXR was not significantly altered at the mRNA level by either the WD or supplementation (Figure 19). The *t*-10, *c*-12 supplementation resulted in a 1.7-fold increase in LXR expression, while the *c*-9, *t*-11 increased expression 3.2-fold. The CLA Mix, as we have often seen, was not intermediate between the individual isomers but rather showed the greatest increase in LXR expression (4.4-fold).

SCD1, which has been previously reported to be down-regulated by CLA supplementation (322;323), had increased expression with each of the individual isomers as well as the CLA Mix although the changes were not significantly different from the WD.

Table 12. Liver lipid mass for LDLr$^{-/-}$ mice.

LDLr$^{-/-}$ mice were fed a WD either supplemented or not with 0.5 % bioactive fatty acid for 11 weeks. Livers were obtained at sacrifice and lipids extracted in chloroform. TG, FC and CE were quantified by gas-liquid chromatography and PL by phospholipid C enzymatic assay. Data are presented as total mass per gram of liver weight (mean ± S.E.M.) (TG, triglycerides; FC, free cholesterol; CE cholesterol esters; PL phospholipids). † $p < 0.01$ vs. WD.

	Diet Supplement				
	WD	LA	*c*-9, *t*-11 CLA	*t*-10, *c*-12 CLA	CLA Mix
TG (mg/g liver)	40.4 ± 14.5	16.2 ± 3.6	55.5 ± 10.1	176.5 ± 20.8†	67.1 ± 10.3
FC (mg/g liver)	4.9 ± 0.5	5.5 ± 0.4	4.4 ± 0.2	4.9 ± 0.3	4.2 ± 0.3
CE (mg/g liver)	8.0 ± 1.9	5.3 ± 0.8	13.0 ± 2.0	19.5 ± 2.2†	8.8 ± 1.4
PL (mg/g liver)	8.6 ± 0.8	9.4 ± 0.4	10.4 ± 0.3	7.8 ± 0.5	9.8 ± 0.4

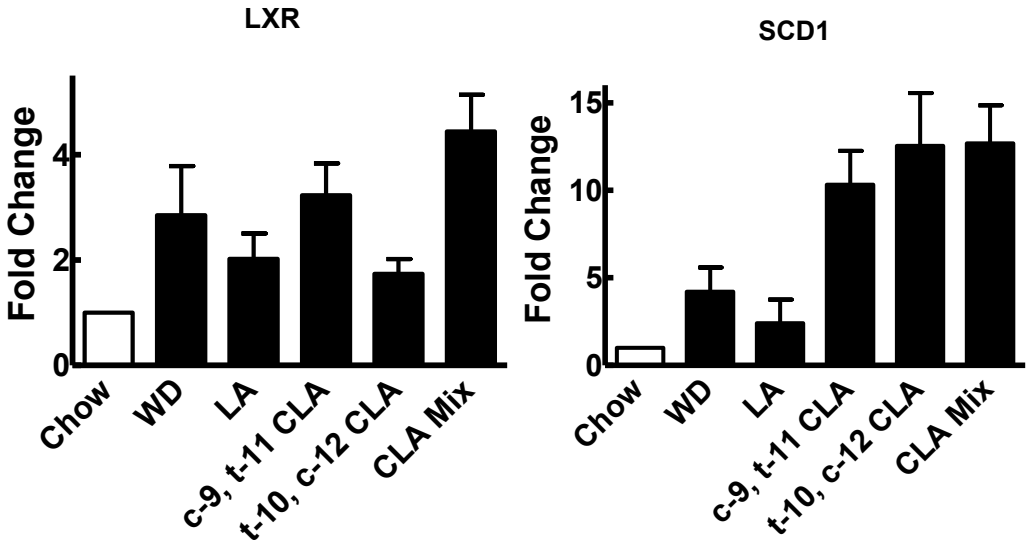


Figure 19. LXR and SCD1 hepatic gene expression in LDLr$^{-/-}$ mice.

Expression of lipogenic genes SCD1 and LXR was measured in the livers of mice fed a WD supplemented with fatty acids. mRNA levels were analyzed by quantitative real-time PCR using specific primers and SYBR green probe. mRNA abundances were calculated as the ratio to the actb mRNA level in each sample and expressed as a fold change, assigning the value in LDLr$^{-/-}$ mice fed a chow diet as 1. Bars represent means ± S.E.M. (n= 5-10 mice).

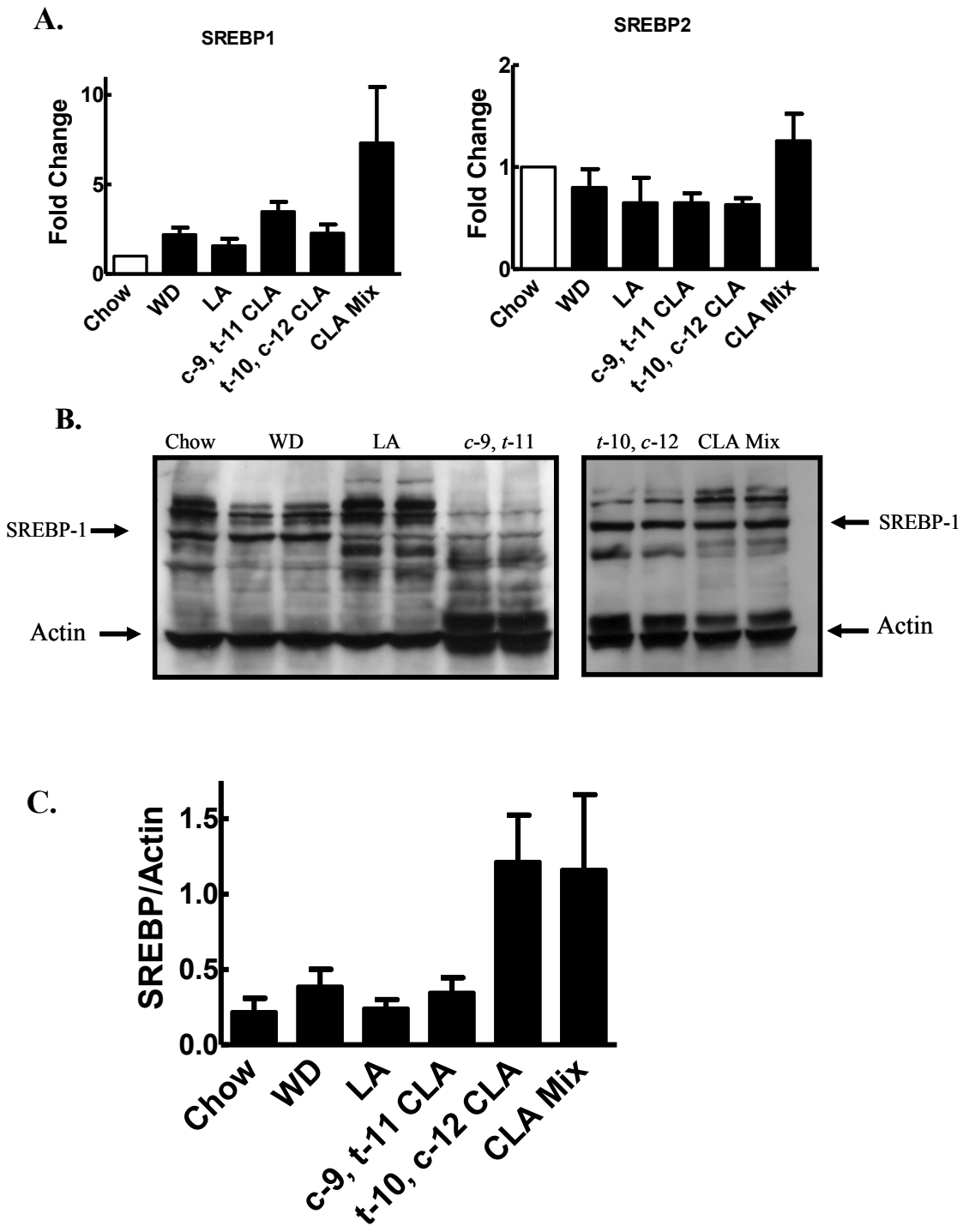
SREBP-1c mRNA expression was increased 7-fold in the CLA Mix supplemented animals (Figure 20A) but because of the large variability within the group the increase was not statistically significant. Immunoblotting of the full length SREBP-1c protein indicated that the level was increased in the *t*-10, *c*-12 CLA and CLA Mix diet groups (Figure 20C) compared to all other diet groups, although again, because of large variability, it did not reach statistical significance.

SREBP-1c is localized on the ER as membrane proteins until cholesterol stores are depleted and the SREBP is moved to the Golgi to be cleaved to the active form. Upon cleavage, the mature SREBP-1c enters the nucleus and activates genes related to fatty acid metabolism. To determine if either the *t*-10, *c*-12 CLA isomer or the CLA Mix influences the maturation of SREBP-1c, nuclear fractions were prepared from fresh liver homogenate from animals fed the WD or supplemented diets for 3 weeks. Immunoblot analysis for the cleaved form of SREBP-1c (n-SREBP-1c) was performed. The WD had reduced levels of the n-SREBP-1c (Figure 21) compared to p-SREBP-1c while both *c*-9, *t*-11 CLA and CLA Mix supplementation had approximately equal amounts of precursor and cleaved forms. In contrast, *t*-10, *c*-12 CLA supplementation induced SREBP-1 cleavage as there was an approximate 2-fold increase in cleaved SREBP (Figure 21).

The PPAR family of transcriptional regulators also showed differences between the individual isomers and the CLA Mix (Figure 22). For PPARα, individual CLA isomers did not increase mRNA expression over the WD alone, but supplementation with CLA Mix resulted in a non-significant 6-fold increase (Figure 22). The ubiquitously expressed PPARβ was largely unaffected by diet supplementation with bioactive fatty

Figure 20. SREBP-1c and SREBP-2 hepatic gene expression and protein mass.

A) Expression of transcriptional regulators SREBP-1c and SREBP-2 was measured in the livers of $LDLr^{-/-}$ mice fed a WD supplemented with 0.5% bioactive fatty acids**.** mRNA levels were analyzed by quantitative real-time PCR using specific primers and SYBR green probe. mRNA abundances were calculated as the ratio to the actb mRNA level in each sample and expressed as a fold change, assigning the value in $LDLr^{-/-}$ mice fed a chow diet as 1. B) Representative western blot for each of the dietary groups as indicated. C) Quantitation of western blots normalized to actin for full length SREBP-1c. Bars represent means ± S.E.M. (n= 5-10 mice).


A.
SREBP1
Fold Change
10
5
0
Chow
WD
LA
c-9, t-11 CLA
t-10, c-12 CLA
CLA Mix
SREBP2
Fold Change
2
1
0
Chow
WD
LA
c-9, t-11 CLA
t-10, c-12 CLA
CLA Mix
B.
Chow
WD
LA
c-9, t-11
t-10, c-12
CLA Mix
SREBP-1
Actin
SREBP-1
Actin
C.
SREBP/Actin
1.5
1.0
0.5
0.0
Chow
WD
LA
c-9, t-11 CLA
t-10, c-12 CLA
CLA Mix

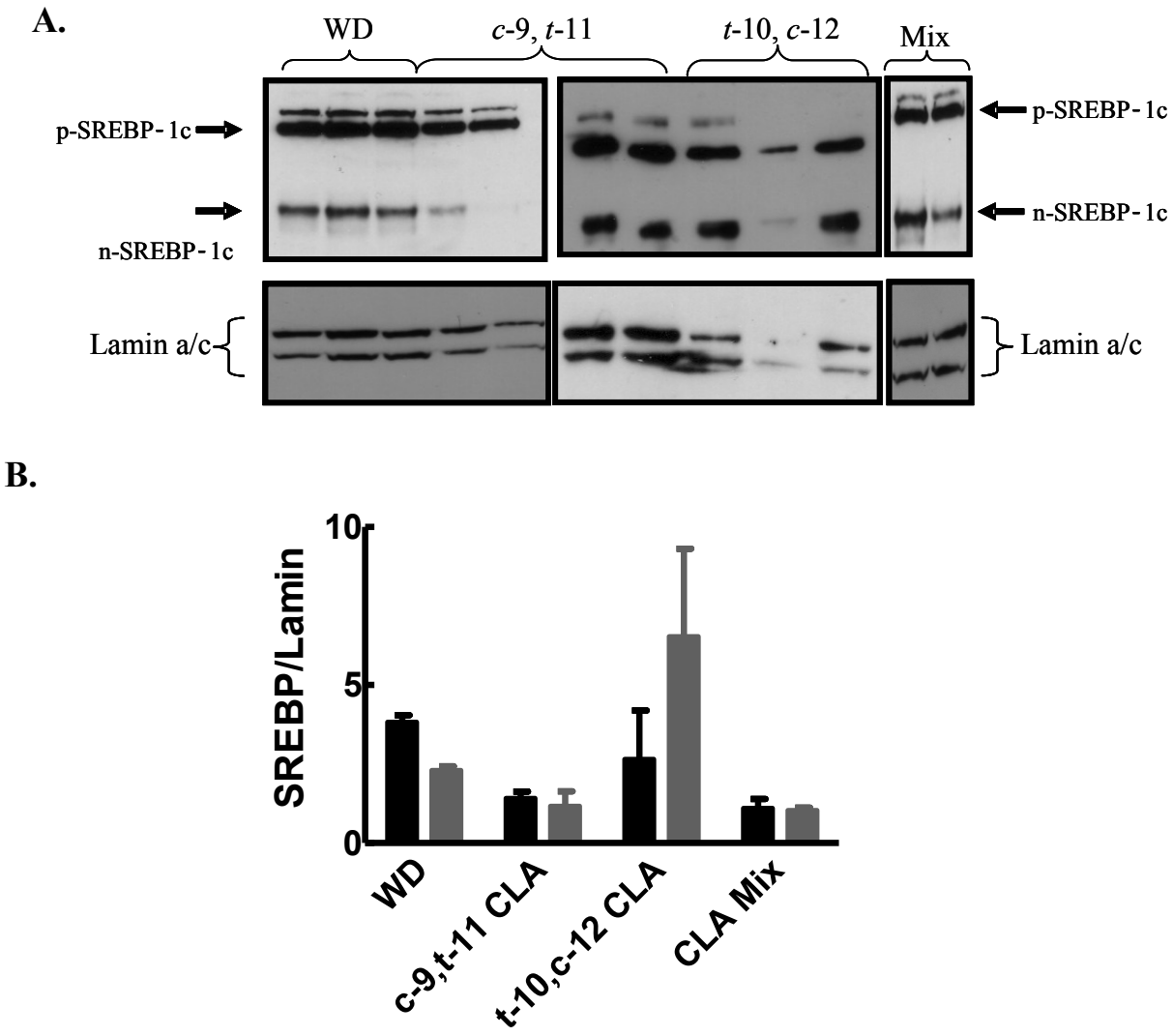


Figure 21. Nuclear SREBP-1c protein mass.

A) Representative western blot for each of the dietary groups as indicated. B) Quantification of western blot for n-SREBP-1c (grey) and p-SREBP-1c (black) normalized to lamin. Bars represent means ± S.E.M. (n= 3-5 mice).

acids. Even though the WD resulted in a 4.9-fold increase in PPARβ mRNA, any bioactive fatty acid supplementation reduced mRNA expression to levels similar to the chow fed animals. PPARγ mRNA expression was significantly increased by the *t*-10, *c*-12 CLA isomer and CLA Mix compared to the WD ($p<0.05$).

PPARα protein levels did not reflect mRNA expression results (Figure 22A and C). There were no differences in mRNA expression between dietary groups yet supplementation with the *c*-9, *t*-11 CLA isomer significantly increased PPARα protein level compared to WD ($p<0.01$). Similarly discrepant results were observed with the PPARγ mRNA and protein expression. Both *t*-10, *c*-12 CLA and CLA Mix supplementation had significantly increased PPARγ mRNA expression compared to WD ($p<0.05$) but it was CLA Mix and *c*-9, *t*-11 CLA supplementation that significantly increased PPARγ protein levels ($p< 0.05$) compared to WD. *t*-10, *c*-12 CLA supplementation significantly decreased PPARγ protein levels compared to WD ($p<0.05$).

Differential regulation of down-stream target genes by PPAR transcriptional regulators may be one of the differences that allowed the animals fed a *c*-9, *t*-11 CLA supplement to maintain normal insulin and adiponectin levels. Further experiments will be needed to elucidate if downstream pathways have been altered by changes in transcriptional regulator expression and what physiological outcomes may have resulted.

