University of Alberta

Trans-11 Vaccenic Acid Favourably Remodels Whole Body Energy and Endocannabinoid Metabolism During Conditions of Dyslipidemia

by

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“You can’t connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future. You have to trust in something – your gut, destiny, life, karma, whatever. Because believing that the dots will connect down the road will give you the confidence to follow your heart even when it leads you off the well worn path; and that will make all the difference.”

— Steve Jobs (Stanford Commencement Address, 2005)

Dedication

To my husband, Hermes Gerardo Cuervo and my family for being my highest inspiration, for sharing my dreams and for always supporting me throughout this journey

Abstract

Introduction: *Trans* 11-18:1 (vaccenic acid, VA) is the *predominant* ruminant-derived *trans* fatty acid (TFA) in the food chain and is also the precursor to endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid (CLA). Literature documents numerous health benefits associated with CLA, which is a *minor* component of natural TFA. A few recent studies have reported hypolipidemic properties of VA. However, independent bioactive properties of VA during dyslipidemia and insulin resistance remain elusive.

Objectives: To determine whether VA can enhance the putative hypolipidemic effects of CLA. To investigate whether enriching dairy fat with VA can favourably modulate whole body energy use/storage.

Methods and Results: Hypolipidemic effects of dietary supplementation with CLA on dyslipidemia were enhanced by the addition of VA in a rat model of metabolic syndrome (the JCR:LA-*cp* rat). VA was also found to reduce total body fat (-6%) while stimulating adipose tissue re-distribution (reduced mesenteric fat (-17%) while increasing inguinal fat mass (29%)). VA fortification increased metabolic rate concomitantly with an increased preference for whole body glucose utilization for oxidation and increased insulin sensitivity (lower HOMA-IR index (-59%)). Additionally, VA re-equilibrated intestinal and hepatic lipid homeostasis (reduced TG secretion) while exerting differential transcriptional regulation in both organs. Targeted lipidomic analyses revealed that VA can regulate concentrations of endocannabinoids in a tissue-specific manner.

Conclusions: VA has hypolipidemic effects independent of CLA and can favorably modulate whole body energy metabolism and lipid homeostasis when added to a dairy-derived diet. The regulatory effect of VA on the endocannabinoid system may have potential therapeutic applications. Based on these results, enriching dairy products with VA (either naturally

or by fortification) may maximize the health value of dairy-derived fats. Findings also support the need for nutrition guidelines focused on eliminating industrial forms of TFA from processed foods, as opposed to all TFA *per se*.

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List of abbreviations

AA: arachidonic acid (n6 C20:4)

AA-5-HT: arachidonoyl-serotonin

ABCA1: ABC-cassette transporter A1

ABCG1: ABC-cassette transporter G1

ABCG5/8: ABC-cassette transporter G1/8

AEA: *N*-acylethanolamine *N*-arachidonoylethanolamide or anandamide

1-AG: 1-arachidonoylglycerol

2-AG: 2-arachidonoylglycerol

ACAT1: acyl CoA acyl transferase 1

ACC-1: acetyl-CoA carboxylase-1

AGPAT: acyl-glycerol-phosphate acyltransferase

ALA: alpha linoleic acid (n3 C18:3)

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ANOVA: analysis of variance

AP-1: activator protein 1

apo: apolipoprotein

ASP: Acylation-stimulating protein

AT: adipose tissue

AUC: area under the curve

BBM: brush border membrane

BCA: bicinchoninic acid

BW: body weight

cAMP: cyclic AMP

C3a: complement factor C3a

CB: cannabinoid

CD: control diet

cDNA: complementary deoxyribonucleic acid

CE: cholesteryl esters

C/EBPα: CCAAT/ enhancer binding protein

Cept1: choline ethanolamine phosphotransferase-1

CETP: cholesteryl ester transfer protein

CHD: coronary heart disease

CLA: conjugated linoleic acid

CM: chylomicron

CMr: chylomicron remnant

CO_2: carbon dioxide consumption

COPII: coat protein complex II

CSF-1: colony-stimulating factor

Ct: cycle threshold

CVD: cardiovascular disease

DBD: DNA binding domain

DAGL: diacylglycerol lipase

DG: diacylglycerol

DGAT: diacylglycerol O-acyltransferase

DHA: docosahexaenoic acid (n3 C22:6)

DHA-5-HT: DHA-serotonin

DIO: diet induced obesity

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EA: elaidic acid

EC: endocannabinoid

ECL: enhanced chemiluminescence

ECS: endocannabinoid system

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immuno sorbent assay

En: energy

EPA: eicosapentaenoic acid (n3 C20:5)

EPA-5-HT: EPA-serotonin

ER: endoplasmic reticulum

ERAD: ER-associated degradation

FA: fatty acids

FAAH: fatty acid amide hydrolase

FABP4: fatty acid protein-4

FAM: fluorescently labeled probe

FAME: fatty acid methyl esters

FAS: fatty acid synthase

FAT/CD36: fatty acid translocase/cluster determinant 36

FATP4: fatty acid transporter 4

FDA: US Food and Drug Administration

FFA: free fatty acids

FC: free cholesterol

GC: gas chromatography

GLUT: glucose transporter

GPAT: glycerol-3-phosphate acyltransferase

GPR119: G protein coupled receptor 119

GPR120: G protein-coupled receptor 120

HDL: high density lipoprotein

HDL-C: high density lipoprotein cholesterol

H&E: hematoxylin and eosin staining

HL: hepatic triacylglycerol lipase

HOMA-IR: homeostasis model assessment-insulin resistance

HPLC: high-performance liquid chromatography

HSL: hormone sensitive lipase

HSPG: heparan sulfate proteoglycans

iAUC: incremental area under the curve

IBD: inflammatory bowel disease

IDF: internatiol diabetes federation

IDL: intermediate density lipoprotein

I-FABP: intestine-type fatty acid binding protein

IKK: IκB-kinase

IKKβ: inhibitory κB kinase β

IL: interleukin

IL-1β: interleukin 1 beta

Ing: inguinal adipose tissue

INSIG: insulin induced gene

IRS: insulin receptor substrate

iTFA: industrially produced *trans* fats

JNK: Jun N-terminal kinases

α-KG: alpha ketoglutarate

LA: linoleic acid (n6 C18:2)

LC-APCI-MS: liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry

LCAT: (lecithin cholesterol acyltransferase)

LC-MS: liquid chromatography-mass spectrometry

LCPUFA: long chain PUFA

LDL-C: low density lipoprotein cholesterol

LDLR: low density lipoprotein cholesterol receptor or apoB/E receptor

LPL: lipoprotein lipase

LRP: LDL receptor-related protein

LXR: liver X receptor

L-FABP: liver-type fatty acid binding protein

LysoPL: sn-1-monoacyl-lysophospholipids

MAGL: monoacylglycerol lipase

MCP-1: monocyte chemoattractant proteins

Mes: mesenteric adipose tissue

MetS: metabolic syndrome

2-MG: sn-2-monoacylglycerol

MGAT: monoacylglycerol acyltransferase

MRM: multiple reaction monitoring

mRNA: messenger ribonucleic acid

MTP: microsomal triglyceride transport protein

MTT: meal tolerance test

MUFA: monounsaturated fatty acids

NaCl: sodium chloride

NAEs: *N*-acylethanolamines

NAFLD: nonalcoholic fatty liver disease

NAPE: N-acyl-PE

NASH: non-alcoholic steatohepatitis

NCEP-ATPIII: National Cholesterol Education Program-Adult Treatment Panel III

NFκB: nuclear factor κB

NMR: nuclear magnetic resonance

NO: nitric oxide production

NPC1L1: Nieman Pick C 1-like protein 1

OA: oleic acid (C18:1)

OEA: oleoylethanolamine

OFC: oral fat challenge

OAA: oxaloacetate

P-407: poloxamer-407

PAI-1: plasminogen activator inhibitor

PAPH: phosphatidate phosphatase

PBS: phosphate buffered saline

PC: phosphatidylcholine

PCR: polymerase chain reaction

PCTV: prechylomicron transport vesicle

PE: phosphatidylethanolamine

PEA: palmitoylethanolamine

PEPCK: phosphoenolpyruvate carboxikinase

Per: perirenal adipose tissue

pHSL: phosphorylated-hormone sensitive lipase

PHVO: partially hydrogenated vegetable oil

PKB: protein kinase B

PL: phospholipids

PPAR: peroxisome proliferator-activated receptor

PPRE: PPAR response elements

P:S: PUFA to SFA ratio

PUFA: polyunsaturated fatty acids

QTRAP: quatrupolo

RER: rough endoplasmic reticulum

RER: respiratory exchange ratio

RNA: ribonucleic acid

ROS: reactive oxygen species

RT°C: room temperature

rTFA: ruminant derived *trans* fats

SAS: statistical analysis software

SAT: subcutaneous adipose tissue

SCD: steroyl-CoA desaturase

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SER: smooth endoplasmic reticulum

SFA: saturated fatty acids

SRB1: scanvenger receptor class B1

SREBP1: sterol response element binding protein 1

Sylon BFT: bis (trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosylane

TAK: transforming growth factor-β-activated kinase-1

TC: total cholesterol

T2DM: type 2 diabetes mellitus

TFA: *trans* fatty acids

TG: triglyceride

TGH: triacylglycerol hydrolase

TLC: thin layer chromatography

TLR4: toll like receptor 4

TNF-α: tumor necrosis factor alpha

Tris-HCL: tris-hydrochloride

TRL: triglyceride rich lipoprotein

TZD: thiazolidinediones

UCP2: uncoupling protein 2

VA: *trans*-11 vaccenic acid (*trans*11 18:1)

VA+CLA: vaccenic acid plus *cis*-9, *trans*-11conjugated linoleic acid

VAT: visceral adipose tissue

VLDL: very low density lipoprotein

VO_2: oxygen consumption

WAT: white adipose tissue

WHO: World Health Organization

Chapter 1 Literature Review

Prelude to Thesis

Trans-11 vaccenic acid (VA) is the most abundant ruminant-derived *trans* fatty acid in the food chain and has sparked major interest due to mandatory labeling on most packaged foods in North America. VA is also the precursor to endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in ruminants and humans; therefore thought to provide health benefits as a result of this conversion. However, in addition to being an alternative source of CLA, there remains a void of information regarding the independent bioactivity of VA. Studies from our laboratory were the first to describe a novel bioactivity for this fatty acid, in particular its lipid modulating properties. In this thesis we provide new evidence that putative hypolipidemic effects of dietary supplementation with CLA on circulating dyslipidemia and fatty liver are enhanced by the addition of VA in a rat model of metabolic syndrome (the JCR:LA-*cp* rat). We also show for the first time new and unique properties of VA to beneficially modulate lipid utilization and adipose storage compartments when supplemented on a background diet containing dairy-derived saturated fat (SFA). Additionally, targeted lipidomic analyses from this thesis demonstrate that VA can selectively regulate tissue concentrations of phospholipid-derived signaling messengers called endocannabinoids, providing a potential novel mechanistic explanation of how VA alleviates hepatic lipid accumulation and intestinal inflammation. Collectively, findings from this thesis provide further insights into the beneficial effects of VA and suggest that enriching dairy products with VA (naturally or by fortification) may be used as an approach to maximize health value of ruminant-derived fats (beef and dairy), especially to those individuals with dysfunctional lipid metabolism. Therefore, results from this thesis have potential to be translated for industrial applications. Additionally, findings from this thesis add to the growing literature that supports the need for nutrition

guidelines/policies focused on eliminating industrial forms of *trans* fat from processed foods, as opposed to all TFA *per se*.