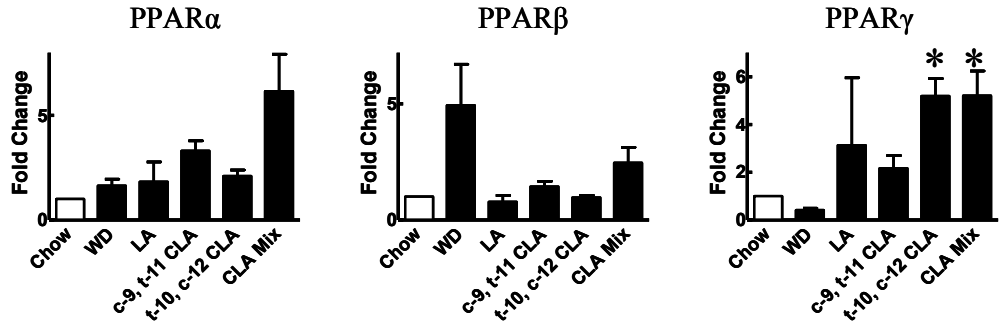
4.3. Western Diet with ω-3 Fatty Acid Supplementation

It has been fairly well established that the consumption of ω-3 polyunsaturated fatty acids, such as ALA, EPA (20:5) and DHA (22:6) is correlated with a reduced risk of

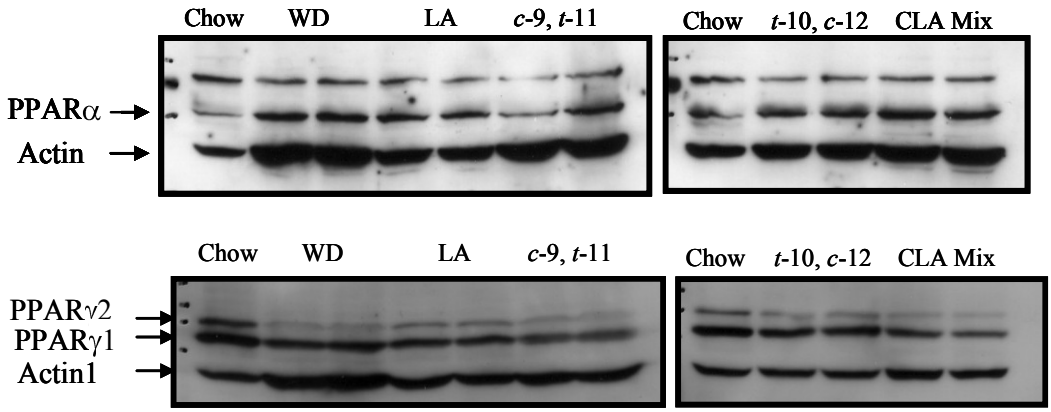
Figure 22. PPARα, PPARδ, and PPARγ hepatic gene expression and protein mass.

A) Expression of transcriptional regulators PPARα, PPARδ, and PPARγ was measured in the livers of $LDLr^{-/-}$ mice fed a WD supplemented with previously described fatty acids. mRNA levels were analyzed by quantitative real-time PCR using specific primers and SYBR green probe. mRNA abundances were calculated as the ratio to the actb mRNA level in each sample and expressed as a fold change, assigning the value in LDLr-/- mice fed a chow diet as 1. B) Representative western blot for each of the dietary groups as indicated. C) Quantitation of western blots normalized to actin. Values represent means ± S.E.M. for 8-10 mice. * $p < 0.05$ vs. WD, † $p < 0.01$ vs. WD.

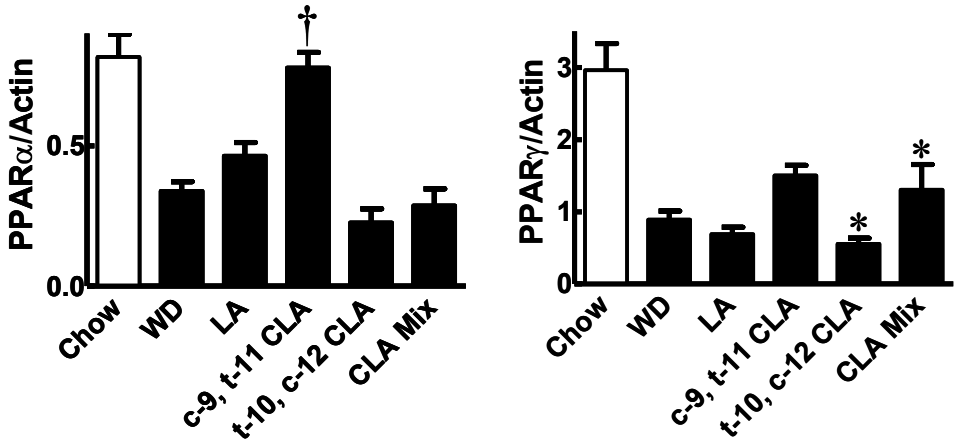
A.


PPARα
PPARβ
PPARγ
Fold Change
Chow
WD
LA
c-9, t-11 CLA
t-10, c-12 CLA
CLA Mix
*
*

B.


Chow
WD
LA
c-9, t-11
Chow
t-10, c-12
CLA Mix
PPARα
Actin
PPARγ2
PPARγ1
Actin1

C.


PPARα/Actin
PPARγ/Actin
†
*
*
Chow
WD
LA
c-9, t-11 CLA
t-10, c-12 CLA
CLA Mix

cancer and cardiovascular disease in clinical and animal studies (216;219). The ω-3 supplementation study was an extension of the work in the $LDLr^{-/-}$ mouse. Many studies have used fish oil (FO) as the treatment supplement. However, depending on the source of FO, the proportion of DHA and EPA in the supplement can be highly variable leading to uncertainty as to the bioactivity of each component fatty acid. Therefore, we set out to investigate each fatty acid individually and in an equimolar mixture.

4.3.1. Body and Liver Weight

There was no significant difference in food consumption or initial body weight between dietary groups (Table 12). $LDLr^{-/-}$ mice fed the Omega Mix gained significantly more weight than either DHA or EPA supplement alone ($p< 0.01$). Despite the increased weight gain, the Omega Mix supplement resulted in a lower liver weight compared to WD, when expressed as a percentage of body weight ($p < 0.05$, Table 12) but the absolute weight of the liver was not different (data not shown) between any of the diet groups.

4.3.2. Plasma Analysis

Fasting blood glucose was not different among dietary treatment groups (Table 13) after 11 weeks on a WD with or without ω-3 fatty acid supplementation. Omega-3 supplementation did not significantly alter fasting insulin levels compared to WD although EPA tended to lower plasma insulin levels.

The concentration of circulating plasma adiponectin is usually proportional to lean tissue mass. Therefore, since DHA supplementation had the lowest increase in body

Table 13. Phenotypic characteristics for LDLr$^{-/-}$ mice on ω-3 supplementation.

LDLr$^{-/-}$ mice were fed a WD either supplemented or not with 1 % ω-3 fatty acid for 11 weeks. Liver weight was determined at sacrifice. *$p < 0.05$ vs. WD.

	Diet Supplement			
	WD	DHA	EPA	Omega Mix
Food Consumption (g/day/animal)	2.7 ± 0.2	3.3 ± 0.8	2.3 ± 0.1	2.6 ± 0.3
Initial Weight (g)	18.3 ± 0.5	20.0 ± 0.7	18.9 ± 0.7	19.7 ± 0.7
Weight Δ (g)	8.4 ± 0.9	6.2 ± 0.6	7.3 ± 0.4	11.9 ± 0.8
Liver wt (% body wt)	4.0 ± 0.1	3.7 ± 0.1	4.0 ± 0.2	3.6 ± 0.1*

Table 14. Plasma measurements for LDLr$^{-/-}$ mice on ω-3 supplementation.

Plasma from LDLr$^{-/-}$ mice fasted for 5 h was obtained after 11 weeks on a WD either supplemented or not with 1 % ω-3 fatty acid. Data are presented as mean ± S.E.M. (n=8-10). * $p < 0.05$ vs. WD.

	Diet Supplement			
	WD	DHA	EPA	Omega Mix
Glucose (mM)	10.7 ± 0.8	9.5 ± 0.6	10.6 ± 0.5	11.8 ± 0.6
Insulin (ng/mL)	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.9 ± 0.1
Adiponectin (μg/mL)	10.5 ± 1.4	18.8 ± 2.6	10.8 ± 1.3	14.7 ± 1.0
TG (mmol/L)	14.4 ± 3.0	3.6 ± 0.2*	3.6 ± 0.2*	3.6 ± 1.1*
TC (mmol/L)	21.2 ± 8.7	8.1 ± 1.5*	7.9 ± 0.8*	11.7 ± 3.4

weight (Table 12) it was not surprising that DHA supplementation resulted in an increase in adiponectin compared to WD ($P = 0.07$) although it was still lower than the level in a chow fed mouse of the same age (27.9 ± 1.3 μg/mL n=3). Previous research has shown that ω-3 supplementation (specifically EPA) has a hypotriglyceridemic effect (220), which is supported in the current study. All ω-3 supplements decreased plasma TG compared to WD (Table 13, $p < 0.05$). Plasma TC was also decreased by DHA and EPA supplementation compared to WD ($p < 0.05$). However the effect was not additive as the Omega Mix supplementation resulted in substantially higher plasma TC than either individual ω-3 ($P=0.07$ vs. DHA and $P=0.06$ vs. EPA).

The lipoprotein profiles were typical of the $LDLr^{-/-}$ mouse, in that the cholesterol profile showed peaks in both the LDL and HDL fractions which were attenuated by ω-3 supplementation to a WD (Figure 23). The decrease in plasma TG and TC that occurs as a result of ω-3 supplementation was associated with a decrease in the LDL fraction (Figure 23).

4.3.3. Quantification of Atherosclerosis

Atherosclerotic lesion development was assessed by quantifying lipid staining of the *en face* preparation (Figure 24A) and the aortic root cross section (Figure 24C) as previously described. The development or reduction in lesion development is the end point measurement as to whether the dietary interventions have been successful in reducing atherosclerotic risk. Given that DHA increased plasma adiponectin and decreased plasma TG and TC, it is not surprising that there was a significant reduction in lipid staining of the *en face* aorta preparation (Figure 24 B; $p < 0.05$) compared to WD.

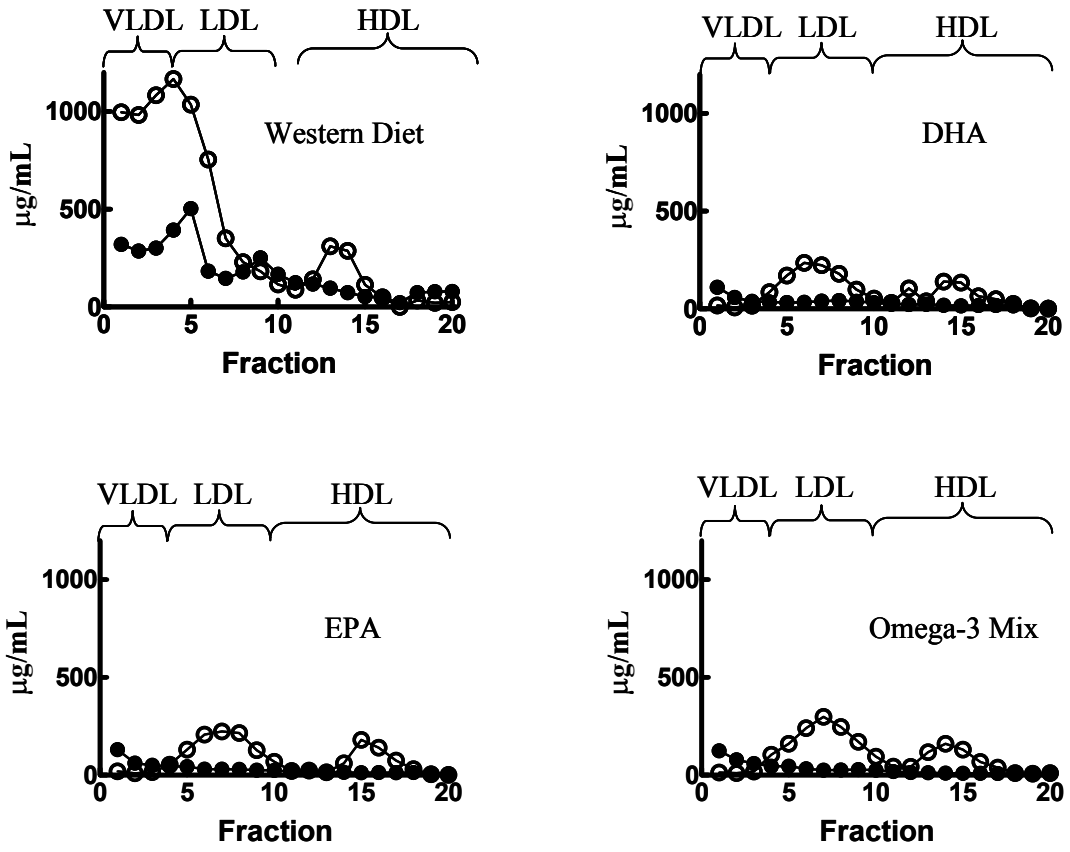


Figure 23. Plasma cholesterol and triglyceride lipoprotein profiles for LDLr$^{-/-}$ with ω-3 supplementation.

After 11 weeks on a WD either supplemented or not with ω-3 fatty acids serum was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and pooled plasma samples (n= 4-5) were separated by density gradient ultracentrifugation. Cholesterol (○) and triglyceride (●) were measured in each fraction.

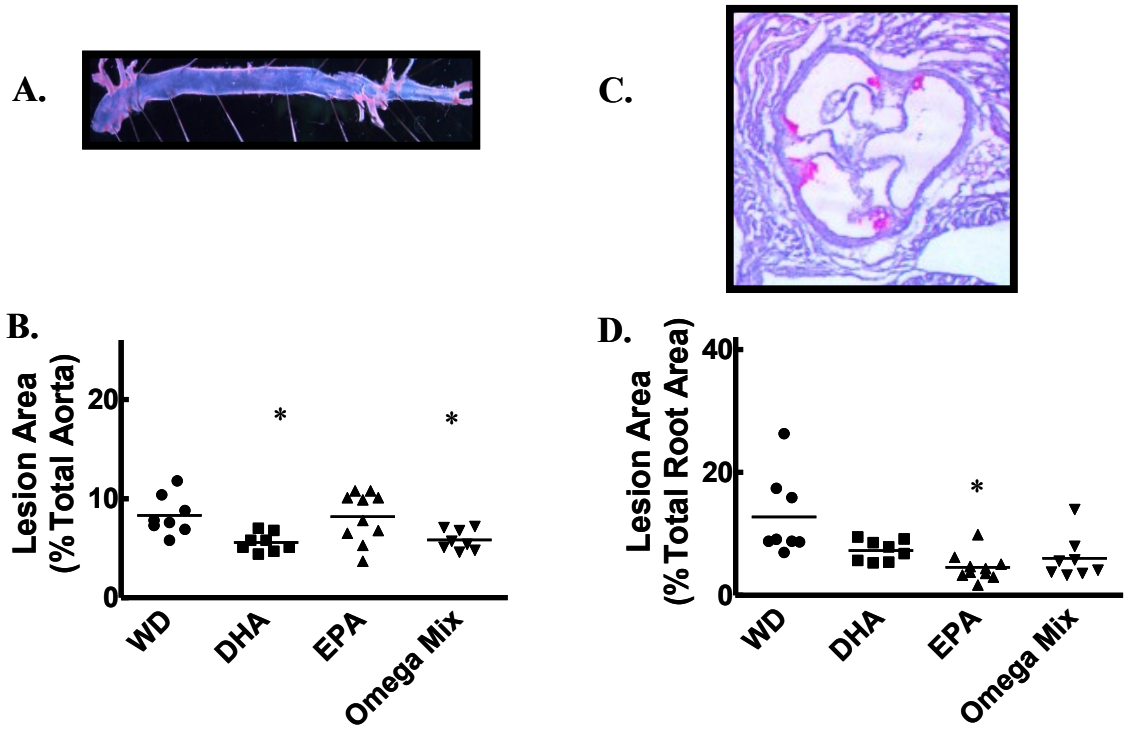


Figure 24. Atherosclerosis lesion measurements in aortic root cross-section and aortic *en face* preparations.

The images show a sample preparation of (A) *en face* and (C) aortic roots from LDLr$^{-/-}$ mice. Mice were fed a WD with or without supplementation as previously described. (B) Quantification of lesion area relative to total aorta area determined as described in the Materials and Methods. (D) Quantification of cross-sectional area of the aortic root occupied by lesion relative to total cross-sectional area determined as described in the Materials and Methods. In (C) and (D), each data point represents the mean of two measurements by independent researchers. Bars represent mean value for each group (n=8-10 animals per group). * $p < 0.05$ vs. WD.

However, even though the lesion area in the aortic root was also decreased compared to WD, this was not significant. EPA, with its similar TG and TC lowering effect, also resulted in a significant reduction in lesions in the aortic root (Figure 24 D; $p < 0.05$) but had similar lipid staining as the WD for the *en face* preparation. Omega Mix supplementation resulted in mixed plasma lipid improvements lowering TG but not TC. The increase in body weight with Omega Mix supplementation may have lowered plasma adiponectin compared to the individual ω-3 fatty acid, but there was still improvement in lesion development in the total aorta (Figure 24 B; $p< 0.05$). This is supported by previous reports that 6% fish oil supplementation to a high fat diet improved lipid metabolism and reduced atherosclerotic lesion development in association with an increase in circulating plasma adiponectin (324).

4.3.4. Liver Lipid Mass

The ω-3 supplementation did not have the same detrimental effects on liver lipid mass as the CLA supplements. Both DHA and EPA significantly decreased hepatic TG stores compared to WD (Table 15; $p< 0.05$). DHA and EPA decreased CE levels (Table 15) substantially compared to WD although this decrease, did not reach statistical significance ($P=0.09$). These results are supported by the fact that the liver appearance was not dramatically different from the WD, unlike the profound changes in liver morphology and size that were found with *t*-10 *c*-12 CLA supplementation (Table 9).

Table 15. Liver lipid mass for LDLr$^{-/-}$ mice on a ω-3 supplementation.

LDLr$^{-/-}$ mice were fed a WD either supplemented or not with 1 % ω-3 fatty acids for 11 weeks. Livers were obtained at sacrifice and lipids extracted in chloroform. TG, FC and CE were quantified by gas-liquid chromatography and PL by phospholipid C enzymatic assay. Data are presented as total mass per gram of liver weight (mean ± S.E.M.) (TG, triglycerides; FC, free cholesterol; CE cholesterol esters; PL phospholipids). *p<0.05 vs. WD.

	Diet Supplement			
	WD	DHA	EPA	Omega Mix
TG (mg/g liver)	41.3± 9.3	7.3 ± 0.9*	10.8 ± 2.6*	31.5 ± 5.3
FC (mg/g liver)	5.3 ± 1.3	4.5 ± 0.3	3.5 ± 0.2	4.4 ± 0.1
CE (mg/g liver)	8.1 ± 2.2	1.9 ± 0.3*	2.0 ± 0.4	4.3 ± 0.7
PL (mg/g liver)	10.9 ± 0.4	11.0 ± 0.7	9.6 ± 1.0	12.7 ± 0.2

4.4 Time Course Study

Given that *t*-10, *c*-12 CLA supplementation induced liver steatosis and hepatomegaly, believed to be related to the significant effect of this isomer on weight gain (Table 9), and plasma adiponectin and insulin (Table 10), a three week study was undertaken. Poirier et al. (295) have previously determined the chronology of alterations in adipose tissue, liver weight, hepatic TG accretion, plasma insulin levels and circulating adiponectin in the C57Bl/6J mouse. Even though our mouse model ($LDLr^{-/-}$) is on a C57Bl/6J background, it was felt that given the metabolic dysregulation that occurs in the absence of a functioning LDL receptor, it was imperative to confirm or establish the chronology for hepatic and plasma measurements.

4.4.1. Body and Liver Weight

Mice were placed on a WD or a WD supplemented with 0.5% *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA or the CLA Mix and monitored for changes in body weight and food consumption. Initial body weight was not significantly different between groups (Figure 25A). Food consumption was measured every 3 days and averaged per animal (Figure 25). In contrast to the 11 week study, where food consumption was unaltered by dietary supplementation, the CLA Mix animals consumed on average 1.8 g/day/animal which was significantly less than the WD (2.2 g/day/animal; $p < 0.001$). Animals were housed in similar conditions as in all previous studies and food was prepared in a likewise manner.

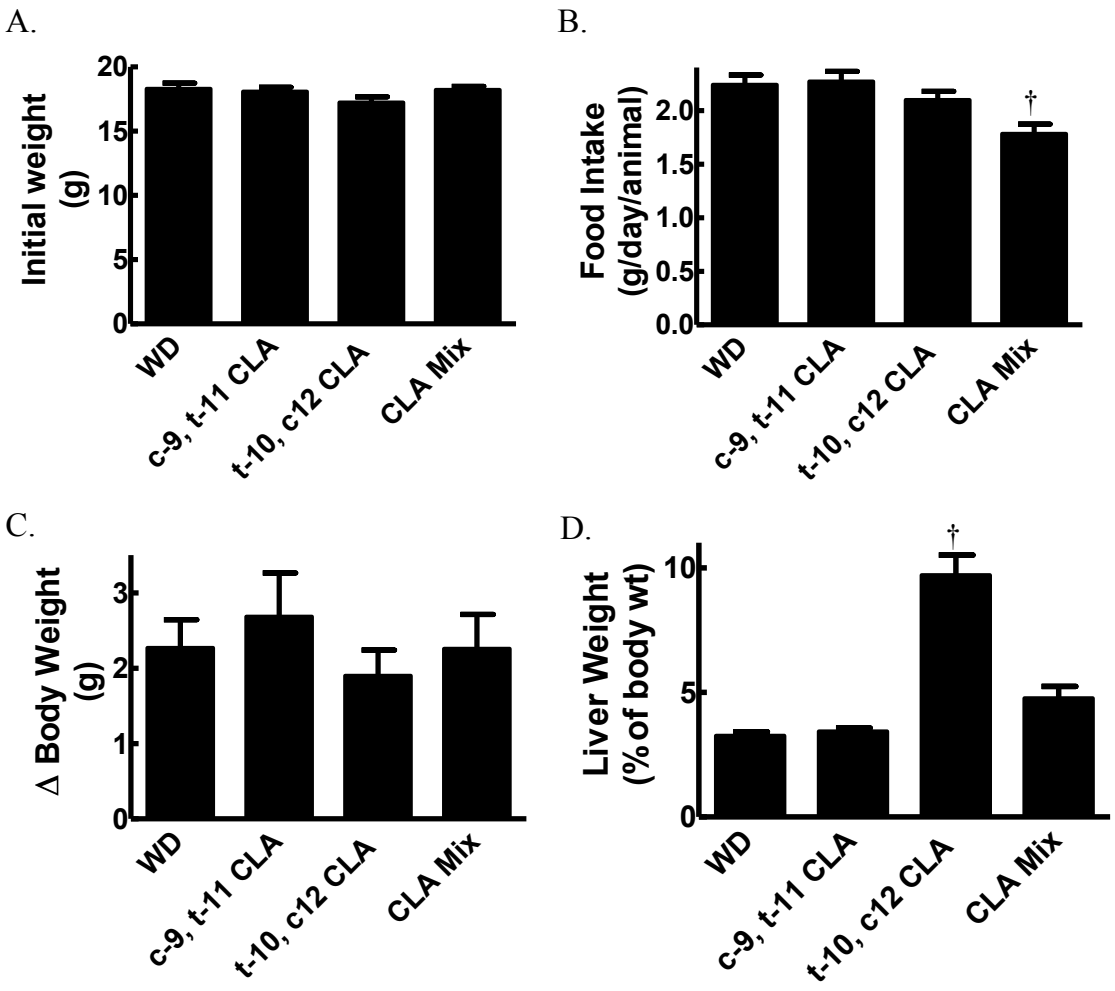


Figure 25. Phenotypic characteristics of LDLr$^{-/-}$ mice after three weeks on CLA supplemented diets.

Initial weight, food consumption, change in body weight and liver weight for animals on a WD either supplemented with or without 0.5% bioactive fatty acid. Each bar represents the mean ± S.E.M; (n = 5-8 animals). † $p < 0.001$ vs. WD.

Mice were monitored for signs of aggression and no signs of fighting or excessive grooming were reported. There have been previous reports that CLA alters food efficiency and therefore food intake but two previous studies in our laboratory did not observe these reductions. Therefore it is unknown as to why this group of animals reduced food consumption. Changes in body weight did not differ among the diet groups at the 3 wk time point (Figure 25 C) or at any early time points (data not shown). At sacrifice liver weight was obtained and the animals from the *t*-10, *c*-12 supplementation group had significantly larger livers (Figure 25 D; $p< 0.001$). Both WD and *c*-9, *t*-11 CLA had liver weights similar to chow fed animals ($3.6 \pm 0.1\%$ body weight; n=3).

4.4.2. Plasma Measurements

At 1 and 3 weeks of diet intervention blood was obtained via a cheek puncture and blood glucose, plasma adiponectin, insulin, TG and TC were measured. There were no changes in blood glucose among the diet groups (data not shown). As expected, plasma cholesterol was significantly increased by the WD (Figure 26A; $p< 0.001$) compared to chow fed animals by 3 weeks but not after one week. The TC in supplementation groups was also increased compared to chow by 3 weeks but not significantly different from the WD. Plasma TG were significantly increased by WD (Figure 26B; $p < 0.05$) compared to chow by 3 weeks. Although the *t*-10, *c*-12 CLA tended to have higher TG then WD and the *c*-9, *t*-11 and mixed isomer CLA groups tended to have lower TG than WD, there were no significant differences among the supplementation groups. As early as three days after initiation of the test diets plasma adiponectin was decreased in the *t*-10, *c*-12 CLA diet group (data not shown) and by one week was reduced by approximately 80% compared to WD (Figure 26C; $p < 0.001$).

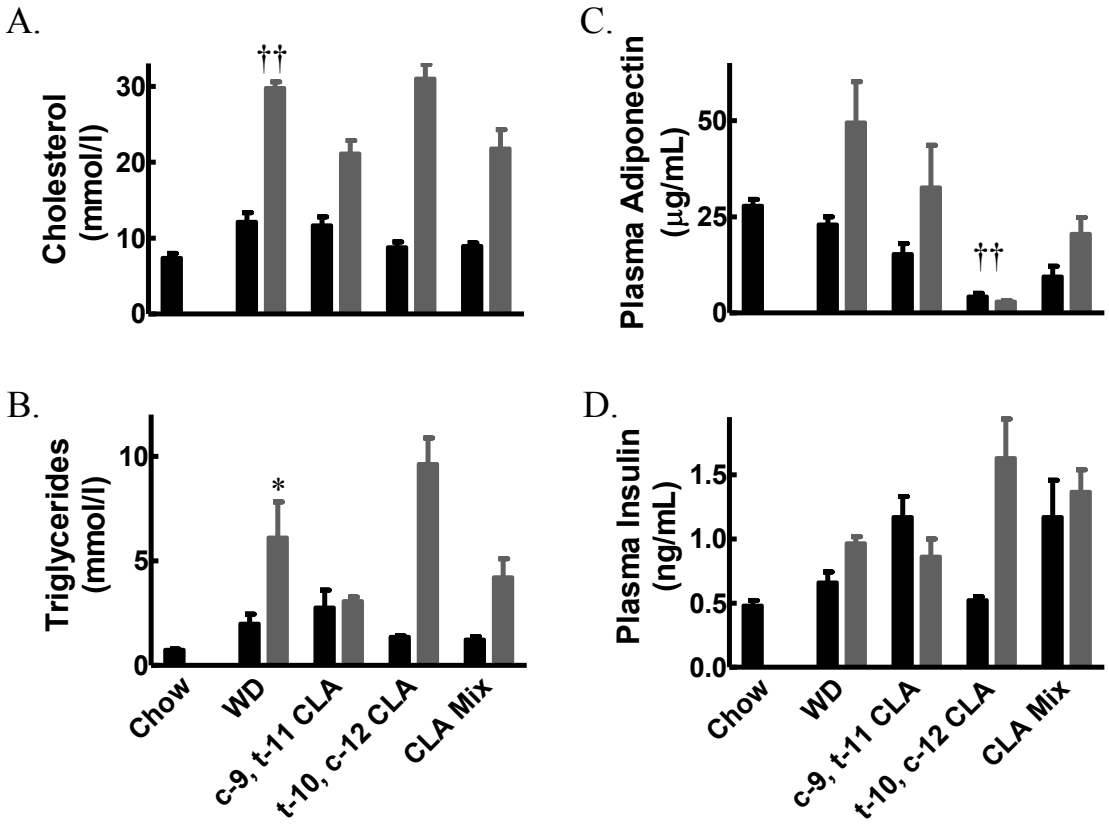


Figure 26. Adverse plasma profiles for *t*-10, *c*-12 CLA supplementation evident at 3 weeks.

After 1 week (black) or 3 weeks (grey) on a WD supplemented with bioactive fatty acids blood was obtained from LDLr$^{-/-}$ mice by cheek puncture. Plasma was separated from the blood cells and TC (A) and TG (B) were measured by enzymatic assay. Plasma adiponectin (C) and insulin (D) were measured by ELISA. Each bar represents the mean ± S.E.M; (n = 5-8 animals). *p< 0.05 vs. WD; ††o p < 0.01 vs. WD.

Interestingly after an initial decrease compared to the chow group at 1 week, the WD, *c*-9, *t*-11 CLA and CLA Mix diet groups all showed an increase in plasma adiponectin at 3 weeks (Figure 26C). The changes in adiponectin with *c*-9, *t*-11 CLA supplementation were not significantly different from WD. Plasma insulin was increased by both the *t*-10, *c*-12 CLA and CLA Mix by 1 week and continued to increase over 3 weeks of supplementation.

4.4. 3. Markers of ER Stress and Bioactive Fatty Acids.

When physiological stress is prolonged it can become a pathological stress condition disrupting ER homeostasis and leading to unfolded or misfolded protein accumulation in the ER lumen (105). Abnormal lipid accumulation or lipid biosynthesis has been associated with induction of a pathological stress (325;326). Ozcan *et al*. (113) have also reported a connection between insulin resistance and ER stress which can be alleviated with a chemical chaperone that can assist protein folding in the ER (127). Exposure to fatty acid and an induced ER stress response have been shown to decrease adiponectin receptor (AdipoR2) expression and decrease pAMPK in the liver (327). Since adiponectin was reduced by 85% within 1 week of initiating *t*-10, *c*-12 CLA supplementation (Figure 26C) levels of the ER stress protein GRP78 were measured in livers of $LDLr^{-/-}$ mice receiving CLA supplementation for 3 weeks. Although it was predicted from the hepatic steatosis and the loss of adiponectin in these animals that there would be an ER stress response, there was no difference in levels of GRP78 protein between *t*-10, *c*-12 CLA supplementation and other diet groups (Figure 27).

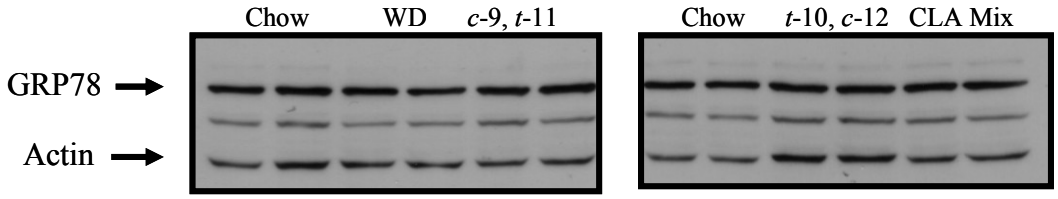


Figure 27. GRP78 protein levels in LDLr$^{-/-}$ mice.

Immunoblot analysis of the ER chaperone GRP78 in livers of LDLr-/- mice fed a HFLC CLA supplemented diet for 3 weeks.

4.5. Discussion

Most studies to date have utilized the C57Bl/6J or ApoE$^{-/-}$ strains to investigate metabolic processes altered by CLA. The LDLr$^{-/-}$ mouse represents a more moderate model of hyperlipidemia than the ApoE$^{-/-}$ and may provide a more responsive phenotype to assess the effect of bioactive fatty acids on plasma lipoprotein profiles, atherosclerotic lesions and hepatic lipid content (241). In addition CLA supplementation, studies were expanded to include ω-3 fatty acids, DHA and EPA, known to have cardioprotective properties. Fish oil, as a mixed compound, was originally attributed the cardioprotective effects, more recently the individual fatty acids, DHA and EPA, have been investigated. Much like CLA, DHA and EPA can have metabolic actions that are substantially different than the mixed compound.

Many of the perturbations associated with the *t*-10, *c*-12 CLA supplementation observed in the ApoE$^{-/-}$ were also apparent in the LDLr$^{-/-}$ mouse. However, caution is required when comparing results between mouse strains (241) due to the substantial differences in lipoprotein profiles caused by the genetic mutation interacting with the dietary manipulation. As previously observed all animals gained weight but the *t*-10, *c*-12 supplementation group gained only 50% of all other groups (Table 9) and had significantly enlarged livers (Table 9). Previous studies utilizing the LDLr$^{-/-}$ mouse model have reported diet induced obesity (241;304;324). Joven et al. (241) reported that ApoE$^{-/-}$ mice consuming a 20% fat (w/w) diet with varying amounts of cholesterol (0, 0.25, 0.5, 1%) were at every time point smaller than corresponding LDLr$^{-/-}$ mice. Interestingly, as dietary cholesterol increased weight gain was diminished (241). Diet-induced obesity was

not observed in the present study which may be a result of slightly lower dietary fat (16% w/w) and cholesterol (0.1%) content.

Many of the plasma changes observed in the ApoE$^{-/-}$ mice were reproduced while others were in complete opposition in the LDLr$^{-/-}$ mice. Plasma TC and TG levels were elevated in the LDLr$^{-/-}$ mice compared to ApoE$^{-/-}$ mice. It has been previously reported that the LDLr$^{-/-}$ strain is more sensitive to dietary factors such as fat and cholesterol content (241). In contrast to the ApoE$^{-/-}$ study, *t*-10, *c*-12 CLA fed LDLr$^{-/-}$ mice had substantially elevated TG and TC compared to all other diet groups (Figures 11 and 15). Both individual CLA isomers increased plasma TC and TG, in the LDLr$^{-/-}$ mice compared to WD but the CLA Mix supplementation, as previously observed, reduced TG and TC in comparison to WD (Figure 15). Given the more pronounced detrimental effect that *t*-10, *c*-12 CLA supplementation had on TG and TC in the LDLr$^{-/-}$ mice it is somewhat surprising that *t*-10, *c*-12 CLA did not increase insulin or decrease adiponectin to the same extent as in ApoE$^{-/-}$ mice (Tables 7 and 10). In many instances the biological activity of the CLA Mix resembled *t*-10, *c*-12 CLA supplementation in the ApoE$^{-/-}$ mouse but in regards to insulin and adiponectin levels in the LDLr-/- mice, CLA Mix had an intermediate effect between the two individual CLA isomers (Tables 7 and 10). The PCA results clearly show that *t*-10, *c*-12 CLA and CLA Mix supplementation result in a distinct clustering of animals based on diet intervention (Figure 17 upper panel). PC3 separates the animals predominately by adiponectin and liver weight while PC2 delineates the animals by TC and insulin (Figure 17 lower panel). Since only approximately 25% of the variation is accounted for by extracted factors othe, other parameters not included in the analysis or possibly not even measured are contributing significantly to the phenotype observed in the *t*-10, *c*-12 CLA supplemented animals. The

high variability in responses of *t*-10, *c*-12 CLA supplemented animals is evident by the horizontal distribution along the PC2 axis. Although the effects of CLA supplementation on adiponectin levels and liver weight were obvious from the raw data the more subtle effects of interactions between variables were more clearly illustrated by PCA.