The introductory sections of this literature review are intended to provide a historical perspective of *trans* fatty acids in the human diet (both from industrial and ruminant sources) and their differential association with cardiovascular disease (CVD) risk. Some of the limitations and implications of current *trans* fat labeling regulations are also discussed below.

1.1 *Trans* Fatty Acids: Historical Perspective and Policy Implications

1.1.1 *Trans* fats in the diet

TFA refer to a class of unsaturated fatty acids with one or more double bonds in the *trans* configuration. Notably, there are two main sources of TFA in our diet; those produced naturally by bacteria present in ruminant animals and found in ruminant-derived products and those produced by partial hydrogenation of vegetable oils (PHVO). It has been estimated that approximately 75-80% of all dietary TFA are derived from industrially produced TFA (iTFA) present in PHVO, while 20-25% are derived from ruminant sources such as dairy products and meats (Alisson *et al*., 1999). PHVO have been the main source of dietary TFA since the second part of the 20th century. Incorporation of industrially produced TFA (iTFA) into the food chain was increased substantially when in response to dietary recommendations, food manufacturers needed to replace saturated fatty acids (SFA) from their formulations while maintaining desirable characteristic in foods. Due to their *trans* configuration, TFA are characterized for having a straight structure (like SFA) resulting in higher melting points, longer shelf life, and greater oxidative stability during deep frying relative to naturally occurring *cis* unsaturated fatty acids. In developed countries, major sources of iTFA include bakery products, deep fried and frozen foods as well as packaged snacks while cooking oils

represent the main source of iTFA in developing countries (Mozzafarian *et al*., 2007). Owing to the presence of TFA in a wide range of commercially available products, it was possible to consume up to 25 g of TFA per day (Stender *et al*., 2006). It was not until the mid-1980's that strong epidemiological evidence showed dietary *trans* fats to be positively associated with incidence of CVD.

1.1.2 Health implications of *trans fats*

Epidemiological data from prospective cohort and case-control studies conducted in the 80's consistently support the strong associations between iTFA consumption and coronary heart disease (CHD). The Nurses' Health Study (Willett *et al*., 1993) was the first and largest (85 095 US women) epidemiological study to show a strong positive association between iTFA consumption and CHD. In contrast, a non-significant inverse association was observed with the intake of ruminant-derived *trans* fats (rTFA). Similar results for the positive association between iTFA intake and CHD were observed in the Boston Area Health Study (Ascherio *et al*., 1994) and the Finnish men cohort (Pietinen *et al*., 1997) studies. A meta-analysis by Mozaffarian *et al*. (2006), which included four prospective cohort studies, concluded that for each 2% (4g, based on a 2000 kcal-diet) increase in energy intake from TFA, there was a 23% higher incidence of myocardial infarction and CHD death. Based on observations from prospective cohort studies, it was estimated that replacement of TFA (7.5% of energy) with butter or canola oil for instance, could result in a 22% or 38% reduction of CHD events, respectively (Mozaffarian and Clarke, 2009). The main physiological effects of TFA as well as the mechanisms by which TFA exacerbate CVD risk have been extensively investigated. Consistent with effects seen in observational studies, in several human clinical trials TFA have been shown to consistently increase low density lipoprotein-cholesterol (LDL-C), triglyceride and the ratio of LDL-C to high density lipoprotein-cholesterol

(HDL-C) (Mozaffarian and Clarke, 2009). Notably, on a per calorie basis TFA have been shown to exert a more deleterious atherogenic lipoprotein profile as compared to SFA (Mensik *et al*., 2003; Mozaffarian *et al*., 2006). Additionally, TFA have been shown to affect other non-lipid markers of metabolic syndrome (MetS) and CVD risk including systemic inflammation, endothelial dysfunction, adiposity and insulin resistance.

1.1.3 Ruminant *trans* fats

Whereas PHVO have been the main source of dietary TFA in the last few decades, naturally occurring TFA that are derived from ruminant animals such as sheep and cows have been estimated to contribute up to 25% in the Western diet (Alisson *et al*., 1999). The most predominant rTFA is *trans*-11 18:1 or vaccenic acid (VA) accounting for up to 70% of total rTFA in dairy products. This differs from the isomeric distribution in iTFA which display non only an assortment of isomers of the 18:1 group but also those of the 16:1, 18:2 and 18:3 groups (De Greyt *et al*., 1996; Aro *et al*., 1998). Additionally, *cis*-9, *trans*-11 CLA, which has been extensively investigated for its purported health benefits, is also formed and found in ruminant-derived fats. Both VA and CLA are produced during the biohydrogenation of linoleic and linolenic acid in the presence of rumen bacteria (Lock and Bauman, 2004). Although CLA is the first intermediate in the pathway, it is only a transitory product and only a small portion of this CLA escapes from the rumen being incorporated into milk and tissues (e.g. meat). In contrast, VA is the major biohydrogenation intermediate that is produced in the rumen and accumulates in the mammary gland and other tissues and therefore it is present in dairy and meat products at higher amounts than CLA. Most of the CLA present in milk fat (over 60%) is derived from endogenous synthesis by Δ9-desaturase (stearoyl-CoA desaturase; SCD) from VA (Griinari *et al*., 2000). Furthermore, VA is the precursor to endogenous synthesis of the *cis*-9, *trans*-11 CLA isomer not only in animals but also in humans. It has been estimated that the

conversion rate of dietary VA into *cis*-9, *trans*-11 CLA is approximately 11% in rodents (Santora *et al*., 2000) and 20% in humans (Turpeinen *et al*., 2002; Kuhnt *et al*., 2006). The synthesis of rTFA in ruminant and tissue compartments is summarized in Figure 1-1.

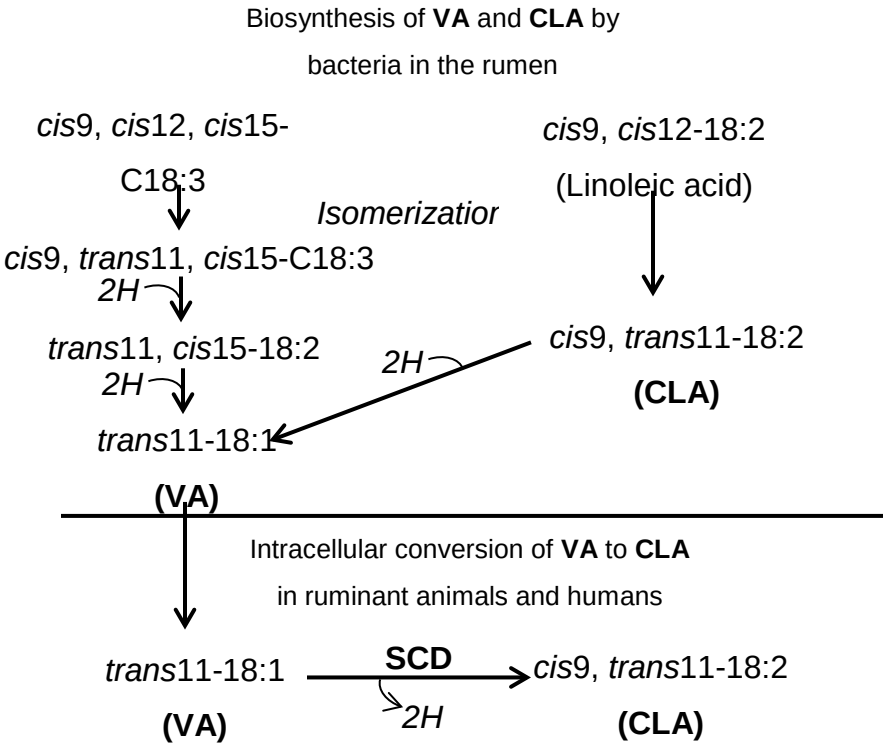


Figure 1-1 Biosynthesis of VA and CLA by bacteria in ruminant animals and conversion of VA into CLA by SCD in tissues of animals and humans (Adapted from Griinari and Bauman, 1999; Chinnadurai and Tyagi, 2011). *SCD1 is the rate limiting enzyme in the desaturation reaction responsible for the addition of a double bond into a saturated acyl chain at the Δ9 position (Ntambi and Miyazaki, 2004).*

While considerable evidence supports a positive association between TFA intake and CVD risk, a few prospective cohort studies have actually evaluated the association between rTFA intake and CVD risk and none to date have shown a significant positive association (Willet *et al*., 1993; Pietinen *et al*., 1997; Oomen *et al*., 2001). Rather, a non-significant trend

toward an inverse association has been observed (Willet *et al*., 1993). Notably, a prospective cohort study in Denmark, a country with typically high per capita dairy intake identified an inverse association between rTFA intake and CHD in women (Jakobsen *et al*., 2008). Since then, it has been proposed that unlike iTFA, naturally occurring TFA may be protective for health; either due to the low level of consumption or due to structural differences (mainly attributed to putative bioactive properties of CLA). However, some inconsistencies regarding the bioactivity of select isomers of CLA and its low concentration in dairy products have questioned the ongoing hypothesis for the health value of CLA in ruminant-derived fats.