The $LDLr^{-/-}$ study was expanded to include ω-3 fatty acids either individually (DHA, EPA) or as an Omega Mix (50:50 EPA, DHA). Most studies examining the cardio-protective effect of FO have used a mix with varying proportions of DHA and EPA. In the current study body weight gain over the 11 weeks was significantly increased with Omega-Mix supplementation (Table 12) compared to DHA or EPA supplementation. Mixed results have been reported in regards to FO supplementation and changes in weight. Previously weight gain with FO supplementation has been reported to not differ from non-supplemented control animals (324;328-330) and in other studies FO has been shown to increase body weight, specifically white adipose tissue (WAT)(221). The preservation of WAT function with ω-3 supplementatin resulted in decreased liver and plasma lipids and ameliorated atherosclerotic lesion formation. It has been suggested that the WAT performs as a buffer for circulating cholesterol, as evidenced by the increased cholesterol content of adipocytes in FO treated animals (221;324) and this was associated with decreased plasma cholesterol and CE in the liver. In the $LDL^{-/-}$ mice the DHA supplemented animals that had similar weight gain as LA and *c*-9, *t*-11 CLA supplemented animals but had substantially higher adiponectin levels (18.8 mmol/L vs. 13.2 mmol/L) suggesting that not only is preserving the mass of adipose tissue beneficial but DHA may stimulate proper adipose tissue functioning. FO has been associated with a 3-fold increase in adiponectin within 15 days in 129 SV mice (331) and a 70% increase in

LDLr$^{-/-}$ *ob/ob* mice (330). The hypolipidemic effects of FO have been well documented (219;221) and are supported by the findings in the current study. Omega Mix, DHA and EPA supplementation significantly reduced plasma TG and TC compared to WD (Table 14) which is in agreement with most FO studies (324;330;331) indicating that either individually or in combination DHA and EPA have lipid lowering properties. In contrast to studies in the LDLr$^{-/-}$ mouse, Xu et al. (328) reported that in the ApoE$^{-/-}$ mouse FO increased plasma TG within 4 weeks of starting supplementation and remained elevated over the next 8 weeks concurrent with no change in plasma TC. As well FO treatment reduced the n-6/n-3 ratio in all plasma lipid species relative to increased TG suggesting that lack of apoE may prevent the positive benefits of FO or specifically DHA/EPA from being expressed (328).

Few studies have reported on the effect of CLA supplementation in the LDLr$^{-/-}$ mouse. A double knockout strain (LDLr$^{-/-}$ApoE$^{-/-}$) was used to study the effectiveness of egg yolks either naturally enriched with CLA or supplemented with CLA on the regression of atherosclerosis (294). Both forms of CLA supplementation were able to significantly reduce the size of atherosclerotic lesion. Both of our current studies observed similar findings in that CLA Mix supplementation was able to reduce lesion development regardless of strain. The ApoE$^{-/-}$ mouse model is highly vulnerable to developing diet induced atherosclerosis lesions (261;262). Being a more moderate model of atherosclerosis was a primary reason for choosing the LDLr$^{-/-}$ mouse. We hypothesized that the CLA supplementation would have a more profound effect on reducing lesions. Overall the LDLr$^{-/-}$ mice were not more resistant to developing atherosclerosis lesions. WD and WD with *c*-9, *t*-11 or *t*-10, *c*-12 CLA supplementation was associated with lesions covering approximately 10-15 % of the total aorta regardless of mouse strain

(Figures 13 and 18) and this was significantly reduced, in both strains, by *t*-10, *c*-12 CLA and CLA Mix supplementation. Only with ω-3 supplementation did the LDLr$^{-/-}$ mice have an overall decrease in lesion development. Interestingly LA had a more profound effect in reducing lesion area in the LDLr$^{-/-}$ mice than the ApoE$^{-/-}$. The LDLr$^{-/-}$ aortic root cross-sections had more area covered by lesions than the ApoE$^{-/-}$ by almost 10% in most groups. These findings are supported by a parallel study in apoE$^{-/-}$ and LDLr$^{-/-}$ mice that showed that lesion area was similar between strains on a high fat diet with or without cholesterol (241). In contrast Wu et al. (304) reported that even in obese LDLr$^{-/-}$ mice there was no increase in lesion development between high fat fed mice, who developed mild hyperglycemia, obesity and insulin resistance, and mice receiving a low fat diet after 20 weeks of feeding.

Overall there was minimal lesion development in DHA, EPA or Omega-Mix animals (Figure 24). Total aortic lesion area (*en face*) was significantly reduced by DHA and Omega-Mix compared to WD while EPA significantly reduced lesion area in the aortic root compared to WD. Saraswathi et al. (324) reported a significant reduction in lesion size with menhaden oil supplement containing a higher proportion of EPA than DHA. In contrast, neither FO (22% EPA, 17% DHA) nor 40% DHA supplementation on a 16% fat diet with 0.25% cholesterol showed reduction in plaque development (329) which was similar to findings in the ApoE$^{-/-}$ mouse (328;332). Lesion development is highly variable and extremely sensitive to dietary manipulations. Therefore it is not surprising that there is disagreement between studies as even within our own work the WD fed mice that served as the control group for the CLA study had on average 16.4 % of the aorta covered in lesions (Figure 18) compared to the WD control for the ω-3 study

where we reported that 8.3 % (Figure 24) of the aorta was covered in lesions. The only distinguishing variable between the two WD groups was a 2-fold increase in plasma TG (Figure 15 vs Table 13) which surprisingly was in the WD group in the ω-3 study.

Linoleic acid has been suggested to have biological activities that may improve lipid profiles and reduce atherosclerosis (284). In the previous ApoE$^{-/-}$ HFHC diet study the influence of LA supplementation was not readily apparent although there were slight reductions in liver TG stores, CE and FC concentrations (274) none were significantly different from WD or CLA supplementation. With the lower dietary cholesterol the reductions were still moderate but consistent in that liver weight was decreased compared to WD and *c*-9, *t*-11 CLA concomitant with a decrease in TG stores compared to both WD and *c*-9, *t*-11 CLA. In the more responsive LDLr$^{-/-}$ mouse model the LA supplementation resulted in decreased weight gain compared to WD (P=0.08) and similar to the ApoE$^{-/-}$ mouse, moderate decreases in liver weight (Table 9) accompanied by significantly reduced CE levels and significantly decreased TG stores (Table 11) compared to *c*-9, *t*-11 and *t*-10, *c*-12 CLA supplementation groups.

The regulation of lipid metabolism involves numerous transcription factors, and multiple signalling pathways that can be influenced by bioactive fatty acids or their metabolites. PPARα is the transcriptional regulator most often associated with CLA biological activities. It has been demonstrated that the individual isomers are potent ligands and activators of PPARα (315). Dietary supplementation with CLA Mix and to a lesser degree *c*-9, *t*-11 CLA, but not *t*-10, *c*-12 CLA, resulted in increased expression of PPARα mRNA. The slight increase in PPARα mRNA expression with *c*-9, *t*-11 CLA was more evident in assessment of protein levels. This divergent activation of PPARα

between individual CLA isomers reflects the physiologic changes in the mice. The phenotype of the *t*-10, *c*-12 CLA supplemented animal closely resembles that of a PPARα knockout animal in that both have increased hepatic TG, especially after fasting (333), increased VLDL production and impaired clearance of TG-rich lipoproteins resulting in increased plasma TG (334). These changes reflect the liver PPARα mRNA and protein levels where there is no substantial influence of *t*-10, *c*-12 CLA on PPARα activation. Some reports have indicated that adiponectin, possibly through PPARα signalling pathways, can increase insulin sensitivity in the liver and muscle (61;66), and thus a lack of PPARα activation, secondary to reduced levels of adiponectin, may be the mechanism for increased insulin resistance in the *t*-10, *c*-12 supplemented mice. In comparison, the *c*-9, *t*-11 CLA supplementation group had lower plasma TG and TC and reduced hepatic TG possibly as a result of increased PPARα stimulated hepatic LPL clearance of TG-rich lipoproteins (316) and subsequent β-oxidation of the fatty acids. PPARγ, another reported target of the CLA isomers (202-204) may also have anti-diabetic effects, as observed in some models (202). Both CLA Mix and *t*-10, *c*-12 CLA supplementation groups had substantially increased levels of PPARγ yet there were no differences between any dietary groups at the protein level. Therefore, more studies will be necessary to elucidate the role of PPAR proteins in the mechanism of CLA action.

SREBP is another key regulator in lipogenesis and its dysregulation has been connected to hepatic steatosis. Inconsistent results have been observed between species as the *t*-10, *c*-12 CLA, but not the *c*-9, *t*-11 CLA isomer, decreased hepatic levels of SREBP-1c mRNA in OLETF rats (335) yet in *ob/ob* mice it was the *c*-9, *t*-11 CLA isomer that was associated with a significant reduction in hepatic SREBP-1c mRNA

expression and improved lipid and glucose metabolism in the liver (292). In contrast, in the current study it was *t*-10, *c*-12 CLA and CLA Mix supplementation that had increased SREBP-1c protein levels which could potentially result in increased plasma lipid levels. Further analysis of SREBP-1c expression, by nuclear fractionation, revealed that there was a 2-fold induction in n-SREBP-1c with *t*-10, *c*-12 CLA supplementation. Under normal physiological conditions, insulin stimulates the lipogenic pathway through increased cleavage of the SREBP-1c protein. Paradoxically, it has been reported that lipogenesis is very active in the livers of *ob/ob* mice even though they are severely insulin resistant as characterized by glucose overproduction and hyperglycaemia. Accumulation of n-SREBP-1c has also been reported in these animals (92). Although not measured directly in the current study, the hyperinsulinemia observed with *t*-10, *c*-12 CLA supplementation is predicted to result in insulin resistance with a decrease in insulin signalling. Yet SREBP-1c maturation seems to proceed unabated which could suggest that this processing is mediated by signalling through an alternate pathway (IRS1 vs. IRS2).

A number of recent reports have emphasized the link between the ER stress response and hepatic lipid metabolism, mainly through genetic knockout studies. Rutkowski *et al*.(336) showed that unresolved ER stress, as induced by tunicamycin, in mice deficient in genes encoding ER stress pathway regulators resulted in hepatic lipid accumulation. Kammoun *et al*. (119) proposed that hepatic ER stress is the link between unabated lipogenesis in insulin resistant animal models. Demonstration that ER stress activated the lipogenic pathway by increasing SREBP-1c cleavage and that it could be inhibited by the over-expression of the ER stress chaperone GRP78 provides insight as to how SREBP-1 cleavage could occur in an insulin independent manner (119). We

investigated the ER stress marker GRP78 and no differences in protein level were detected between any of the dietary groups. The WD may be enough of an assault on the liver to induce an ER stress response and that further perturbations with CLA supplementation are too subtle to detect with a single marker of ER stress.

With the dramatic reduction in adiponectin and increased liver weight already evident at 3 weeks with *t*-10, *c*-12 CLA supplementation, further investigation is warranted to understand the connection between lipogenic gene regulation, insulin resistance and ER stress response. The differences in phenotypes between the *c*-9, *t*-11 CLA and the *t*-10, *c*-12 CLA supplementation groups may result not because of an ER stress response, but rather due to the lack of an ER stress response. *c*-9, *t*-11 CLA supplementation resulted in marginally elevated plasma TG and TC and hepatic TG while the *t*-10, *c*-12 isomer had significantly elevated plasma TC and hepatic TG stores and marginally elevated TG along compared to WD animals. Hepatic transcriptome studies conducted in the ApoE3 Leiden mouse (337) suggested that three phases of adaptation may occur in the liver in response to high fat feeding. The early response phase was shown to involve the induction of genes typical of lipid metabolism PPARα, suggesting that the liver was increasing β-oxidation to accommodate the increased flux of FFA to the liver. Increased expression of PPARγ and SCD-1mRNA, the authors suggested, indicated an adipogenic transformation of the hepatocytes (337). From the current study our data suggests that *c*-9, *t*-11 CLA supplementation may be increasing the expression of PPARα mRNA in an adaptive response to the FFA flux (Figure 22C). In contrast, the *t*-10, *c*-12 CLA group is already in the second phase of the response, showing signs of ectopic accumulation of hepatic TG stores, with concomitant increased expression of SCD-1 and

PPARγ at the mRNA level. The response to lipid management appears to be a concerted activation of both lipolysis and hepatic lipid storage and the balance may be tipped by the actions of bioactive fatty acids.

CHAPTER FIVE –STUDIES IN THE SYRIAN GOLDEN HAMSTER

5.1. *In vitro* Studies

The hamster may be particularly appropriate for studies that have translational application to humans because of their similar lipoprotein systems (249). While most rodents carry plasma cholesterol primarily in the high density lipoprotein (HDL) fraction, the hamster can transport substantial amounts of cholesterol in the LDL and VLDL lipoproteins when the diet contains cholesterol (240;249;338). In addition, hamster and human livers both produce VLDL containing only apolipoprotein B100 (apoB100) (339), while the rat and mouse produce both apoB100- and apoB48-containing VLDL particles. As in the human system, the hamster has plasma cholesterol ester transfer activity (340), whereas the rat and mouse do not. Unlike other rodents, when challenged with a high fat high cholesterol diet (western diet), the hamster carries the additional plasma cholesterol in the very low density lipoprotein (VLDL), rather than the high density lipoprotein (HDL) (240;249). Previously, we have reported that HFHC-fed hamsters have significantly elevated plasma TC, VLDL-TG and VLDL-TC compared to chow-fed animals (253). Further, we were unable to show a significant improvement in these parameters with CLA supplementation but there was substantial improvement in atherosclerotic lesion development (253) even though these lesions were very small. In conjunction with an animal model of insulin resistance, we wished to extend these studies and compare the effects of individual bioactive fatty acids on the synthesis and secretion of hepatic VLDL. The fructose-fed hamster model has proven to be very informative in studying the effects of dietary manipulations in a hepatic insulin resistant animal (341).

Fructose feeding for a 2 week period induces insulin resistance accompanied by increases in plasma TC, TG and FFA in the absence of obesity (341). Fructose-induced insulin resistance is associated with a significant increase in the synthesis and secretion of VLDL-TG in the hamster (341). In the current study we evaluated the effects of bioactive fatty acid supplementation on both a HFLC and 60% fructose diet in hamsters.

5.2. VLDL Assembly in the Presence of Bioactive Fatty Acids

Once they are taken up by the hepatocyte, fatty acids can be metabolized via the β-oxidation, or esterification pathways and the disposition will depend to some extent on the nutritional status of the animal. For our purposes esterification into PC and TG was examined in the context of the role of these lipids as lipid sources for VLDL assembly. It is known that fatty acids of differing chain lengths and degrees of unsaturation are metabolized differently (342) and therefore we investigated whether this fate would be altered by the bioactive fatty acids.

The normal human plasma concentration of unesterified fatty acid is between 0.4 and 0.7 mM (343) and rat hepatoma cells have been cultured at fatty acid concentrations between 0.2 and 0.8 mM. A concentration of 0.4 mM fatty acid was utilized as this concentration is shown to maximally stimulate apoB synthesis and is not toxic to primary hepatocytes (Section 2.3.4.1.).

VLDL assembly requires two components; a properly folded apoB protein and a sufficient lipid supply. Both of these components were examined *in vitro* in hamster hepatocytes. Hamster hepatocytes were isolated from fasted chow-fed animals and

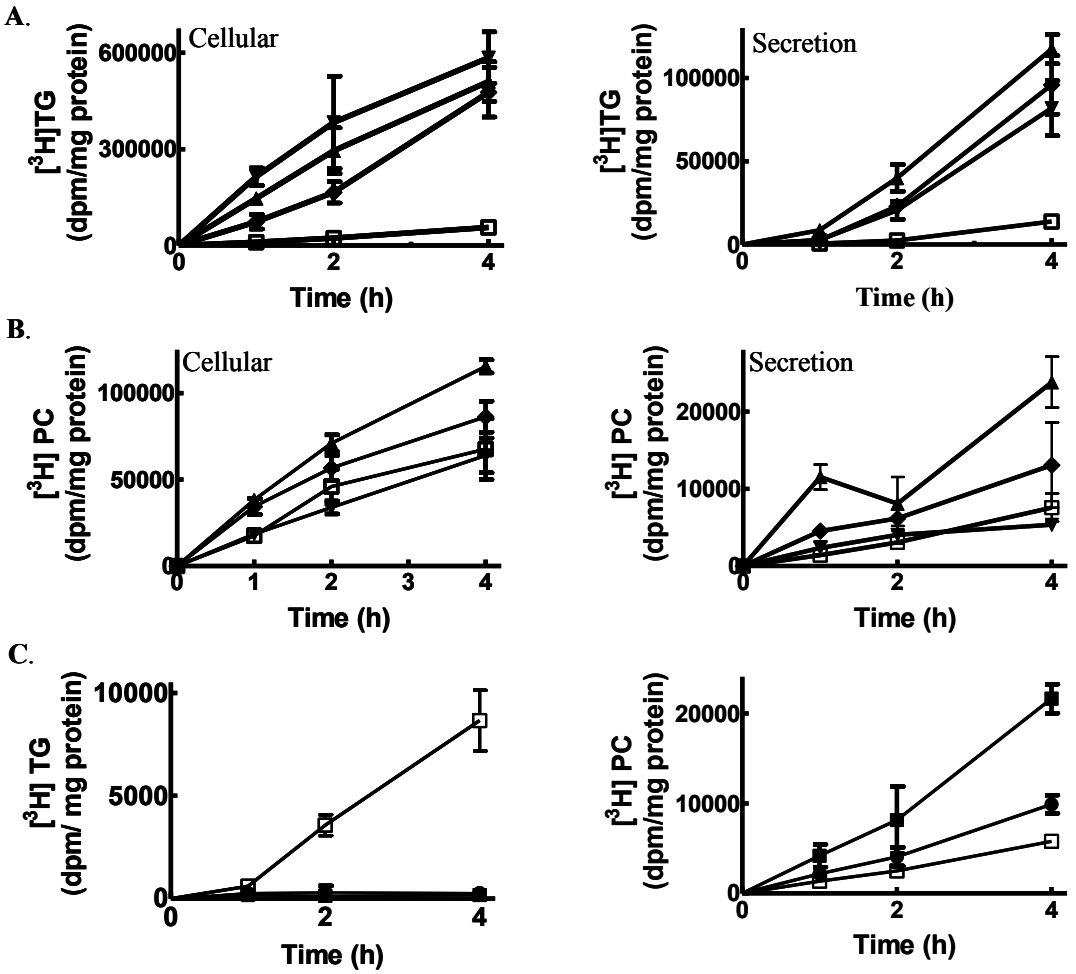


Figure 28. Incorporation of [^{3}H]glycerol into lipids.