1.1.3.1 Conjugated linoleic acid

CLA is a term that refers a group of geometrical and positional isomers of linoleic acid (18:2n-6) with conjugated bonds in either the *cis* or *trans* configuration. The group of CLA includes a number of isomers with two of these isomers (*cis*-9, *trans*-11 18:2 and *trans*-10, *cis*-12 18:2) known to possess bioactivity. Notably, while the *cis*-9, *trans*-11 CLA is the predominant CLA isomer found in ruminant-derived fat (90% of all isomers), the *trans*-10, *cis*-12 CLA is produced by alkali isomerization of linoleic acid and is only found in natural sources in trace to low amounts. CLA can be produced from safflower oil rich in linoleic acid and thus is commercially available in the supplement form which usually contains 80% of the two isomers (*cis*-9, *trans*-11 CLA to *trans*-10, *cis*-12 CLA, 1:1 ratio). Supplemental CLA has been accepted as generally safe by the US FDA up to a daily consumption of 3 g and 3-5 g up to 6 months by Health Canada. At the same time, the current Codex definition for *trans* fat excludes fatty acids that contain conjugated double bonds in the *trans* configuration (which include all CLA isomers, an issue that will be raised later in this thesis).

Health benefits of CLA have been extensively investigated under experimental conditions and it has been associated with reducing the risk of cancer, atherosclerosis, improving insulin resistance, dyslipidemia and inflammation. However, there has been a growing concern based on scientific evidence suggesting potential adverse effects with the use of supplemental CLA (particularly at high doses). Early experimental studies investigating effects of individual CLA isomers have shown that the *cis*-9, *trans*-11 CLA is associated with improved insulin sensitivity (Roche *et al*., 2002), anti-atherogenic effects (Arbonés-Mainar *et al*., 2006) and improved blood lipid profile (Valeille *et al*., 2005). Conversely, the *trans*-10, *cis*-12 CLA isomer has been shown to regulate body weight by reducing adipose tissue mass (Kennedy *et al*., 2010), but at the same time to cause insulin resistance and hepatic steatosis in mouse models (Roche *et al*., 2002, Cooper *et al*., 2008), probably due to increased mobilisation of fatty acids from adipose tissue into other tissues (Kennedy *et al*., 2010; Obsen *et al*., 2012). In addition, clinical studies evaluating supplementation of CLA (3 to 20 g/day) have reported adverse effects on blood lipids (Wanders *et al*., 2010), insulin resistance (Moloney *et al*., 2004; Raff *et al*., 2009) as well as inflammation and oxidative stress (Risérus *et al*., 2004; Steck *et al*., 2007). A recent study has reported no effect of supplemental CLA on reducing body weight or body fat mass in overweight men, which has also been disappointing considering body weight reduction is one of its major health claims (Joseph *et al*., 2011). On the other hand, the *cis*-9, *trans*-11 CLA isomer from ruminants in the form of moderately enriched dairy products (0.5-1.3 g/day) appears to be associated with neutral to beneficial health outcomes in humans (Tricon *et al*., 2006; Venkatramanan *et al*., 2010; Pintus *et al*., 2012). *These data have led to suggestions that the fatty acid profile (as opposed to single components) of enriched dairy products may influence the health value of dairy fat.*

1.1.3.2 TFA-enriched dairy products

It is generally assumed that the contribution of naturally occurring TFA in a typical diet is low. For instance, rTFA constitute a relatively small portion of the fat in dairy products (2-5%) and beef and lamb (3-9%) (O'Donnell-Megaro *et al*., 2011; Mendis *et al*., 2008; Aro *et al*., 1998). However, the fatty acid composition of ruminant-derived products is highly dependent on feeding practices as well as geographical and seasonal changes. Ruminants that are exclusively pasture fed produce greater levels of VA and *cis*-9, *tran*-11CLA compared to those fed indoors (e.g. grain and silage rations) (White *et al*., 2001; Kraft *et al*., 2003). Also, the content of rTFA in milk generally increases in the summer as opposed to the winter grazing period (Hydamaka *et al*., 2012). Thus, the content of rTFA is particularly high in countries with pasture feeding practices. For instance, it has been estimated that rTFA intake represents 63-75% of total TFA in European and Australian diets (Huth, 2007). Furthermore, there has been a growing interest to increase the concentrations of naturally occurring TFA in meat and dairy products. This has been initially motivated by interest in the purported health benefits of consuming CLA-enriched butter, particularly those associated with reducing the risk of cancer (Ip *et al*., 1999; Corl, *et al*., 2003). However, feeding practices that increase the concentration of CLA in dairy foods also increase the concentration of VA (Cruz Hernandez *et al*., 2007). For instance, the amount of VA in ruminant sources can range from 1.5 g/100g of fat with a standard feeding regimen to as high as 12 g/100g of fat in VA/CLA enriched dairy products (Bell *et al*., 2006). *Despite VA being the most predominant ruminant-derived fat accounting for up to 3 fold higher amounts than CLA, prior to my thesis, very little was known about its own bioactivity. This in turn has formed the foundation for this thesis to investigate the independent bioactivity of VA.*

Emerging evidence from epidemiological and pre-clinical studies suggest that unlike iTFA, rTFA may be benefitial to health. However, as discussed

below, current labeling regulations consider all TFA (regardless of the source) to be under the same category of deleterious TFA.

1.1.4 Trans fat regulations

Based on convincing evidence demonstrating that while *trans* fatty acids from PHVO provide no nutritional benefit, rather representing a substantial health risk, some government policies have focused on limiting dietary intake of *trans* fats to as low as possible (Reviewed by Field, 2008). In 2002, the Institute of Medicine and the National Cholesterol Education Program (NCEP)-ATPIII recommended *trans* fat consumption to be as low as possible in a nutritionally adequate diet. Subsequently, in 2003 the World Health Organization (and then the American Heart Association in 2006) recommended limiting TFA consumption to less than 1% of total energy intake. Additionally, in 2006 the Trans Fat Task Force (co-chaired by Health Canada and the Heart and Stroke Foundation of Canada), recommended a TFA limit of 5% of total fat in all products sold to consumers and 2% for commercial margarines and spreads. At the same time, regulatory bodies from different countries have proceeded to adopt similar recommendations. In 2003, Denmark was the first country to set a maximum of 2% TFA in all food ingredients (as opposed to whole foods), excluding those of animal origin (Leth *et al*., 2006). Canada and the U.S. Food and Drug Administration (FDA) introduced mandatory labeling of TFA for packaged foods and supplements, effective as of December 2005 and January 2006, respectively. Nonetheless, foods with less than 0.2 g of TFA/serving in Canada and 0.5 g of TFA/serving in the United States can be listed as 0 g TFA and can carry the claim to be '*trans* fat free'.

As discussed below, implementation of mandatory labeling regulations in North America has resulted in a substantial decline in trans fat intake. However, because current labeling regulations do not distinguish between

iTFA and rTFA, a decrease in TFA intake may not be limited to industrial forms but may also impact the intake of TFA from ruminant sources.

1.1.5 Dietary intake of industrial *trans* fats after 2006

An increasing consumer awareness and reformulation of products by food manufacturers have paralleled a decline in TFA intake in the last two decades. It was estimated that in the early 1990's, average daily consumption of TFA in the United States was approximately 6 g (2.6% of energy) with individual consumption varying from 1 to 29 g/day (Allison *et al*., 1999). Similar intakes of TFA were reported in Canada in 2002 (about 7g/day) (Peng, 2004). In Iran, where PHVO were used extensively for cooking, TFA accounted for 4.2% of all calories consumed (12.3 g/day) between 2001 and 2003 (Mozzafarian *et al*., 2007). In contrast, in countries where diets have traditionally contained less TFA such as Mediterranean and eastern Asian countries, the estimated average intake of TFA was approximately 1-3 g/day (Craig-Schmidt, 2006). Notably, there has been a worldwide decline of total TFA consumption from an average of 10 g/day to 3-4 g/day in North America (Ratnayake e*t al*., 2004) and northern European countries such as Denmark and the Netherlands (Craig-Schmidt, 2006). It was reported recently in a subset of non-Hispanic white adults in the United States that plasma levels of TFA (both from industrial and ruminant sources) were substantially lower (58%) in 2009 than in 2000 (Vesper, 2012). Thus, with the introduction of labeling regulations in Canada and the United States after 2006, a significant decline in TFA intake is expected in the next few years.

1.1.6 Controversies in *trans* fat labeling regulations

Although current labeling regulations represent an effective approach to reduce the availability of deleterious iTFA in the food chain, there appear to be limitations that need to be resolved. There have been some

concerns regarding the limit set by regulatory agencies to claim '*trans* fat free' food products (Remig *et al*., 2010). Allowing all products with less than 0.2 g (in Canada) or 0.5 g (in the United States) of TFA/serving to be labeled as '*trans* fat free' can be misleading. Consumers perceive the message on a '*trans* fat free' product as 0 g of *trans* fat in that specific product and therefore interpret this as being healthy. However, under this current labeling regimen, consuming 5 daily servings of foods with close to 0.5 g of TFA per serving would cumulatively exceed the recommended 1% of total energy intake from TFA (which equates to less than 2 g/day). In contrast, this would not be the case for rTFA (dairy plus beef) since their daily intake is usually well below the recommended limit (2g/day). Given the cumulative evidence regarding the health effects of individual CLA isomers (currently excluded from the TFA definition) as well as putative differential effects of ruminant versus industrial TFA, public health and regulatory agencies are now required to consider highlighting the two primary sources of TFA. *Indeed, there is a growing body of evidence to indicate that TFA derived from ruminant and industrial sources have differential bioactivity*.

Evidence from epidemiological, clinical and pre-clinical studies collectively demonstrates neutral or beneficial health effect of rTFA at normal consumption levels. However, enriching dairy products with rTFA would result in a relatively high daily intake of rTFA possibly exceeding the recommended limit. Furthermore, the SFA content in ruminant-derived fat may be a sufficient reason to limit its intake. Given recent evidence for the health effects of VA and those of CLA, it is plausible to suggest that increasing the proportion of these ruminant trans fats could provide an additional health value to animal-derived fats. Only a few clinical studies have explored these effects and the main findings from these studies are summarized below.

1.1.7 Effects of naturally occurring *trans* fats on biomarkers of CVD risk

There are a handful of studies investigating the effects of VA on MetS and CVD risk. Specifically, there have been a few highly controlled clinical intervention trials exploring the potential health benefits of dietary VA-enriched ruminant fat; however no conclusive evidence is currently available. These studies have failed to show consistent improvements in dyslipidemia but it has been proposed that the hypolipidemic activity of rTFA may be limited to study participants that have severe hyperlipidemia. It is also possible that insufficient statistical power may have reduced the ability to detect of treatment effects in these studies.