Hamster hepatocytes were labelled up to 4 h with the indicated fatty acid (400 μM) and 10 μCi of [^{3}H] glycerol. Cells and medium samples were collected at the indicated time and lipids extracted, separated by thin layer chromatography and radiolabel in lipid species were quantified by liquid scintillation counting **(A.)** Cellular and secreted TG and **(B.)** cellular and secreted PC with CLA supplementation. **(C.)** Secreted TG (Left panel) and PC (Right panel) with ω-3 supplementation. □ Control, ▲, LA; ▼, *c*-9, *t*-11 CLA; ◆ *t*-10, *c*-12 CLA, ■ EPA, ● DHA. Results are expressed as mean ± S.D of triplicates.

bioactive fatty acid supplementation was initiated after cells were allowed to adhere for 4 h. Viability of hepatocytes was between 70-85% and all experiments were completed within 24 h of isolation. To observe the effect of fatty acid treatment on newly synthesized lipids, hamster hepatocytes were labelled with [^{3}H] glycerol and 10% BSA alone (Control) or containing 0.4 mM of LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, DHA or EPA for 4 h. Newly synthesized and secreted TG and PC were separated by TLC and quantified by scintillation counting (Figure 28A and B). All fatty acid treatments resulted in nearly linear incorporation of [^{3}H]glycerol into TG over the 4 h time period. However, there were some differences in the extent of radiolabel incorporation depending on the fatty acid supplement. When no fatty acid was present in the medium (Control, Figure 28A or C) there was almost no [^{3}H]glycerol incorporation into cellular or secreted TG. In contrast, when exogenous fatty acid was present in the medium there was rapid incorporation into cellular TG and 15-25 % of the radiolabelled TG was secreted (Figure 28A). This would suggest that increased TG synthesis does not result in an immediate increase in secretion; rather it increases the intracellular pool of TG, leaving the hepatocyte at risk of increased TG accumulation. Thus, continuous exposure to fatty acids may eventually lead to steatosis. [^{3}H]glycerol incorporation into secreted TG was severely diminished by the presence of either DHA or EPA in the medium (Figure 28C) while the control (no fatty acid) showed linear secretion over the 4 h incubation period.

Phospholipids are an integral component of every membrane in the cell in addition to their role in lipoprotein assembly. Therefore, interference in the synthesis or secretion of PL can disrupt cellular homeostasis. All fatty acids treatments resulted in nearly linear incorporation of [^{3}H]glycerol into phosphatidylcholine (PC) over the 4 h time period (Figure 28B). When no fatty acid was present in the medium there was

minimal [^{3}H]glycerol incorporation into cellular or secreted PC (Control) which surprisingly, was similar to *c*-9, *t*-11 CLA. LA, when provided as the exogenous fatty acid, rapidly increased the incorporation of [^{3}H]glycerol into PC and overall had the greatest incorporation into radiolabelled PC of all treatments. Analysis of PC secretion was complicated by the high turnover rate of PC in membranes and a minimal amount (5%) of [^{3}H]glycerol labelled PC was secreted.

In order to begin to understand the potential role of bioactive fatty acids in hepatic lipoprotein metabolism, metabolic labelling studies were used to investigate the synthesis, secretion and degradation of apoB100. Using pulse labelling, the level of apoB100 synthesis was determined and by pulse-chase experiments the degradation and secretion of apoB100 were followed over time. For each experimental condition, hamster hepatocytes were subjected to a 1 h pre-treatment with the appropriate fatty acid. Following the pre-treatment cells were incubated in medium containing [^{35}S]methionine/cysteine and the appropriate fatty acid for 1 h. For the chase experiments the radiolabelling medium was then removed and replaced with medium containing the test fatty acid mixture and excess unlabelled methionine/cysteine and chased for up to 2 h. ApoB was recovered from the cell lysate and medium by immunoprecipitation, and radioactivity associated with apoB100 was quantified. The stability curve (Figure 29A) shows that over the course of 2 h approximately 50% of the apoB100 was secreted and 50% remained in the cell. This stability/secretion profile is representative of many studies performed in our lab and shows that minimal apoB degradation occurs in the primary hepatocyte. This was also true in the presence of supplemented fatty acid. As shown in

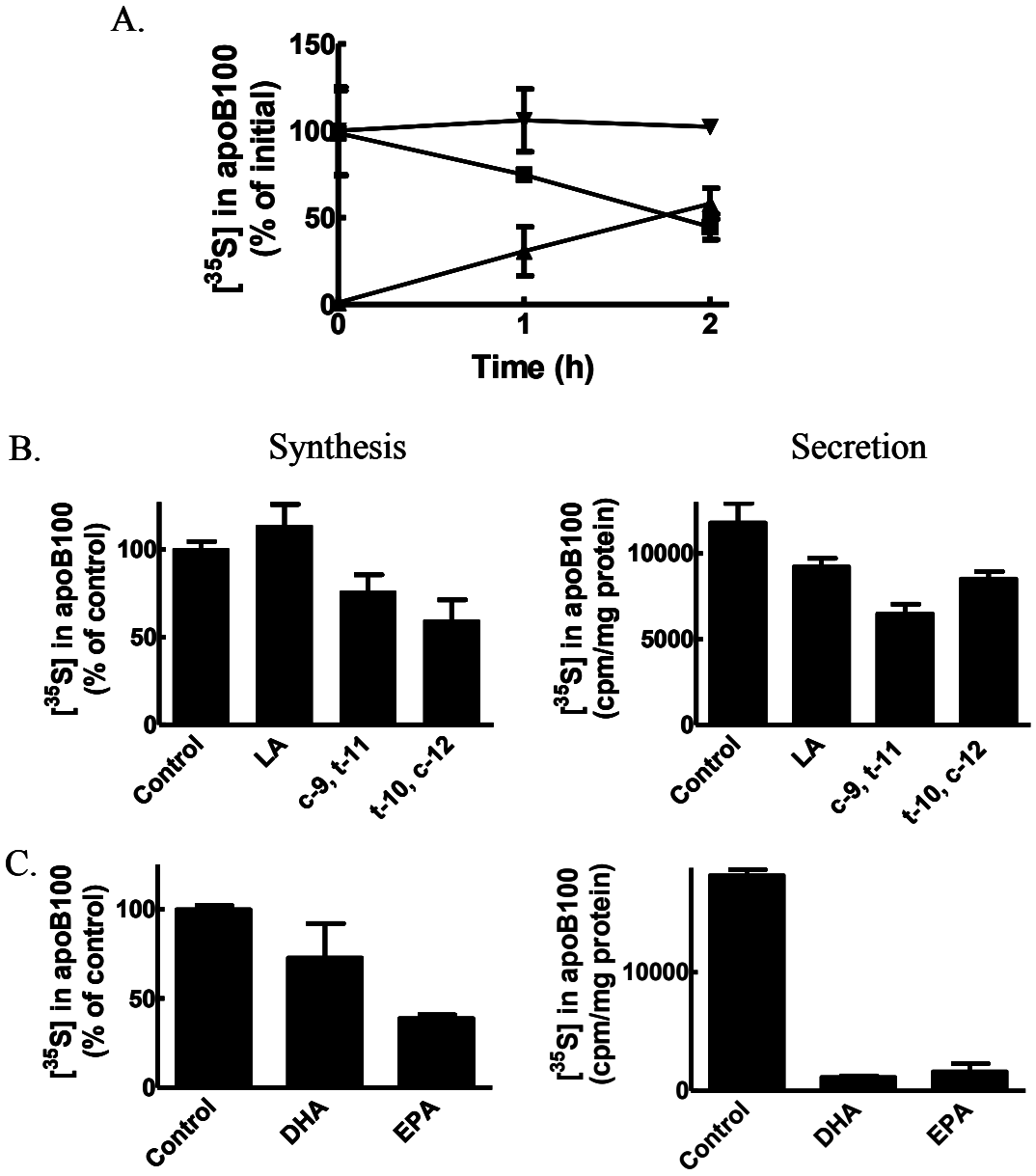


Figure 29. Bioactive fatty acids decrease ApoB100 synthesis and secretion.

Hamster hepatocytes were pulsed-labelled for 60 min with [^{35}S] methionine/cysteine in the presence of 400 μM of the indicated fatty acid. (**A).** Representative apoB100 stability curve for control conditions; ▼, Total apoB 100; ▲, secreted apoB100; ■, cellular apoB100. (**B.) and (C.)** Synthesis: ApoB was recovered from cell lysate by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity associated with apoB100 was quantified by liquid scintillation counting. Secretion: The labelling medium was removed and replaced with chase medium containing 400 μM of the indicated fatty acid. Two h after initiating the chase, apoB was quantified as with cells**.** Results are expressed as the mean ± S.D. of triplicates.

Figure 29 (B and C), each of the five fatty acids stimulated incorporation of [^{35}S]methionine/cysteine into apoB100 to varying degrees. Incubation with the CLA isomers resulted in approximately 25-30% reduction in labelling as compared to control. LA stimulated radiolabel incorporation into apoB100 even greater than fatty acid free medium by about 14%. DHA and EPA both reduced apoB100 synthesis substantially compared to control. These observations suggest that bioactive fatty acids may suppress hepatic synthesis of apoB100.

Figure 29 (B and C, right-hand panels), shows the radiolabelled apoB100 secretion over a two hour time period. When the medium contained LA, approximately 20% less apoB100 was secreted and with *t*-10, *c*-12 CLA approximately 30% less apoB100 was secreted compared to control. In agreement with the glycerol labelling studies where *c*-9, *t*-11 CLA substantially decreased TG secretion, both apoB100 synthesis and secretion were reduced. DHA and EPA substantially decreased apoB secretion as minimal apoB radiolabel was detected in the medium at 2 h (Figure 29C).

As it is both the composition and the number of apoB-containing lipoprotein particles that influences the development of atherosclerosis, radiolabelled apoB100 was analysed by density gradient ultracentrifugation. From the studies of [^{3}H]glycerol incorporation into secreted TG and the [^{35}S]methionine/cysteine labelling of secreted apoB100, it was predicted that the number of apoB100 containing particles would be reduced by the bioactive supplements, but it was possible that the particles would be more dense due to a decrease in TG content compared to control. The density gradient analysis (Figure 30) indicated that *t*-10, *c*-12 CLA fatty acids stimulated secretion of apoB100 containing

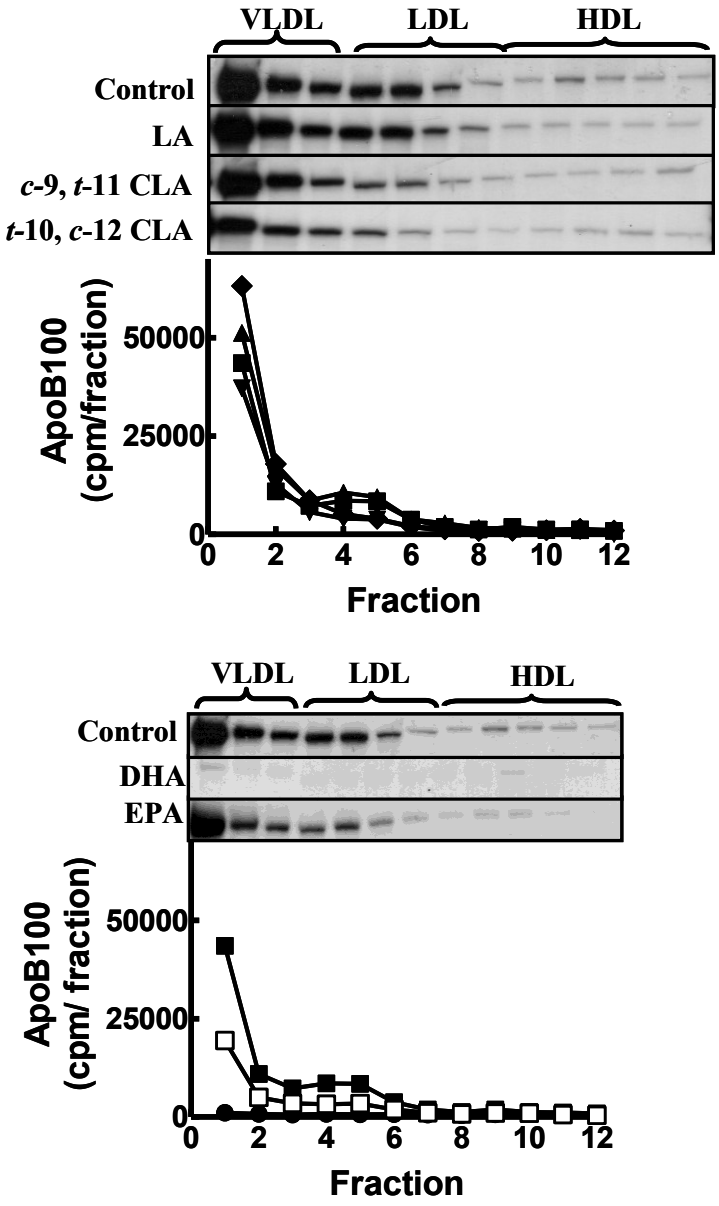


Figure 30. ω-fatty acids decrease VLDL assembly but CLA isomers have little effect on VLDL assembly.

Hamster hepatocytes were isolated and pulse-labelled for 60 min with [^{35}S] methionine/cysteine followed by a 24 h chase with fatty acid in medium. Lipoproteins were separated by sucrose density gradient ultracentrifugation into 12 equal fractions from the top (fraction 1) of the gradient. Radioactivity associated with apoB100 was quantified by liquid scintillation counting. ■ Control, ▲LA, ▼ *c*-9, *t*-11 CLA, ♦ *t*-10, *c*-12 CLA, □ EPA, ● DHA.

VLDL, whereas LA and *c*-9, *t*-11 CLA had little effect compared to control. In contrast, EPA and DHA decreased the amount of apoB100 in the VLDL density region of the gradient. None of the fatty acids promoted the secretion of lipoproteins of higher density.

5.3. Dietary Supplementation with Bioactive Fatty Acids

We have previously collected data on the HFHC-fed hamster and shown that the deranged lipoprotein profiles are evident at two weeks (253). In the current study, two dietary conditions were examined: HFLC and 60% fructose, each supplemented with either an individual CLA isomer, with DHA or with EPA. The HFLC study involved the feeding of 2 animals per dietary treatment for 6 weeks in order to analyze apoB100 synthesis and secretion, and VLDL assembly. In the fructose-fed hamster study, 9 animals were used per dietary treatment group for a total of 6 weeks. Every 2 weeks, 3 animals were subjected to glucose and insulin tolerance testing along with collection of plasma. One animal was designated for hepatocyte preparations while for the others the liver was flash frozen and utilized for further analysis.

5.3.1 Food Consumption and Body Composition.

After 1-2 weeks of acclimation to the animal unit on chow diet, animals were separated into diet groups and housed two per cage. Food and water were available *ad libitum* and the hamsters were weighed each week and food weighed and replaced every three days. Unlike our previous HFHC study, in which the high fat-fed animals adjusted their food intake, resulting in similar daily caloric intake to chow, the animals consuming

Table 16. Phenoytpic characteristics of fructose-fed Syrian Golden Hamsters.

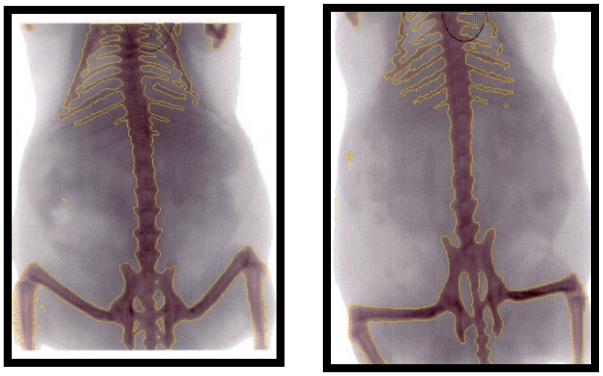
	Dietary Supplementation				
	Base	**Fructose**	***c*-9, *t*-11 CLA**	**DHA**	**EPA**
Food Consumption (g/animal/day)	6.1± 0.5	8.1± 0.3*	7.5± 0.4	8.0± 0.3*	7.7± 0.2
Liver Weight (% BW)	4.9 ± 0.3	4.9 ± 0.3	5.3 ± 0.4	4.8 ± 0.4	4.1± 0.3
TG (mmol/L)	4.4 ± 0.6	5.1 ± 0.2	3.1 ± 1.3	4.1 ± 0.3	5.4 ± 0.2
TC (mmol/L)	3.7 ± 0.2	3.2 ± 0.4	3.4 ± 0.1	3.1 ± 0.1	3.5 ± 0.1

Liver weight (n-=9), plasma TG and TC were obtained at 6 week sacrifice (n=3). * $p<0.05$ vs. Base.

a diet supplemented with either fructose or DHA all had significantly increased food intake ($p<0.05$, Table 15). A mixed design ANOVA was utilized to test for interactions between diet supplementation and changes in body weight and body fat. All diet supplement groups had a significant increase in body weight over the six weeks but there was a non-significant main effect of diet $F(1,10)$, $P=0.68$. There was a significant main effect of time as all animals, regardless of diet group, weighed significantly more at four weeks ($F(1,10) =43.7$, $p<0.001$) and six weeks ($F(1,10)=29.5$, $p<0.001$) compared to 2 weeks. There was no diet supplementation x time interaction ($F(4,10)=0.64$, $p=0.6$). Although both the DHA supplemented and fructose only animals increased their percentage body fat by 10.5% over the 6 weeks as measured by DEXA, there was a non-significant main effect of diet supplementation alone on body fat accumulation ($F(1,10) =3.49$, $P=0.05$). Percent body fat, regardless of dietary supplementation, was significantly increased at 4 weeks ($F(1,10) = 10.7$ $p< 0.01$) and at 6 weeks ($F(1,10)=18.7$, $p< 0.01$) compared to 2 weeks. Similar to body weight, there was no diet supplementation x time interaction ($F(1,4)=2.8$, $P = 0.08$).