Some of these clinical studies have investigated the effect of foods (e.g. VA/CLA enriched dairy products *versus* control dairy products) with different fatty acid compositions (e.g. SFA and MUFA) (Pintus *et al*., 2012; Chardigny *et al*., 2008; Tardy *et al.,* 2009; Tholstrup *et al*., 2006). However, different fatty acids (alone or in combination) can exert independent bioactive effects. Therefore, results from these studies cannot be attributed to the effect of increasing VA *per se*. A few other studies have been actually designed to compare the effect of *cis versus trans* MUFA or the effect of *trans* MUFA from either ruminant or industrial sources (Motard-Belanger *et al*., 2008; Lacroix *et al.,* 2012). In these studies, the fatty acid composition of VA/CLA enriched and control dairy products was adjusted and the resulting test diets were matched with respect to SFA. Regardless of the study design, findings so far have suggested that moderate intake of rTFA (<1.5% of energy, 3 g/d), which would be attainable with consumption of a very high-dairy diet, may not significantly affect CVD risk factors at least in healthy individuals. Table 1-1 summarizes evidence from clinical studies that investigated the physiological effects of VA either using VA/CLA enriched dairy products or purified forms of the fatty acid.

While findings from clinical trials are still inconclusive, pre-clinical studies so far suggest that VA (either in the natural or purified form) has lipid-lowering effects during conditions of dyslipidemia and MetS. *However*, *whether VA per se provides an additional benefit when found in VA/CLA enriched products remains to be elucidated. Additionally, literature to date has only documented effects of purified VA on a vegetable oil background diet and whether VA can actually provide an additional health value to animal-derived fats is still unknown. Indeed, these questions provided the premise for my thesis. The initial approach for my thesis was to consider the potential impact to health value of VA-containing foods. For example, this could be translated in the development of specialized dairy products (such as cheese and yogurt) that have high concentrations of these natural trans fats.*

Table 1-1 Effects of VA on biomarkers or risk factors of CVD and MetS in humans (adapted from Gebauer *et al*., 2011).

Evidence from studies using VA/CLA enriched dairy products						
Study description	**Participants**	**Intervention**		**Results**	**Notes**	**Ref.**
		Treatment	Length			
Randomized double-blind parallel design	Healthy (n=42) (Danish) men	**Test**: VA-butter (3.6 g/d) **Control**: low-VA butter	5 wk	VA-butter decreased TC and HDL-C. No differences in TC/HDL ratio	FA composition of diets was not matched. Basal diets were not controlled.	Tholstrup *et al*., 2006
Randomized- double blind parallel design	Overweight (French) women (n=63)	**Test**: rTFA (4.8 g/d), iTFA (5.6 g/d); **Control**: low TFA (0.54 g/day)	4 wk	No effects on lipids and lipoproteins or insulin sensitivity	FA composition of diets was not matched.	Tardy *et al.,* 2009
Randomized-double blind controlled crossover design	Healthy (n=40) (French) men and woman	**Test**: rTFA (11-12 g/d, 5% En); **Control**: iTFA (11-12 g/d, 5% En)	3 wk, 1-wk washout	rTFA increased LDL-C and HDL-C in women only	FA composition of diets was not matched. Basal diets were not controlled. High amount of TFA fed.	Chardigny *et al*., 2008
Randomized-double blind crossover design controlled feeding	Healthy (n=38) (Canadian) men	***High** (10.2 g/3.7% En); ***Moderate** (4.2 g/d, 1.5% En); ***Low** (2.2 g/d, 0.8 % En). *From either rTFA or iTFA	4 wk	LDL-C higher after high rTFA diet vs control or moderate rTFA diet. HDL-C lower after high rTFA diet vs moderate rTFA diet. No difference between moderate rTFA vs control	Moderate rTFA diet has no detrimental effects. High rTFA diet increases CVD risk markers	Motard-Belanger *et al*., 2008
Randomized-double blind crossover design controlled feeding	Healthy young (Canadian) women (n=61)	**Test**: Moderate (3.7g/d, 1.5% En); **Control**: Low (0.9 g/d, 0.3% En)	4 wk	No effect on TC, LDL-C, apoB, apoA1, and TG. Small decrease in HDL-C in woman with BMI ≥ 25		Lacroix *et al*., 2012
Randomized-single blind, controlled crossover design	Hypercholesterolemic (Italian) mean and women (n=42)	Test: rTFA (2.8 g/d); Control: Low rTFA (1 g/d)	3 wk	rTFA cheese reduced LDL-C vs control cheese. No effects on inflammatory markers	Fatty acid composition of diets was not matched.	Pintus *et al*., 2012
Evidence from studies using purified or semi-purified fatty acid						
Randomized parallel design	Healthy (n=24) (German) men and women	**Test**: VA (3g),+t12-18:1 (3 g); **Control**: Oil (18:1 (50%), C16:0, C18:2)	6 wk	No effect on inflammatory markers (IL-6, IL-8, TNF-α)	VA and t12-18:1 oils had 60% purity. Basal diets were not controlled	Kuhnt *et al.*,2006

TC, total cholesterol; En, energy; rTFA, includes VA plus other C18:1 *trans* isomers; FA, fatty acid

1.1.8 Synopsis: Understanding the role of VA in ameliorating metabolically aberrant conditions

A major focus for this thesis will be to investigate the effect of VA supplementation on intestinal and hepatic lipid metabolism, lipid utilization and storage during conditions of MetS and insulin resistance. In order to study putative lipid-lowering properties of VA, it is of utmost importance to appreciate the most relevant physiological aspects of basic lipid metabolism. The following literature review is therefore intended to summarize these concepts. Specifically, emphasis has been given on how the liver and the intestine play complementary and coordinated roles in the regulation of lipoprotein metabolism during the transition from the fasted to the fed state. A discussion on how normal lipid functions become aberrant during conditions of MetS and insulin resistance and the implications of this on increased CVD risk is also presented in later Sections. Because of the putative direct lipid-lowering actions of VA in the intestine, particular focus will be on the importance of non-fasting lipemia during insulin resistance and increased CVD risk. Additionally, a review on the fundamental role of insulin on lipid metabolism and how these regulatory effects of insulin become blunted during obesity-induced insulin resistance is also presented in subsequent Sections. This is followed by a summary of the most relevant characteristics of the animal model of MetS used in the dietary intervention studies conducted in this thesis. Finally, in order to study the potential mechanisms of action by which VA may exert its health benefits, a discussion on how bioactive fatty acids differentially regulate lipid metabolism and insulin pathways with respect to major transcription factors is also provided. Novel properties of fatty acids to potentially regulate lipid signaling pathways are also discussed at the end of this review.

1.2 Plasma Transport of Dietary and Endogenous lipids

1.2.1 Lipoprotein composition and function

Lipoproteins are macromolecules that contain both lipids and proteins to enable the transport of insoluble lipids in the aqueous blood plasma. Each lipoprotein particle consists of a hydrophobic core surrounded by a monolayer surface containing amphipathic lipids and proteins. The hydrophobic core of lipoproteins is comprised of triglycerides (TG) and cholesteryl esters (CE) while the amphipathic surface consist of phospholipids (PL), small amounts of free cholesterol (FC) and proteins (Ginsberg *et al*., 2005). As shown in Table 1-2, circulating lipoproteins can be differentiated based on density, size and composition. Each lipoprotein contains characteristic apolipoproteins that not only function as structural components but also facilitate respective physiological functions.

Table 1-2 Physical properties of major classes or lipoproteins. Adapted from Ginsberg *et al*., 2005

	Nascent CM	CM-r	VLDL	LDL	HDL
Density (g/mL)	<0.95	1.006-1.013	0.95-1.006	1.019-1.063	1.063-1.21
Size (nm)	100-1200	45-60	40-70	18-25	5-12
Lipid (%)					
TG	80-95	70	55-80	5-15	5-10
Cholesterol	2-7	13	5-15	40-50	15-25
PL	3-9	11	10-20	15-25	20-30

CM, chylomicrons, CM-r, chylomicron remnants, LDL, low density lipoprotein; HDL, high density lipoprotein.

Apolipoproteins are the proteins on the surface of lipoproteins providing structural stability. They also play critical roles in the regulation of plasma lipids and lipoprotein transport by acting as ligands for cell surface lipoprotein receptors or activating enzymatic activity in the plasma. For instance, the enzyme lipoprotein lipase (LPL), which catalyzes the hydrolysis of circulating TG, is activated by apoC-II while apoC-III inhibits its activity. Table 1-3 shows the major apolipoproteins (apo), their association with lipoprotein classes and their metabolic functions.

Table 1-3 Characteristics of major apolipoproteins and their metabolic functions. Adapted from Ginsberg *et al*., 2005

Apolipoprotein	Lipoprotein	Metabolic function
apoA-1	HDL, CM	Structural component of HDL, LCAT activator
apoA-II	HDL, CM	Modulates TG metabolism possibly by reducing lipases activity
apoA-IV	HDL, CM	Unknown, possibly facilitates transfer of apos between HDL and CM
apoA-V	HDL	Associated with lower TG levels; mechanism unknown
apoB-48	CM	Necessary for the assembly and secretion of CM from the small intestine. Also secreted from the liver of rats and mice
apoB-100	VLDL, IDL, LDL	Necessary for the assembly and secretion of VLDL from the liver; ligand for the LDL receptor
apoC-I	CM, VLDL, IDL, HDL	May inhibit hepatic uptake of CM, VLDL remnants
apoC-II	CM, VLDL, IDL, HDL	Activator of lipoprotein lipase
apoC-III	CM, VLDL, IDL, HDL	Inhibitor of lipoprotein lipase and of uptake of CM and VLDL remnant by the liver
apoE	CM, VLDL, IDL, HDL	Ligand for binding of several lipoproteins to the LDL receptor, LRP and proteoglycans
apo(a)	Lp (a)	Composed of LDL, apoB linked covalently to apo(a): function unknown but is an independent predictor of CVD

CM, chylomicrons, VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein, LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT; lecithin cholesterol acyltransferase; LRP: LDL receptor-related protein.

1.2.2 Introduction to lipoprotein metabolism

Lipoprotein metabolism involves the transport and redistribution of dietary and endogenous lipids between lipoproteins and tissues in the blood. See Figure 1-2 for a schematic representation of lipoprotein metabolism. Under normal fasting conditions in humans, LDL and HDL are the most abundant circulating lipoproteins in plasma while CM are only found at very small amounts as they are rapidly cleared from the circulation (Sul and Storch, 2006). However, in the presence of compromised metabolic conditions such as insulin resistance, a combination of dyslipidemic characteristics are present and the liver and the intestine play complementary and coordinated roles in regulating these changes. One of the major foci of this thesis will be to investigate whether VA retains its lipid lowering effects on intestinal versus hepatic triglyceride secretion when supplemented on a background diet that includes dairy fat. Therefore a brief review of the key factors regulating basic lipoprotein metabolism will be discussed in this Section. Particularly, special emphasis is on the regulation of assembly and secretion of triglyceride-rich lipoproteins (TRL) and their role on delivering dietary and endogenous lipids to peripheral tissues during fasting and post-prandial conditions.

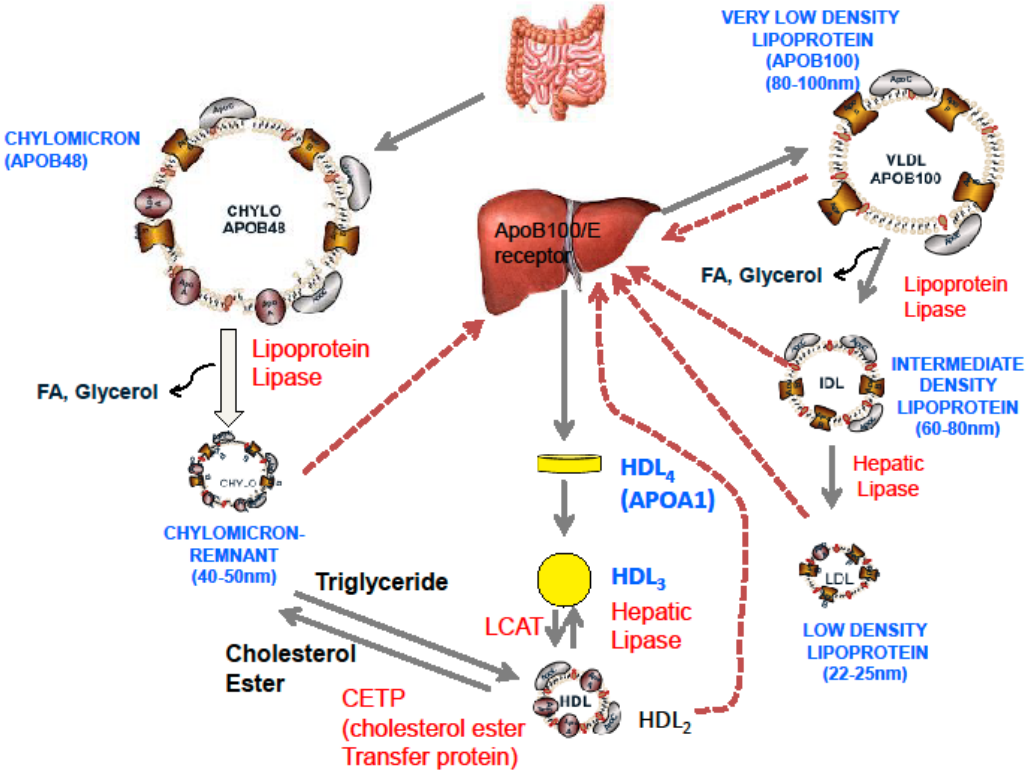


Figure 1-2 Schematic representation of lipoprotein metabolism under normal physiological conditions (*Adapted from Sul and Storch, 2006; Rader and Daugherty). The intestine absorbs dietary fat and packages it into apoB-48 containing-CM which are transported to peripheral tissues through the blood. The liver also packages lipids onto apoB-100 and secretes VLDL. Both triglyceride-rich lipoproteins (TRL) undergo lipolysis by the enzyme lipoprotein lipase (LPL), a triglyceride lipase that is bound to the luminal surface of the capillary endothelium of adipose and muscle tissues. Most of the dietary and endogenously produced-fatty acids are taken up locally by these tissues, although some escape 'spill-over' into the circulation. The resulting particles, CM and VLDL remnants (CM-r and IDL, respectively) can be subsequently taken up by tissues, predominantly by the liver through binding to the LDL receptor (LDLR, also called apoB/E receptor) and other receptors. Another lipase, hepatic triacylglycerol lipase (HL), further hydrolyses IDL triglyceride at the endothelial surface of the*

liver while transferring apoE to HDL. The resulting LDL particles can be also taken up by the liver through the LDLR at the apoB binding site.

The liver and the intestine can also secrete lipid-free apoA-1, the main apolipoprotein constituent of HDL, independently of VLDL/CM secretion. ApoA-1 recruits FC from these organs via ABC-cassette transporter A1 (ABCA1), thus forming nascent HDL (lipid poor HDL). Nascent HDL particles permeate through the vascular bed to the extravascular fluid of peripheral tissues promoting cholesterol efflux by accepting FC and PL from peripheral tissues, including macrophages, via ABCAI. After continuous lipidation, nascent HDL becomes enlarged into disc-shaped discoidal HDL and re-enters the plasma compartment via the lymph flow. Within the plasma, LCAT (lecithin cholesterol acyltransferase), an enzyme activated by apoA-I, esterifies the FC with a sn-2 fatty acid from phosphatidylcholine, forming mature HDL (Jonas, 2000). Mature HDL also promotes the efflux of cholesterol but through ABC-cassette transporter G1 (ABCG1). The cholesterol in HDL is then returned to the liver both directly through uptake by the scavenger receptor B1 (SRB1) and indirectly via transfer to LDL, VLDL and CM through the cholesteryl ester transfer protein (CETP).

1.2.3 Lipid sources for lipoprotein synthesis and secretion

1.2.3.1 Dietary and fatty acids released from adipose stores

A major difference between CM and VLDL production is the source of fatty acids used for TG synthesis and lipoprotein production. While diet is the primary source of fatty acids and glycerol synthesis in the ER of the enterocyte, circulating FFA from lipolysis of adipose tissue is the main source of TG synthesis and VLDL production in the liver.

CM secretion from the intestine primarily follows ingestion of a fat-containing meal; however, native CM particles are also produced and

secreted by the intestine at a basal rate during the fasted state. Accordingly, it has been demonstrated that CM production is stimulated not only by increased availability of dietary lipids but also by non-dietary sources of endogenous fatty acids (circulating free fatty acids) (Duez *et al.,* 2008). However, the extent by which these endogenous fatty acids contribute to the TG pool for CM production is not known.

Studies using stable isotope methodologies have provided quantitative evidence for the contribution of fatty acid sources to hepatic lipid secretion in healthy subjects (Barrows and Parks, 2006). During the fasted state, adipose tissue-derived FFA may account for up to 77% to the total TG secreted as VLDL from the liver. In addition to FFA from adipose tissue (44%), dietary lipids from CM lipolysis and their remnants can contribute to approximately 26% of the VLDL-TG pool during the fed state. Thus, under normal physiological conditions, VLDL secretion from the liver occurs during both fasting and post-prandial states.

1.2.3.2 De novo synthesized fatty acids

Although the liver has the highest capacity for fatty acid synthesis in humans, the intestine also possesses significant activity. Early studies in lymph cannulated rats demonstrated that during lipid absorption a substantial fraction (>50%) of mesenteric lymphatic TG is derived from endogenously synthesized sources (Shiau *et al*., 1985). Both CM and VLDL can incorporate newly synthesized (*de novo*) fatty acids using metabolites from dietary carbohydrates.

The synthesis of fatty acids is tightly controlled by hormonal and nutritional conditions, being mainly determined by insulin concentrations. *De novo* lipogenesis is stimulated by feeding, especially if the diet is high in carbohydrates, whereas fasting or high-fat diets inhibit the rate of *de novo* formation. Yet, *de novo* synthesis contributes to a minor extent to the VLDL-TG secreted from the liver under normal physiological conditions

(Barrows and Parks, 2006). Figure 1-3 shows the schematic representation of the pathways involved in the synthesis of fatty acid in the cellular cytoplasm.

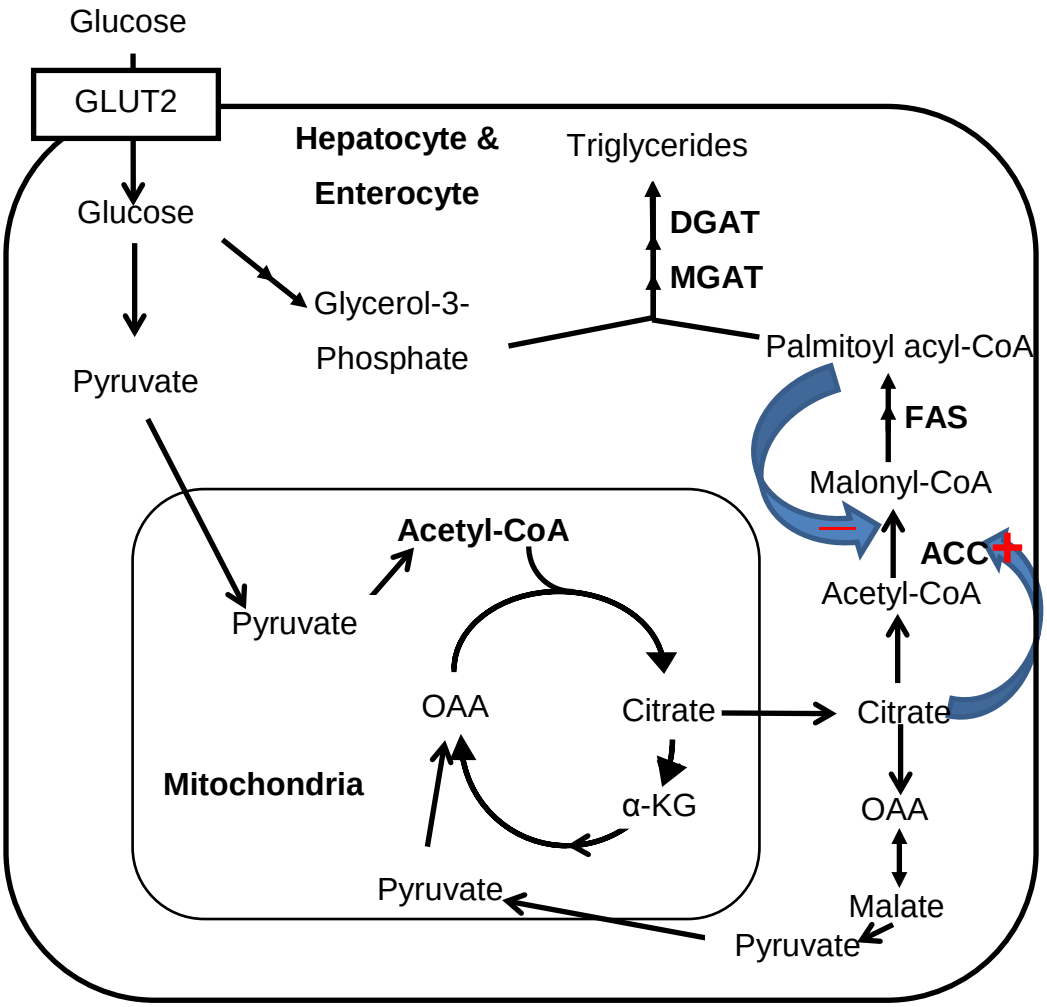


Figure 1-3. Schematic representation of de novo synthesized fatty acids in the cellular cytoplasm *(adapted from Sul, 2006). The enzyme acetyl CoA carboxylase (ACC) catalyzes the conversion of acetyl CoA to malonyl-CoA, the first rate-limiting step in fatty acid synthesis. The activity ACC is allosterically stimulated by citrate. The substrate for fatty acid synthesis, acetyl CoA, is formed from pyruvate during the citric acid cycle in the mitochondria. The inner mitochondrial membrane is impermeable to acetyl CoA. However, increased production of acetyl CoA leads to accumulation of intramitochondrial citrate which unlike acetyl CoA, can be*