5.3.2 Plasma Measurements

Fructose-fed hamsters had higher plasma TG and TC (Table 15) compared to chow-fed animals (0.84 and 1.04 mmol/L, respectively) but this increase was due almost exclusively to differences between chow and the semi-purified base diet. While the fructose-fed animals had a 5-fold increase in TG compared to chow, there was only a modest increase in TG over the base diet (Table 15). Similarly, the TC levels in fructose-fed animals were not different from the base diet. Therefore the base diet that was used for the fructose supplementation was used as the control group instead of chow.



31.5 % BF 37.6 % BF

	Diet Supplementation				
			c-9, *t*-11		
	Base	Fructose	CLA	DHA	EPA
	Δ Body wt (g)				
2 week	15.5 ± 7.7	22.3 ± 0.7	23.4 ± 1.6	23.8 ± 10.4	25.8 ± 6.6
4 week	24.9 ± 9.7†	38.0 ± 1.7†	35.9 ± 3.0†	40.5 ± 7.2†	35.1 ± 7.8†
6 week	42.4 ± 7.3†	47.5 ± 3.6†	34.3 ± 9.4†	53.4 ± 5.1†	45.0 ± 11.7†
	Δ Body fat (%)				
2 week	-1.6 ± 3.3	5.4± 0.6	3.8 ± 0.5	0.6 ± 1.6	1.8 ± 1.4
4 week	-2.5 ± 3.0*	6.7 ± 1.3*	6.5 ± 1.3*	4.5 ± 0.6*	2.7 ± 0.9*
6 week	2.1 ± 2.3*	10.5 ± 2.7*	3.1 ± 2.6*	10.5 ± 1.9*	5.6 ± 2.0*

Figure 31. Body composition changes associated with bioactive fatty acid supplementation.

Body weight and percentage body fat of Syrian Golden hamsters fed fructose diets supplemented with bioactive fatty acid. **A.)** Sample DEXA images from which weekly body fat was calculated. **B.)** Change in body weight and change in percentage body fat. For each diet group n=3. † $p<0.001$ vs. week 2 for each diet group; * $p<0.01$ vs. week 2 for each diet group.

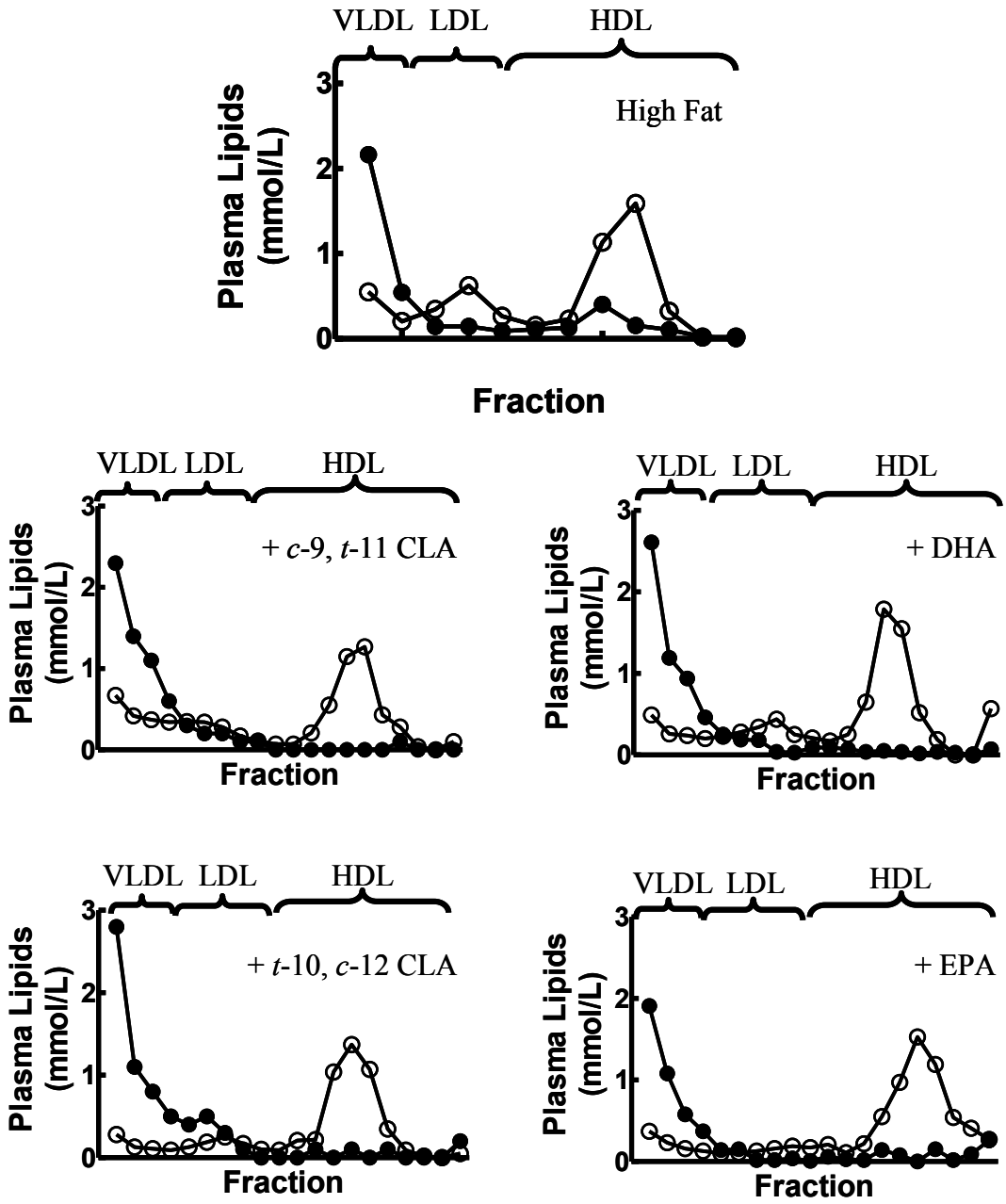


Figure 32. Plasma cholesterol and triglyceride lipoprotein profiles for Syrian Golden Hamsters.

Hamsters were fed a HFHC diet supplemented with or without 1% bioactive fatty acid for 6 weeks. Serum was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and pooled plasma samples (n=2) were separated by density gradient ultracentrifugation. Cholesterol (○) and triglyceride (●) were measured in each fraction.

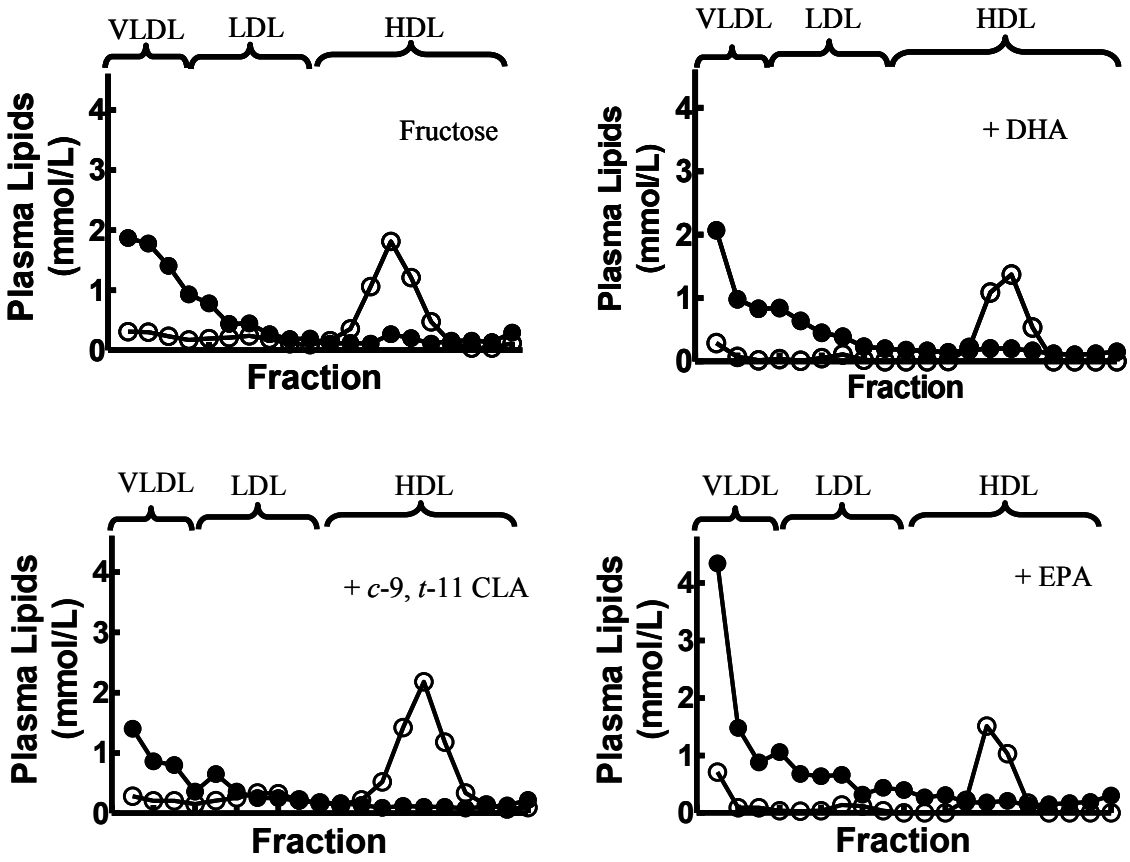


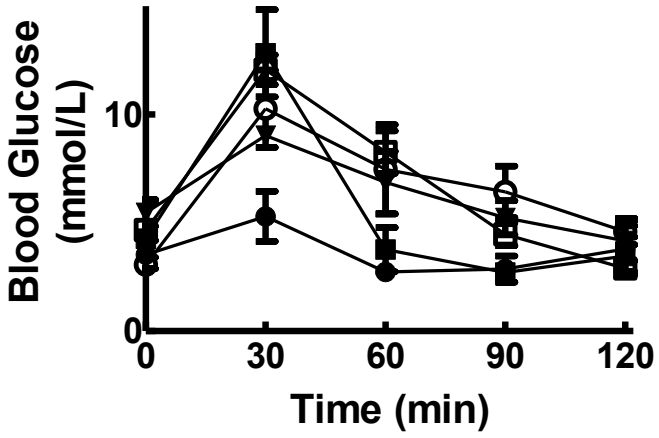
Figure 33. Plasma cholesterol and triglyceride lipoprotein profiles for Syrian Golden hamsters.

Hamsters were fed a fructose diet supplemented with or without 1% bioactive fatty acid for 6 weeks. Serum was obtained from mice by cardiac puncture. Plasma was separated from the blood cells and pooled plasma samples (n=2) were separated by density gradient ultracentrifugation. Cholesterol (○) and triglyceride (●) were measured in each fraction.

Supplementation with *c*-9, *t*-11 CLA only slightly decreased TG levels compared to fructose and all other plasma parameters were unaffected by fatty acid supplementation. HFHC animals had a 10-fold increase in plasma TC over chow (10.2 mmol/L) but only a nominal increase in plasma TG (1.23 mmol/L) over the chow group. The lipoprotein profiles (Figures 32 and 33) clearly show the effect of added cholesterol to the diet. With the exception of EPA supplementation to the HFHC diet, all of the HFHC groups lipoprotein profiles have a second cholesterol peak in the LDL fractions (Figure 32) while the fructose-fed animals have a singular peak, as expected, in the HDL fractions (Figure 33).

Hyperglycemia and insulin resistance are precursors to the metabolic syndrome. Although insulin resistance was not measured, fasting blood glucose, glucose tolerance and insulin tolerance, which can be considered surrogate measures of the insulin resistant state, were measured at 4 and 6 weeks in the fructose-fed hamster. At 4 and 6 weeks, IPGTT were performed on overnight fasted hamsters. Between week 4 and 6 only the DHA supplemented animals showed a substantial improvement in glucose tolerance, represented by a 50% reduction in AUC, which was a significant improvement compared to Fructose feed animals ($p<0.05$, Figure 34). The fructose-fed animals had a 20% reduction in AUC between weeks 4 and 6. All other supplementation groups remained unchanged between weeks 4 and 6.

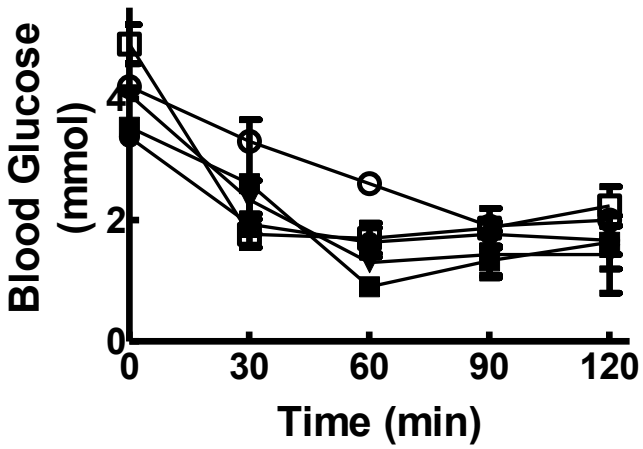
Insulin tolerance tests were also performed at week 4 and 6 (Figure 35). Three of the dietary groups, Base, *c*-9, *t*-11 CLA and EPA all showed improvement in insulin tolerance between week 4 and 6 as evident by the 20% reduction in AUC (Figure 35). Similar to glucose tolerance, DHA supplementation resulted in the largest improvement in AUC (34%) suggesting improvement in insulin sensitivity.



Dietary Treatment	AUC – 4 weeks (mM-min)	AUC – 6 weeks (mM-min)
Base	639.5 ± 116.1	695.0 ± 98.6
Fructose	1085.2 ± 89.2	857.5 ± 18.9
c-9, *t*-11 CLA	796.0 ± 26.8	778.0 ± 73.2
DHA	863.5 ± 56.3	437.0 ± 58.9*
EPA	845.0 ± 116.9	840.0 ± 105.8

Figure 34. Effect of bioactive fatty acid supplementation on glucose tolerance.

Hamsters were maintained for 6 wk on a fructose diet with or without bioactive fatty acid supplementation. Blood was collected following an overnight fast. An intraperitoneal glucose tolerance test (IPGTT) (section 2.2.12.) was performed at 4 and 6 weeks. IPGTT at 6 weeks ■ Base, □ Fructose, ▼ *c*-9, *t*-11 CLA, ● DHA, ○ EPA. Area under the curve (AUC) values following feeding for 4 and 6 wk. Data is presented as mean ± S.E.M. AUC 4 week n=3, AUC 6 week n=3. *$p<0.05$ vs. Fructose.



Dietary Treatment	AUC – 4 weeks (mM-min)	AUC – 6 weeks (mM-min)
Base	275.0 ± 17.8	222.5 ± 45.4
Fructose	264 ± 25.3	267.0 ± 11.7
c-9, *t*-11 CLA	292.0 ± 28.2	235.0 ± 29.4
DHA	356.0 ± 172.4	235.5 ± 7.1
EPA	325.0 ± 96.1	257.5 ± 52.5

Figure 35. Effect of bioactive fatty acid supplementation on whole body insulin sensitivity.

Hamsters were maintained for 6 wk on a fructose diet with or without bioactive fatty acid supplementation. Blood was collected following an overnight fast. An intraperitoneal insulin tolerance test (IPITT) (section 2.2.12.) was performed. IPITT at 6 weeks ■ Base, □ Fructose, ▼ *c*-9, *t*-11 CLA, ● DHA, ○ EPA. Area under the curve (AUC) values following feeding for 4 and 6 wk. Data is presented as mean ± S.E.M. AUC 4 week n=3, AUC 6 week n=3.

5.3.3. VLDL Assembly.

To examine the effects of diet supplementation with bioactive fatty acid on VLDL assembly in the hamster, hepatocytes were prepared from diet-supplemented animals after 6 weeks. A HFHC diet with no additional supplementation increased apoB100 synthesis by approximately 50% compared to hepatocytes from chow animals (Fig 36A). Supplementation with either CLA isomer resulted in a two-fold increase in apoB100 synthesis. The second component, an adequate lipid supply, was assessed using [^{3}H]glycerol labelling to monitor TG synthesis and secretion. TG synthesis in primary hepatocytes isolated from hamster supplemented with either CLA isomer was reduced compared to hepatocytes from the HFHC control diet (Figure 36 A). Despite a lower stimulatory effect on synthesis both isomers significantly stimulated TG secretion compared to HF alone (Figure 36B). Density gradient ultracentrifugation analysis (Figure 36C) indicated that VLDL secretion was stimulated by both CLA isomers. Interestingly the *t*-10, *c*-12 isomer appeared to stimulate the formation of more particles in the intermediate to low density fractions compared to high fat alone or with *c*-9, *t*-11 supplementation. This would suggest that the *t*-10, *c*-12 diet may stimulate the secretion of lipoproteins with less TG content (Figure 36B).