translocated to the cytosol and then converted back to acetyl CoA. In contrast, ACC activity is allosterically inhibited by long chain acyl CoA, the level of which is decreased during the fed state when fatty acid synthesis is stimulated. Further, the activity of ACC is also regulated by phosphorylation. When cellular energy state is low, AMP-activated protein kinase is allosterically activated by adenosine monophosphate (AMP) that in turn phosphorylates ACC resulting in a decrease in its activity. In addition, ACC is also regulated by changes in protein concentration, which is controlled at the transcriptional level. Insulin and glucose increase whereas glucagon (via cyclic AMP) decreases the rate of its transcription. The second and final committed step in fatty acid synthesis is catalyzed by a multifunctional enzyme complex, fatty acid synthase (FAS) which is responsible for the serial addition of two-carbon fragments to a growing chain until a 16-carbon fatty acid (palmitate) has been formed. The activity and abundance of FAS is also regulated at the transcriptional and post-transcriptional level being higher in the fed and lower in fasting state. Fatty acids from both dietary and de novo sources are subject to further chain elongation and subsequent desaturation by the action of the enzyme SCD1. Fatty acyl CoA are further esterified into TG, an enzymatic reaction catalyzed by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltrasnferase (DGAT). OAA, oxaloacetate; *α-KG: alpha ketoglutarate.*

1.2.4 Synthesis of triglyceride from dietary and endogenous sources and regulatory enzymes

The liver and the intestine synthesize triacylglycerols by two basic pathways; the glycerol phosphate pathway and the monoacylglycerol pathway. The glycerol phosphate pathway is a major source for *de novo* TG synthesis in the liver and is also responsible for all synthesized TG in the intestine when sn-2-monoacylglycerol (2-MG) is not available (for example during fasting conditions). In contrast, the monoacylglycerol pathway predominates in the small intestine and is the major source of TG

synthesis during dietary fat absorption. Figure 1-4 shows the two metabolic pathways, in which the enzymes MGAT and DGAT catalyze two consecutive steps of enzyme reactions in TG biosynthesis. MGAT is mainly expressed in the small intestine while DGAT is expressed in most tissues, including the liver and the intestine (Yen *et al*., 2008).

MGAT and DGAT play fundamental roles in regulating fat absorption, storage and lipoprotein secretion and have direct implications in obesity, insulin resistance and hepatic steatosis. In the liver, DGAT1 appears to be required for esterifying exogenous fatty acids (circulating FFA from adipose or CM lipolysis) and contributes to increased hepatic TG storage induced by high-fat feeding and prolonged fasting (Villanueva *et al*., 2009). Studies using genetically modified mice have demonstrated that MGAT2 and DGAT1 have a crucial role in the assimilation of dietary fat in the intestine and subsequent accretion of body fat. MGAT2$^{-/-}$ and DGAT1$^{-/-}$ mice are protected against developing obesity, glucose intolerance and fatty liver. Interestingly, deficiency of MGAT2 (Yen *et al*., 2009) does not cause malabsorption but presents a delayed rate of dietary fat uptake into the circulation, shifting the bulk of absorption from the proximal to the distal small intestine. A similar phenotype has been described in the DGAT1$^{-/-}$ mouse (Smith *et al*., 2000) which presents a slower assembly and secretion of CM and results in the accumulation of large neutral lipid droplets in the enterocyte, particularly when acutely or chronically challenged by high amounts of dietary fat (Buhman *et al*., 2002). Interestingly, while whole body DGAT1$^{-/-}$ is resistant to fat accumulation in the liver and adipose tissue, restoring DGAT1 expression in the intestine reverses the resistance to diet-induced fatty liver and obesity (Lee e*t al*., 2010). This study demonstrated that altering the secretion of dietary fat out of the enterocytes subsequently affects the fate of dietary fat in specific tissues, highlighting the importance of CM secretion on diet-induced obesity.

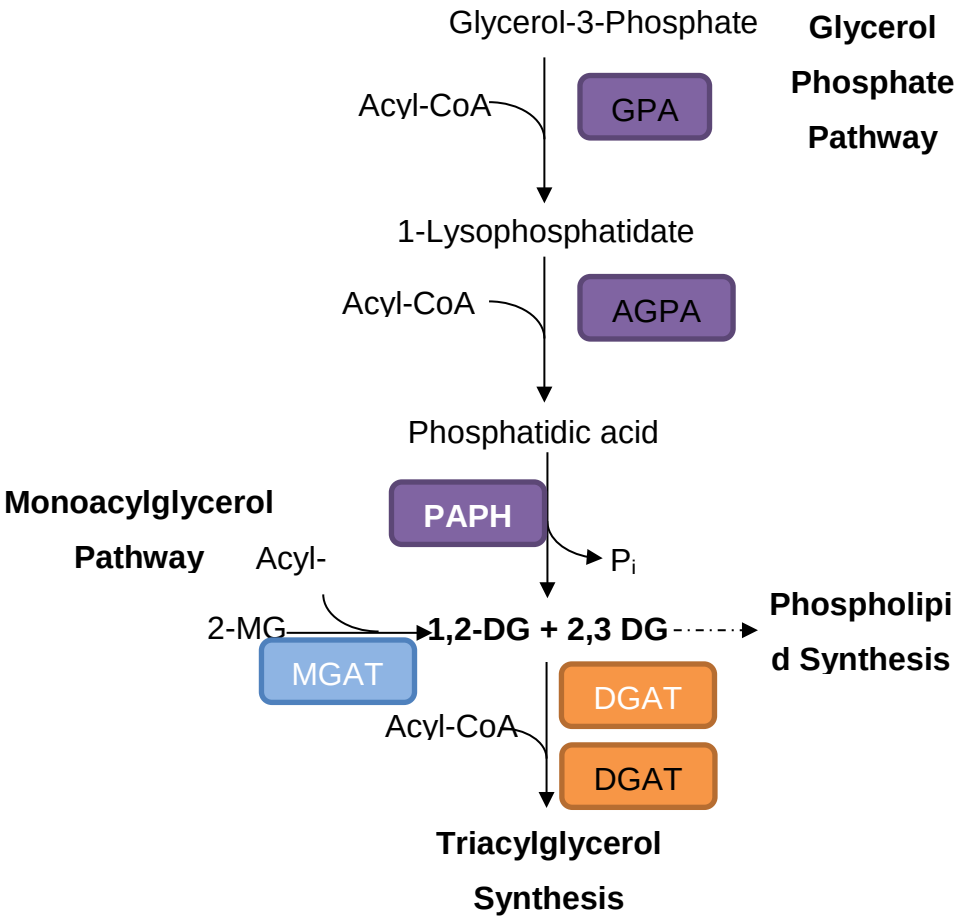


Figure 1-4 The metabolic pathways and enzymes for TG synthesis (adapted from Yen et al., 2008). Both pathways use the activated forms of fatty acids, fatty acyl-CoA (synthesized by intracellular acyl-CoA synthases) as acyl donors. In the final reaction of both pathways, a fatty acyl CoA and diacylglycerol (DG) molecule are covalently joined to form TG, a reaction catalyzed by DGAT at the surface of the endoplasmic reticulum (ER) bilayer membrane. DG is also used as a substrate for the synthesis of phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Newly synthesized TG are then channeled into cytosolic lipid droplets or secreted into nascent lipoproteins. GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acyl-glycerol-phosphate acyltransferase; PAPH, phosphatidate phosphatase.

1.2.5 Synthesis and secretion of triglyceride rich lipoproteins

ApoB is the dominant non-exchangable apolipoprotein of CM and VLDL. Measuring apoB secretion is indicative of particle secretion as there is only one apoB molecule per CM or VLDL particle. In humans, apoB-48 is transcribed from the gene for apoB-100 in the small intestine. The apoB mRNA is edited to the truncated apoB-48 form in which the cytosine in position 6666 (CAA) encoding for glycine, is deaminated to a uracil resulting in an in-frame UAA stop codon. The translation to apoB-100 stops at this codon, which is 48% translation of the whole apoB (apoB-100) gene (Chen *et al*., 1987). With the exception of the human liver, apoB-100 is edited in both the liver and the intestine of a number of species such as mouse and rat. Thus, while apoB-48 is secreted exclusively from the intestine in CM in humans, it is secreted from both organs in rodents.

The synthesis and secretion of intestinal and hepatic-derived TRL requires coordinated synthesis of lipids and a number of intracellular factors that facilitate the transfer of these lipids onto the apoB protein (Lehner *et al*., 2012). The current model for the core lipidation of apoB-containing lipoproteins proposes a two-step process. In the first step, apoB protein is cotranslationally lipidated while translocated across the rough endoplasmic reticulum (RER) to form a primordial apoB particle. In the second step, triglyceride-rich lipid droplets lacking apoB are assembled within the lumen of the smooth endoplasmic reticulum (SER) of hepatocytes and ribosome poor regions of enterocyte ER. Subsequent fusion of the two particles leads to core expansion of primordial apoB and synthesis of nascent CM or VLDL. These nascent lipoproteins are then exported to the Golgi where they undergo further maturation by acquiring other apolipoproteins on the surface. However, how these other apoproteins are synthesized or sourced in the enterocyte/hepatocyte or incorporated into the CM/VLDL is not known in detail. Then, nascent CM

and VLDL are secreted into the mesenteric lymphatic system or the hepatic vein, respectively (Hamilton *et al*., 1998). See Figures 1-5 and 1-6 for the schematic representation of CM and VLDL synthesis, respectively.