The *in vitro* radiolabelling studies showed that although there seemed to be an adequate lipid supply (TG synthesis Figure 28) apoB100 secretion was significantly less in the presence of DHA (Figure 29) which was further supported by the density ultracentrifugation results (Figure 30) that show minimal secretion of apoB containing particles. Therefore it was somewhat surprising that plasma lipoprotein profiles of fructose-fed DHA supplemented animals were similar to animals fed only fructose (Figure 33). To examine lipoprotein expression more thoroughly, we performed apoB

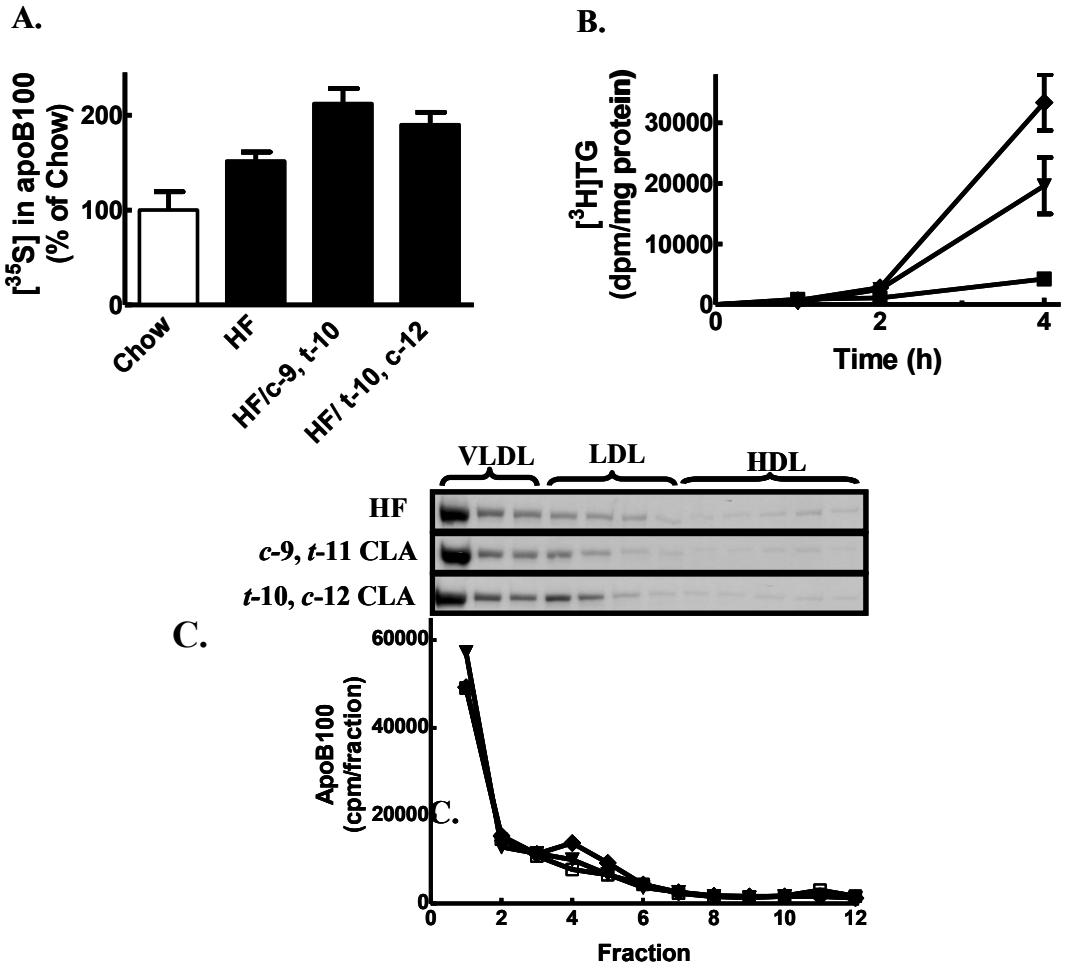


Figure 36. VLDL assembly and secretion.

Hepatocytes from hamsters fed a high fat diet supplemented with 1% *c*-9, *t*-10 CLA or *t*-10,*c*-12 CLA for 6 wks were isolated. A.) Hepatocytes were labelled for 60 min with [^{35}S]methionine/cysteine and the ApoB100 protein was recovered and quantified as described section 2.3.4.2. B.) Hepatocytes were labelled up to 4 h with 10 μCi of [^{3}H] glycerol and collected. Lipids were quantified as described in section 2.3.2. C.) Hepatocytes were pulsed-labelled for 60 min with [^{35}S] methionine/cysteine followed by a 24 h chase. Lipoproteins were separated by sucrose density gradient ultracentrifugation into 12 equal fractions from the top (fraction 1) of the gradient. Radioactivity associated with apoB100 was quantified by liquid scintillation counting as in section 2.3.4.2. ■ HF, ▼ HF/*c*-9, *t*-11 CLA, ◆ HF/*t*-10, *c*-12 CLA. Results are expressed as means ± S.D of triplicates.

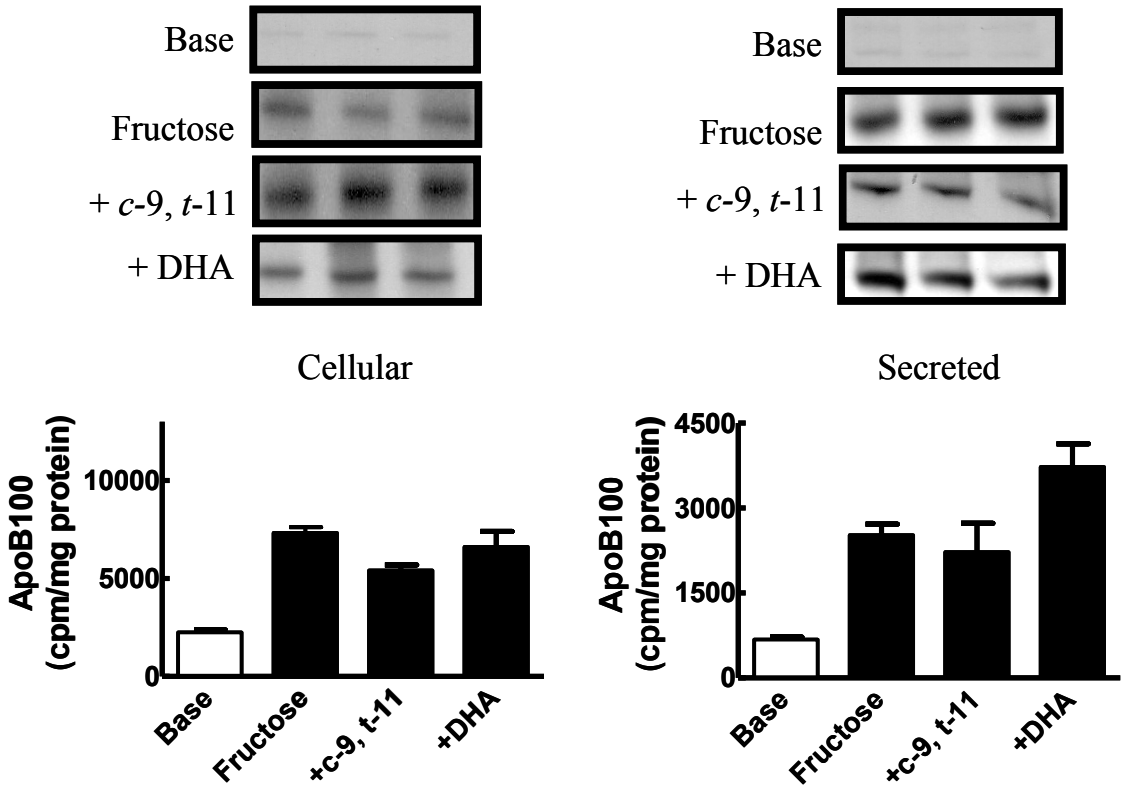


Figure 37. ApoB100 synthesis and secretion.

Hepatocytes from hamsters fed a fructose diet supplemented with 1% *c*-9, *t*-10 CLA or DHA for 6 weeks were isolated. Hepatocytes were pulse-labelled for 60 min in the presence of [^{35}S]methionine/cysteine. Synthesis: ApoB100 was recovered from cell lysate by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity associated with apoB100 was quantified by liquid scintillation counting. Secretion: The labelling medium was removed and replaced with chase medium. 2 h after initiating the chase, apoB100 was quantified as with cells Results are expressed as means ± S.D of triplicates.

radiolabelling studies in hepatocytes prepared from fructose fed animals. A fructose diet with no additional supplementation increased apoB100 synthesis by three-fold compared to hepatocytes from base-fed animals (Fig 37). Supplementation with *c*-9, *t*-11 CLA or DHA resulted in only a two-fold increase in apoB100 synthesis compared to chow fed animals. Surprisingly, given the previous results *in vitro* (Figure 29C) with DHA supplementation, apoB secretion in the presence of DHA was increased compared to fructose alone or *c*-9, *t*-11 CLA supplementation. TG synthesis and secretion and density gradient ultracentrifugation analysis to determine the size and number of particles were not performed.

5.4. ER Stress and the Fructose Fed Hamster

ER stress has been recently implicated as the connecting factor between hepatic steatosis and insulin resistance as well as metabolic disease and inflammation (109;113;344-346). Although, unlike the mouse studies, we did not observe hepatic steatosis in hamsters with any of the bioactive fatty acid supplementations we did observe dyslipidemia (Table 15) and decreased insulin sensitivity (Figures 34 and 35) therefore we investigated three ER markers. HSP 70 and calnexin are not direct markers of ER stress pathways rather they are an indication of misfolded protein load in the ER. Livers of hamsters fed with the fructose only or supplemented diet for 2, 4 and 6 weeks were extracted and homogenized as described in section 2.2.9. GRP78 has been identified as a major chaperone protein involved in all three ER stress pathways (336). DHA supplementation resulted in a time dependent increase in each of the three markers (Figure 38A and B) whereas there was no change in any of the markers with EPA supplementation (Figure 38A and C), fructose only or *c*-9, *t*-11 CLA supplementation

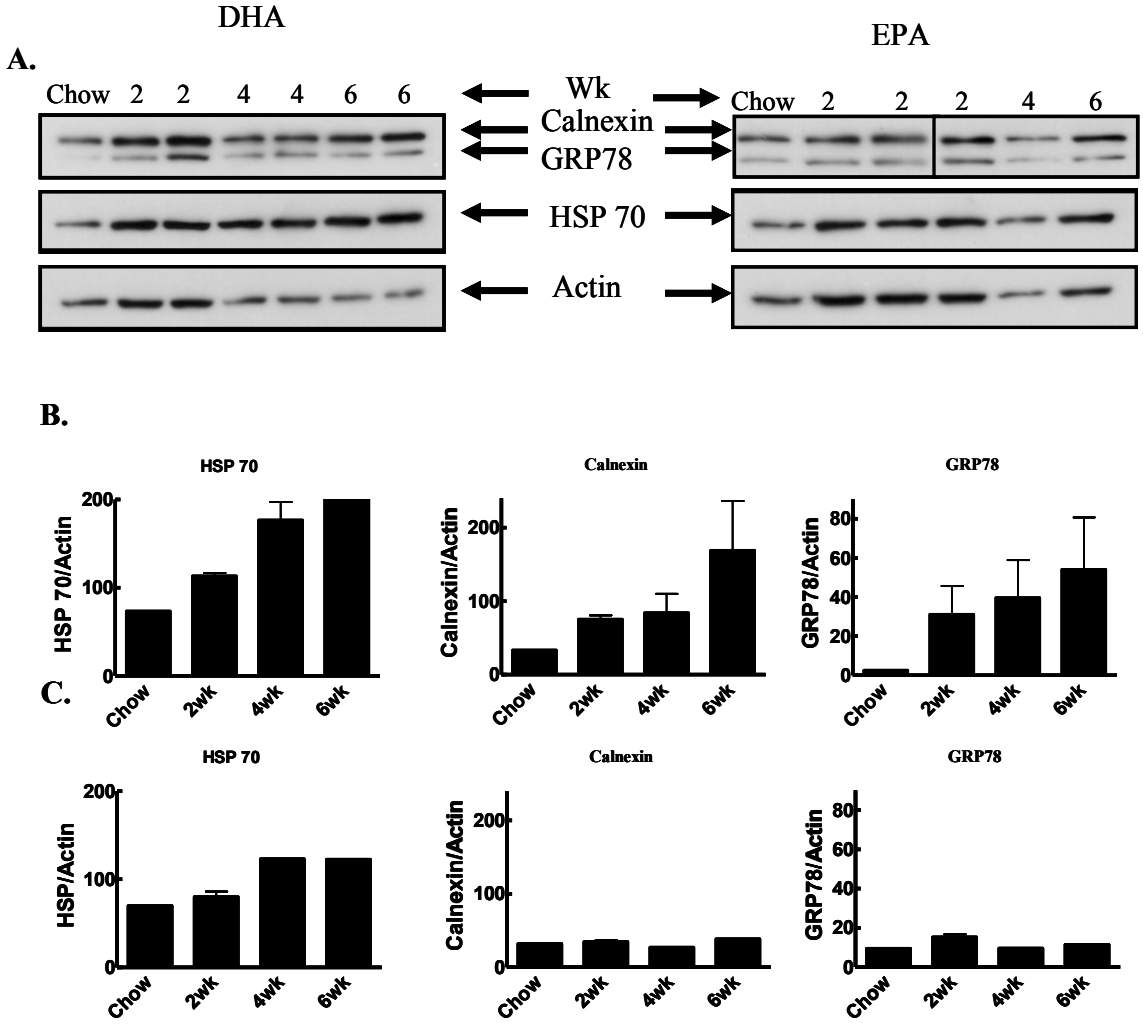


Figure 38. Expression of ER Stress markers in the livers of fructose-fed hamsters supplemented with 1% DHA or EPA for 6 wk.

A.) Representative western blot for the expression of Hsp70, calnexin and GRP78. **B.)** Levels of Hsp70, calnexin and GRP78 in the presence of DHA and **C.)** in the presence of EPA as determined by scanning densitometry, relative to actin. (mean ± S.E.M., n=2-3 per group).

(data not shown). Sample size precludes a statistical analysis but it is interesting to note that the increase in ER markers coincides with improvement in glucose and insulin tolerance (Figures 34 and 35). These preliminary observations suggest that DHA induces an ER response that may alleviate ER stress and maintain homeostasis compared to other bioactive fatty acids.

5.5 Discussion

Recent interest in fructose metabolism has been fuelled by the realization that as human fructose consumption increases so does the prevalence of obesity (347). Fructose perturbations in the cell may involve signalling and inflammatory pathways that are at the crux of metabolic homeostasis (348). The only animal model that has shown to be resilient to fructose-induced insulin resistance is the C57Bl/6 mouse; hamsters, rats and other species of mice have all been utilized for dietary studies of fructose metabolism (347). In the current study the fructose-fed hamster was used as a model for the human conditions of dyslipidemia and insulin resistance, both components of the metabolic syndrome (349). DHA and EPA have been shown to have hypolipidemic effects, specifically lowering of TG, in numerous clinical studies (219) and a prescription strength omega-3 preparation is available for the treatment of hypertriglyceridemia in conjunction with dietary modifications (219), even though we lack a complete mechanistic understanding as to how omega-3 fish oils lower TG. From our previous work in the HFHC fed hamster (253) we determined that individual CLA isomers did not substantially improve plasma lipoprotein metabolism although atherosclerotic lesion development was improved. We extended these studies to a model of insulin resistance

(fructose-fed) and expanded the supplemented bioactive fatty acid to include ω-3 fish oils (DHA, EPA).

Previous studies have reported that when hamsters are challenged with an increase in calories, they will reduce food consumption to maintain a constant caloric intake (253;350). However this was not observed in the current study of a high fructose diet. With the addition of fructose to the diet animals consumed on average significantly more food. Although DHA supplementation was associated with the greatest weight gain, body fat increase and liver weight (Table 15), no significant effect of diet on body weight or body fat was observed. In contrast to our studies in the $LDLr^{-/-}$ mice, neither DHA nor EPA had a substantial effect on plasma TG or TC (Figure 14) and lipoprotein profiles were not changed from the fructose only diet group (Figure 33). Although not previously used in conjunction with a fructose diet, it is still surprising that improved plasma lipoprotein profiles were not observed. This may be indicative of the small sample size or length of the dietary intervention. ω-3 supplementation has been previously shown to improve plasma and VLDL-TG concentrations in hamsters but only in the absence of cholesterol (351) With as little as 0.5% cholesterol added to the diet there was no significant improvement with ω-3 supplementation (351). This effect of cholesterol has been reported before in the fructose-fed hamster (352). The severity of metabolic derangements such as dyslipidemia and insulin resistance in the fructose-fed hamster were cholesterol concentration dependent (352). Plasma TC in the HFHC hamsters were 3-fold higher than any of the fructose based diet groups. Anti-diabetic properties of DHA and EPA have not been consistently shown (219) but it has been suggested that it is related to their triglyceride lowering properties. Therefore, since we did not observe any

improvement in plasma TG levels, improvement in glucose tolerance may not be expected. DHA supplementation was associated with an improvement in both glucose tolerance (50%) and insulin sensitivity (34%) as measured by reductions in AUC (Figure 34 and 35). Future studies are necessary to elucidate how DHA supplementation improves insulin and glucose metabolism without improvement in adiposity or plasma lipoprotein profiles. One possibility is that DHA is able to induce the ER stress response in an effective manner that allows for proper function of the ER. Only DHA supplementation induced a time dependent increase in 3 markers of ER function (Figure 38) and this coincided with improved glucose and insulin tolerance. Specific ER stress pathway regulators need to be investigated to determine if ER stress response is involved in DHA action.