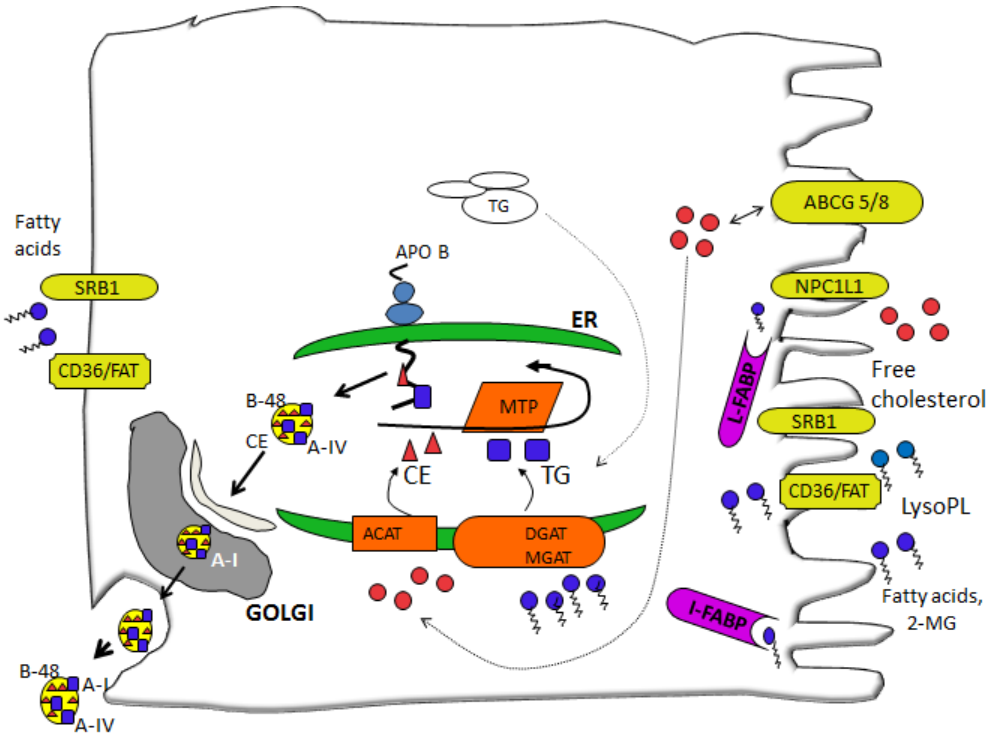


Figure 1-5 Schematic representation of CM synthesis in the enterocyte. Dietary lipids, mostly TG are emulsified and hydrolyzed within the lumen of the small intestine to fatty acids (FA) and 2-MG by pancreatic TG lipase. *Additionally, FC and sn-1-monoacyl-lysophospholipids (LysoPL) mainly from biliary cholesterol and to a lesser extent dietary PL (primarily PC), are also presented to the brush border membrane (BBM) of the enterocyte. These digestion products are efficiently and rapidly absorbed across the BBM, putatively transported into the enterocyte by a number of lipid transporters (CD36/FAT, NPC1L1, SRB1, ABCG5/8). Absorbed lipids are then chaperoned to the ER by intracellular trafficking proteins (L-FABP, I-FABP and FATP4). It has also been proposed that the enterocyte can uptake circulating free fatty acids and cholesterol from the basolateral*

membrane via CD36/FAT, SRB1 and other transporters. Fatty acids, 2-MG and lysophospholipids are re-esterified into TG and PL by the action of MGAT and DGAT prior to their incorporation into CM. FC is also re-esterified into CE in the ER by the action of acyl CoA acyl transferase 1 (ACAT1). These newly synthesized lipids are incorporated onto a single apoB-48 by the action of microsomal triglyceride transport protein (MTP). This particle is subsequently coated by apoA-IV, which then buds from the ER surrounded by a membrane as the prechylomicron transport vesicle (PCTV), an ATP-dependent process facilitated by CD36 and L-FABP. The PCTV translocates to and fuses with the Golgi complex, where apoA-I presumably attaches to the prechylomicron. Then a mature CM containing apoAI, apoA-IV and apoB-48 is secreted in large transport vesicles fusing at the basolateral membrane of the enterocyte.

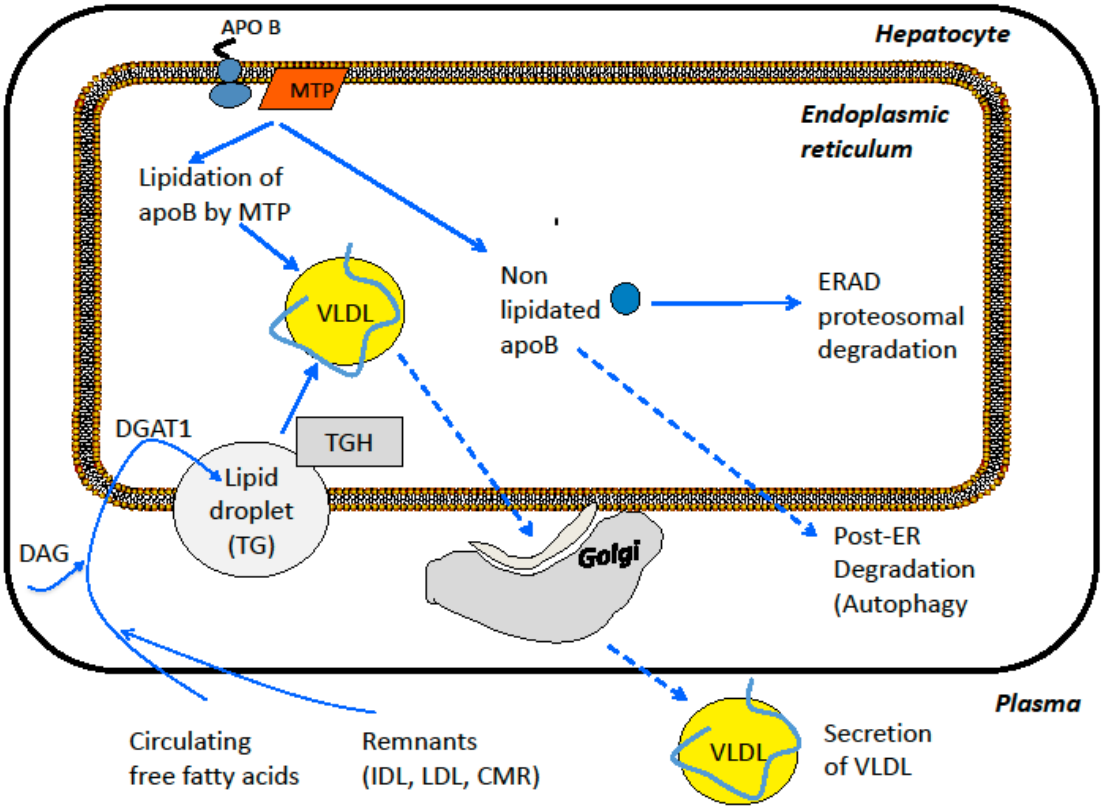


Figure 1-6 Schematic representation of VLDL synthesis in the hepatocyte (adapted from Xiao et al., 2011). The biosynthesis of VLDL follows similar pathways of CM packaging in the intestine, but hepatic TG may be derived from circulating FFA (main source), lipoprotein remnant uptake and lipid storage pools in the liver. Circulating FFA are preferentially esterified into TG by DGAT1, while triacylglycerol hydrolase (TGH) is involved in the lipolysis of luminal lipid droplets that after re-esterification load onto nascent apoB-containing particles. Hepatic apoB synthesis and secretion is regulated by intracellular degradation. If apoB100 is not lipidated in the ER, it can be co- or post-translationally targeted for degradation via the ubiquitin-proteasome pathway (ER-associated degradation, ERAD) (Fisher et al., 1997). Lipid overload which induces ER stress can also drive apoB100 to proteasomal or non-proteasomal (autophagic; Pan et al., 2008) degradation. In contrast, if core lipids are available (in the absence of ER stress), nascent VLDL buds from the ER membranes in the VLDL

transport vesicles that contain coat protein complex II (COPII), to be transported to the Golgi along the secretory pathway.

The mechanism that controls the synthesis and number of particles secreted from the intestine is not fully understood. However, there is evidence to suggest that similar to hepatic VLDL, CM assembly and secretion is mainly driven by lipid substrate availability. In the absence of sufficient lipid substrates, apoB may be targeted to degradation thus resulting in reduced CM and VLDL secretion (Haidari *et al*., 2002).

1.2.6 Metabolism of triglyceride rich lipoproteins

As soon as CM and VLDL enter the circulation, they acquire apoCII, apoCIII and apoE from HDL while apoA-I is transferred from nascent CM and VLDL to the HDL. At the same time, the enzyme CETP enables CM and VLDL to take up CE from HDL while donating TG to the HDL. As CM and VLDL circulate in the plasma, core TG are hydrolyzed by LPL present on the capillary of the endothelium of adipose and muscle tissues and FFA and glycerol are released. These lipolytic products can be taken up by the adipose tissue and muscle, where they are used for storage or as energy sources, respectively. Some FFA can also spill-over into the plasma as albumin-bound FFA and circulate back to the liver and the intestine for uptake by free diffusion or via plasma membrane transporters.

The resulting remnant particles following lipolysis (CM-r, small VLDL and IDL) are smaller, TG-poor and enriched in apoE and CE. These remnant particles can be cleared from the plasma by all tissues, but predominantly by the liver through the apoB/E receptors, which have a higher affinity for apoE than apoB. Unlike CM-r, VLDL remnants (IDL) can undergo further lipolysis by HL at the endothelial surface of the liver while transferring apoE to HDL. The resulting LDL particle, lacking of apoE, can be also taken up by the liver through the apoB/E receptor at the apoB binding site.

The apoE-containing lipoproteins (CM-r, small VLDL and IDL) can also be taken up by tissues through binding to the LDL receptor-related protein (LRP), which only recognizes apoE but not apoB. This alternative remnant clearance involves the initial sequestering of remnants by negatively charged surface heparan sulfate proteoglycans (HSPG), which not only anchor LPL on the capillary of the endothelium but also facilitate the binding of apoE to LRP (Mahley, 1999). However, the metabolism of CM via this alternative pathway was shown to be significantly slower as compared to the LDLR pathway, probably due to slow internalization of remnants (Mortimer *et al*., 1995).

CM and VLDL share common, saturable metabolic pathways and therefore, compete for LPL and cell surface receptors for removal from the circulation (Brunzell *et al*., 1973). Postprandially, increased delivery of exogenous dietary lipids promotes hepatic secretion of VLDL (Barrows and Parks, 2006) and delayed clearance of LDL due to increased competition with CM for removal. Therefore, the mechanism by which postprandial lipemia increases CVD risk was thought to be primarily as a consequence of impaired LDL kinetics. However, there is now accumulating evidence that intestinal-derived remnant lipoproteins, including nonfasting postprandial remnants can also promote CVD risk. This risk is substantially increased during compromised metabolic conditions such as insulin resistance and metabolic syndrome.