Previously it has been reported that the hepatic over-production of apoB in the insulin resistant state may be a combination of increased lipid supply and decreased apoB degradation (349). From our *in vitro* studies it was clearly evident (Figure 28 and 30) that DHA decreases the synthesis of apoB and substantially decreases both TG and apoB secretion. This is further supported by the almost complete disappearance of apoB containing lipoproteins in the density gradient ultracentrifugation analysis (Figure 30). In contrast the plasma levels of lipids in DHA supplemented animals were not altered compared to any other diet group (Table 15). As well the *in vivo* studies reveal that DHA does not reduce apoB protein levels in hamster hepatocytes compared to *c*-9, *t*-11 CLA supplementation or fructose only diet (Figure 37) and, in contrast to the *in vitro* analysis, may actually stimulate apoB secretion to a greater extent than fructose alone. Nevertheless DHA has been previously associated with marked decreases in apoB secretion in rat hepatoma cells (353). In these studies lipid peroxidation was suggested to

be the signal that marked apoB for degradation. Discrepancies in results may be related to difference in lipoprotein metabolism between hamsters and rat. Similar to humans, the hamster liver secretes only full length apoB100 while rats and mice secrete both apoB48 and apoB100.

Transcriptional control is not believed to be the major regulatory step in apoB synthesis in the liver. It appears that a steady state level of apoB mRNA is expressed and translated. It is post-translationally that alterations in apoB production are regulated. Assembly of apoB containing lipoproteins is affected by variations in fatty acid composition, such as omega-3 or CLA. These fatty acids may affect different steps in the assembly of VLDL (Figure 39). From the *in vitro* studies it appears that both CLA isomers and DHA and EPA can decrease the synthesis of apoB or although not specifically measured, increase apoB degradation. In contrast both HFHC and 60% fructose diets with or without supplementation increased apoB synthesis and secretion compared to chow or base diet. Further studies are needed to fully understand the regulation of apoB lipoprotein synthesis and secretion and how bioactive fatty acids may have positive or negative effects on multiple regulatory steps.

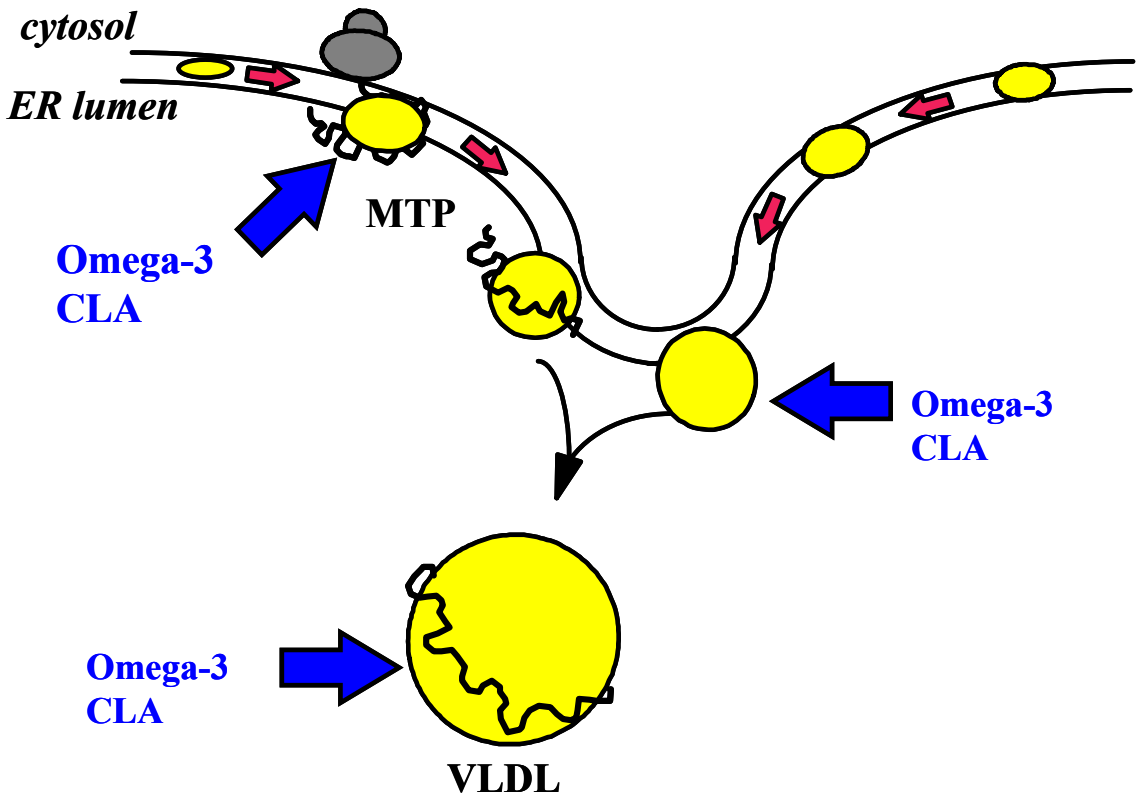


Figure 39. Bioactive fatty acids may affect both steps of VLDL assembly.

VLDL assembly requires two essential components: a properly folded apoB lipoprotein and an adequate lipid supply. If either component is not present or it is incomplete the particle will be targeted for degradation. Both ω-3 fatty acids and CLA isomers have been shown to decrease apoB protein synthesis and secretion *in vitro* while *in vivo* results show that both types of fatty acids support VLDL assembly.

CHAPTER SIX –CONCLUSIONS

6.1. Summary

Although the data is not conclusive, there has been significant public health promotion to the effect that lipids, namely saturated and trans fatty acids, are detrimental to the cardiovascular health of Canadians and therefore dietary intake should be limited (354). Lipids provide essential fatty acids, fat soluble vitamins and energy; they are vital for the structural integrity of cell membranes and act as messengers within the cell. Eliminating a specific type of fatty acid is neither feasible nor advisable. Conversely, functional foods have garnered attention as a more "natural way" to get health benefits even though they may contain "trans fat" (CLA).

Both CLA and ω-3 fatty acids have been investigated as potential supplements to foods. Their benefits are proclaimed adamantly even though their mechanisms of action are not clearly understood. The current studies were undertaken to investigate both CLA and ω-3 fatty acids, in various rodent models, to not only ascertain if the benefits outweighed the risks but also the mechanisms by which bioactive fatty acids exert these effects and the relevance to health.

Given the complex nature of the metabolic syndrome, specifically that it is a grouping of pathologies such as glucose intolerance, insulin resistance, obesity and dyslipidemia; it is not surprising that determining the positive or negative role of dietary fats on these risk factors is also complex. The potential roles for bioactive fatty acids are summarized in Figure 40. The results from the current studies, using genetic or dietary manipulation of animal models, were able to substantiate many of these suggested roles

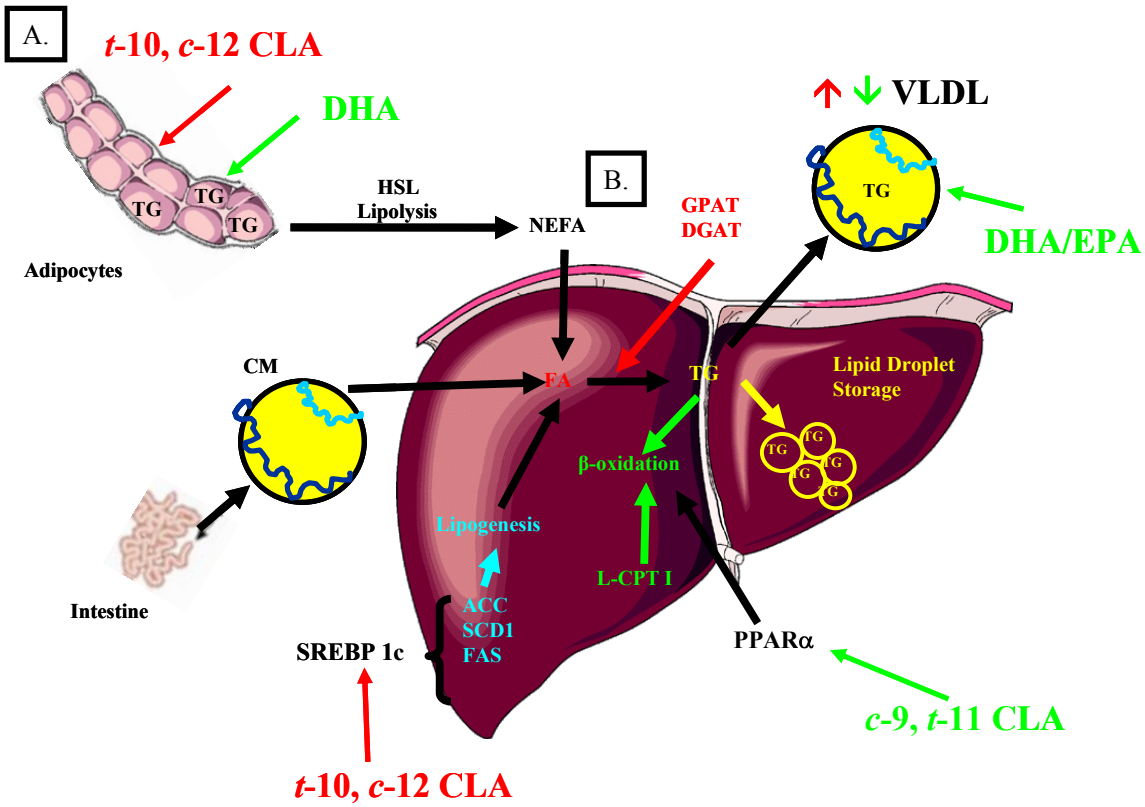


Figure 40. Summary of potential roles of bioactive fatty acids in tissue metabolism.

A. Adipose tissue: *t*-10, *c*-12 CLA decreased while DHA increased circulating levels of adiponectin. **B**. Hepatic gene expression of SREBP-1c was increased in the presence of *t*-10, *c*-12 CLA possibly contributing to hepatic steatosis and increased plasma TG/TC levels by increasing the flux of FA available for VLDL synthesis. *c*-9, *t*-11 CLA increased hepatic gene expression of PPARα thereby possibly reducing the flux of FA available for VLDL secretion leading to decreased hepatic lipid stores and plasma TG/TC.

while others were not clearly delineated. It is also apparent from the current research that the effects of fatty acids are multi-system and may include, but are not limited to, the adipose tissue, liver, endothelial cells and macrophages. It is also evident that the effect on one tissue or cell type may oppose the actions at another site such as the effects of CLA on reducing atherosclerotic lesion development while simultaneously being associated with hepatic steatosis. Adding complexity to the interpretations of results is the translation from rodent models to humans. With the availability of human cell culture models it will be imperative to confirm these mechanisms of action in the most relevant system. All of these factors must be considered when deciding what role bioactive fatty acids have in human health.

The apoE$^{-/-}$ mouse model provided the opportunity to delve into the role of dietary fat and cholesterol on the development of atherosclerosis. Given the profound development of lesions on a chow diet the cholesterol content in the current study was reduced 12.5-fold compared to the previous study from oyr laboratory with the HFHC diet (274). This reduction in cholesterol resulted in only a 10% decrease in plasma cholesterol but more importantly, a significant reduction in atherosclerotic lesions with *t*-10, *c*-12 supplementation. This improvement in atherosclerosis was also observed in the CLA Mix supplemented animals. What brought about this improvement? It may be related to the decrease in plasma lipids as CLA Mix supplementation improved both plasma TG and TC compared to all other dietary groups. Although not specifically studied, CLA as an anti-inflammatory agent must also be considered. Both CLA isomers are known to be PPARγ ligands through which improvements in atherosclerosis have been reported without concomitant improvements in plasma lipids. Further investigation

into the role of CLA and macrophage infiltration is required. Although the current studies did not assess whether CLA supplementation altered macrophage function, previous research has shown that not only is macrophage infiltration reduced but the levels of PPARα/γ are also increased in atherosclerotic lesions without an accompanying reduction in plasma TG (355).

The modest improvements in plasma lipids are overshadowed by the myriad of negative effects such as hyperinsulinemia, hypoadiponectinemia, increased liver weight and hepatic steatosis. These profound deleterious changes with *t*-10, *c*-12 CLA supplementation were not confined to the ApoE$^{-/-}$ mouse model. Utilizing a less severe model of hypercholesterolemia and atherosclerosis, the LDLr$^{-/-}$ mouse, we determined that regardless of genetic disruption, *t*-10, *c*-12 CLA supplementation was associated with hyperinsulinemia, hypoadiponectinemia, increased liver weight and hepatic steatosis and in the LDLr$^{-/-}$ mouse hyperlipidemia.

The common thread in these two studies and the 3 week time course is the drastic and profound depletion of circulating adiponectin. Marked reduction in plasma adiponectin precedes differences in weight gain, increased insulin levels and any significant increase in plasma TG and TC. Shortly thereafter liver weight is increased suggesting that within 3 weeks of starting supplementation hepatic TG accretion is occurring. Our understanding of the depth and breadth of the role of adiponectin in hepatic metabolism is murky at best. The loss of functional adipose tissue has been suggested to be the instigating event (308) with continued suppression of adiponectin synthesis, most likely through CLA-mediated down regulation of PPARγ (313). Liu *et al*. (286) reported on the combined effects of rosiglitazone, a PPARγ agonist and CLA mix

supplementation. In the absence of rosiglitazone, CLA decreased adiponectin levels which were restored with rosiglitazone treatment suggesting that CLA may, directly or indirectly, prevent PPARγ activation to maintain circulating levels of adiponectin.

Insulin resistance and hypoadiponectinemia have been closely associated not only in the current study but in others as well (66;67;72;312). Subsequent or in parallel with the hyperinsulinemia, hepatic steatosis has been observed. It is currently unclear whether insulin resistance causes the excess accumulation of TG in liver or whether or not the increase in TG plays a causal role in the development of hepatic insulin resistance. Recent studies favour the hypothesis that the accumulation of intrahepatic lipid precedes the insulin resistance (356;357). This hypothesis is supported by the time course study that indicates that by 3 weeks a significant liver weight increase has occurred while only a nominal increase in plasma insulin is observed. Determining the mechanism of the increased liver weight at three weeks through liver lipid analysis will provide further insight.

Gene expression analysis provided insight into possible differential regulation of lipogenesis between the two CLA isomers. One must be cautious when interpreting gene expression changes as these may not result in alterations in protein level or activity. Changes in transcriptional regulators such as the PPAR family and SREBP-1c need to be confirmed by analysis of changes in down-stream targets. Although it appears that *c*-9, *t*-11 CLA supplementation may up-regulate β-oxidation through up-regulation of PPARα mRNA it is imperative that these findings be corroborated by assessing changes in down-stream targets such as liver carnitine palmitoyltransferase I (L-CPT1). Similar studies are needed to elucidate the specific role that SREBP-1c may play in the development of

hepatic steatosis associated with *t*-10, *c*-12 CLA supplementation. SCD1 mRNA expression was not altered with CLA supplementation but this does not rule out changes in activity. Further analysis to determine the fatty acid species esterified to the hepatic glycerolipids and cholesterol esters may provide insight into possible changes in the desaturation ratio another potential contributor to hepatic steatosis.

Omega-3 fatty acids were studied in both the $LDLr^{-/-}$ mouse and the fructose fed hamster models with inconsistent results. In the $LDLr^{-/-}$ mouse the hypolipidemic effects of both DHA and EPA were clearly evident with significant reductions in plasma TG and TC, while plasma adiponectin was increased with DHA supplementation. In contrast, in the hamster, DHA supplementation was associated with increased body weight, body fat gain and increased liver weight but with no decrease in plasma TG or TC. Despite these potential harmful effects, insulin sensitivity and glucose tolerance were improved. These findings reinforce the complex nature of *in vivo* studies and suggest that improvements in metabolic markers of cardiovascular risk may still be evident with adipose tissue expansion.

The possible roles of ER stress either as a causative or a defence mechanism in the development of hepatic steatosis was briefly investigated in both mice and hamsters. Further studies of the role of the ER stress response in liver lipid metabolism are an important avenue for further investigation.

6.2 Conclusions

The hypothesis that bioactive fatty acids will improve hepatic lipid and lipoprotein metabolism and reduce the development of atherosclerosis has been partially addressed. It appears that a reduction in atherosclerosis comes with a price in mouse models. *t*-10, *c*-12

CLA supplementation reduced weight gain, decreased plasma TG and decreased atherosclerotic lesions but with hypoadiponectinemia, hyperinsulinemia, increased hepatic TG accretion and hepatomegaly. In contrast, DHA supplementation in the fructose-fed hamster improved glucose and insulin tolerance but at the cost of increased weight and body fat gain and increased liver weight.

Conflicting results have been demonstrated in many trials designed to determine the effect of CLA and omega-3 fatty acids on transcriptional regulation of lipid and glucose metabolism. Since efficacy and direction of action in pathophysiological states varies, depending on the model, the therapeutic value of CLA is still unclear. Given all of the negative findings – is CLA useless as a nutraceutical or food supplement? Or do we still not understand the optimal dose or mechanism sufficiently to recommend its use. Perhaps the most appropriate delivery route for these bioactive ingredients is whole food rather than pure supplement.

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