1.3 Metabolic Syndrome, CVD Risk and Non-fasting Lipids

1.3.1 Introduction

The continuous provision of energy via select macronutrients in the Western diet may differentially contribute to the development of CVD risk. By definition, CVD are a group of disorders of the heart and the vessels and include coronary heart disease, cerebrovascular disease, peripheral artery disease, reumathic heart disease, congenital heart disease, deep

vein thrombosis and pulmonary embolism. Amongst these, stroke and coronary heart disease contribute to the highest rate (up to 78%) of deaths (WHO, 2011). The most common cause of these events is atherosclerosis, which is a condition in which the build-up of lipids on the arteries leads to plaque formation resulting in the narrowing and hardening of the arteries. This ultimately leads to a blockage in the vessels that carry oxygen-rich blood to the heart and the brain.

A significant component of CVD risk can be prevented by addressing risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity, raised blood pressure, diabetes and raised lipids (WHO). Despite significant progress through management of blood lipid levels with dietary changes and pharmacological therapies, CVD remains the leading cause of mortality attributing to approximately 30% of all global deaths (WHO, 2011). Unhealthy diets and physical inactivity clearly remain as the major underlying risk factors of this public health burden.

1.3.2 Pathophysiology of the metabolic syndrome

The rise in daily caloric intake and physical inactivity in the last few decades corresponds with a dramatic increase in the prevalence of obesity, type 2 diabetes (T2DM) and metabolic syndrome (MetS) (Wright *et al*., 2004). The MetS is a clinical condition predisposing affected individuals to the development of T2DM and CVD risk. There are several definitions of the MetS (see Table 1-4 for the diagnostic criteria of the MetS for some of the established definitions). It is important to note that although obesity is one of the main underlying risk factors for the MetS, absolute mass of adiposity does not necessarily account for all metabolic abnormalities (Wildman *et al*., 2008). Rather than total adiposity, visceral and/or ectopic fat is the core clinical component of the MetS contributing to the development of insulin resistance (Bremer *et al*., 2012).

Insulin resistance or the resistance of tissues (e.g. skeletal muscle, adipose tissue) to insulin mediated glucose uptake has been postulated to be the fundamental defect during MetS. Further, during insulin resistance a compensatory hyperinsulinemia will be needed in order to maintain glucose homeostasis and tissue uptake. If this compensatory increase is not enough to overcome insulin resistance to allow glucose uptake by tissues, then progression to T2DM will occur (Reaven, 1988).

Insulin resistance underlies a number of the manifestations and cardiovascular complications of T2DM and is considered itself a CVD risk factor (Rader, 2007). See Figure 1-7 for the metabolic risk factors linking insulin resistance and CVD risk. Although individuals with coexisting MetS and diabetes have the highest prevalence of CVD, the risk of CVD begins to increase with insulin resistance even before the onset of diagnosed diabetes (Alexander *et al*., 2003; Hu *et al*., 2002). Thus, preventive management of insulin resistance via lifestyle modifications that target visceral adiposity can potentially reduce the progression to T2DM and CVD risk.

A major focus for this thesis will be to investigate the effect of VA supplementation on adipose tissue distribution and ectopic (hepatic and intestinal) lipid accumulation. Additionally, the emphasis of this thesis will be on the putative ability of VA to favourably regulate intestinal lipid metabolism during conditions of insulin resistance and MetS. Therefore, a brief review of the role of non-fasting remnant dyslipidemia in increased CVD risk during the pathophysiology insulin resistance will be discussed in the following Sections.

Table 1-4 Clinical criteria for the diagnostic of the metabolic syndrome.

International Diabetes Federation (IDF) (Alberti *et al*., 2005)	**Adult Treatment Panel (ATPIII) (Grundy *et al.,* 2005)**
The patient must have: **Elevated waist circumference *(as a measure of central obesity)*** †*Europids*: *Men*: ≥ 94 cm *Women*: ≥ 80 cm **South Asian and Chinese*: *Men*: ≥ 90 cm *Women*: ≥ 80 cm *Japanese*: *Men*: ≥ 85 cm *Women*: ≥ 90 cm †Also for Sub-Saharan Africans, Eastern Mediterranean & Middle East populations *Also for South & Central Americans **Plus any 2 of the following:** ***Elevated triglyceride*** ≥ 1.7 mmol/L (150 mg/dL), or Specific treatment for this lipid abnormality ***Reduced HDL- cholesterol (HDL-C)*** *Men:* <1.03 mmol/L (40 mg/dL) *Women*: <1.3 mmol/L (50 mg/dL) or Specific treatment for this lipid abnormality ***Elevated blood pressure*** ≥ 130 mm Hg (systolic) ≥ 85 mm Hg (diastolic) ***Elevated fasting glucose*** ≥ 5.6 mmol/L (100 mg/dL) or Previously diagnosed type 2 diabetes	**Measure (any 3 of 5 criteria constitute diagnosis for MetS):** **Elevated waist circumference** *Men*: ≥ 102 cm *Women*: ≥ 88 cm *Non-Asian (e.g. White, Black, Hispanic) at increased risk:* *Men*: 94-101 cm *Women*: 80-87 cm *Asian:* *Men*: ≥ 90 cm *Women*: ≥ 80 cm ***Elevated triglyceride*** ≥ 1.7 mmol/L (150 mg/dL) ***Reduced HDL cholesterol (HDL-C)*** *Men:* <1.03 mmol/L (40 mg/dL) *Women*: <1.3 mmol/L (50 mg/dL) or On drug treatment for reduced HDL-C ***Elevated blood pressure*** ≥ 130 mm Hg (systolic) ≥ 85 mm Hg (diastolic) ***Elevated fasting glucose*** ≥ 100 mg/dL or On drug treatment for elevated glucose

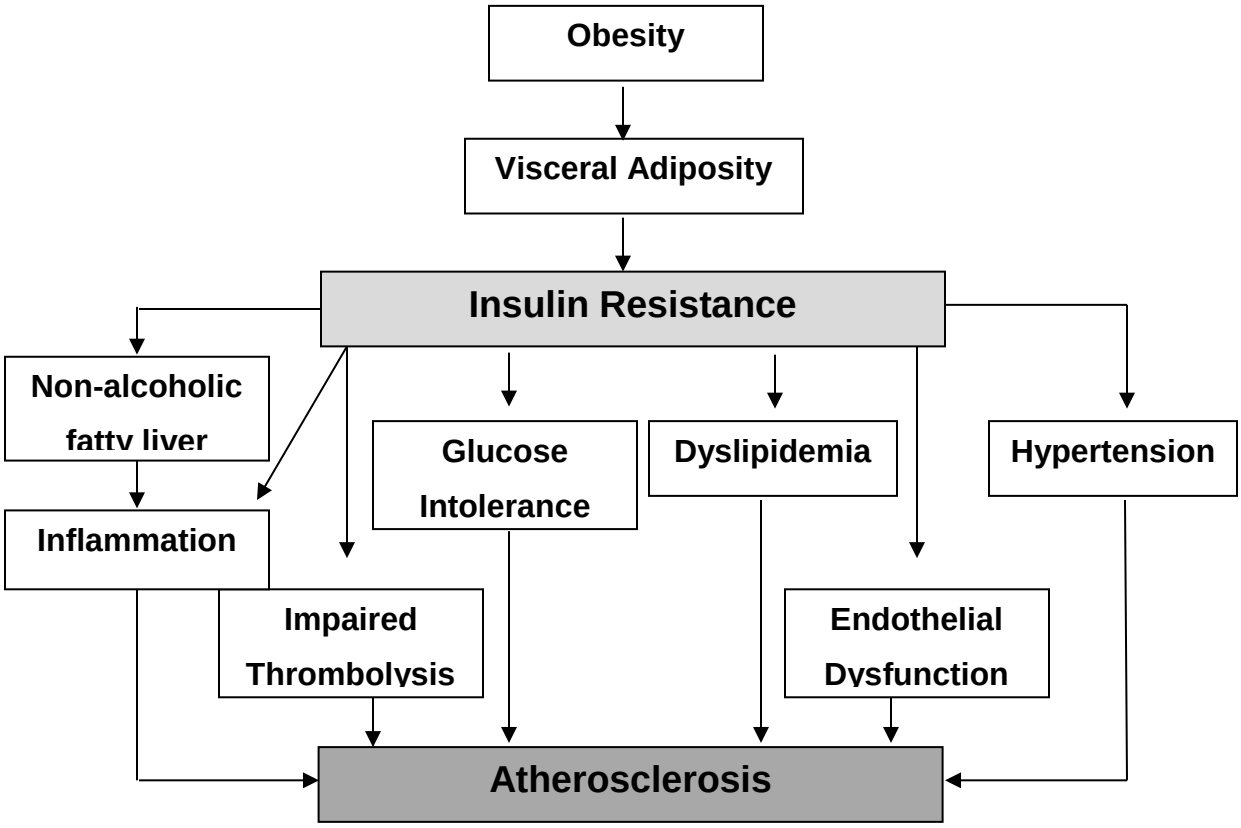


Figure 1-7 Association of insulin resistance with CVD risk factors (adapted from Rader, 2008)

1.3.3 Non-fasting lipemia as an independent predictor of CVD risk

The dyslipidemia in insulin resistance has been largely attributed to the overproduction of VLDL from the liver; however, there is now accumulating evidence that production of intestinal-derived remnant lipoproteins, including non-fasting postprandial remnants are also significantly elevated during these conditions (Duez *et al*., 2008b). Physiologically, it is also important to consider the often hyperphagic behavior observed during obesity which contributes to a sustained secretion of CM-associated TG and increased circulating TG levels. Additionally, delayed clearance of these lipoproteins is likely during these

conditions which in turn results in the accumulation of CM-r even in the fasted state (Curtin *et al*., 1996; Hogue *et al*., 2007).

1.3.3.1 Indirect effect of TRL on increased CVD risk

The MetS is significantly associated with a 1.5 to 3 fold increased risk of atherosclerotic CVD (Eckel, 2007; Hu *et al*., 2005). It is important to note that despite the established role of LDL-cholesterol as a CVD risk factor, none of the definitions of MetS include LDL-cholesterol (Table 1-4). Rather, elevated plasma triglyceride is included as a diagnostic criterion suggesting the importance of hypertriglyceridemia as a surrogate marker to evaluate CVD progression. Much of this increase in CVD risk is associated with the presence of a combination of risk factors that include elevated TG, decreased HDL-C and relatively normal concentrations of LDL-C carried in small, dense, LDL particles. This characteristic dyslipidemic profile of decreased HDL-C concentrations and abnormal LDL composition is largely dependent on the metabolism of TRL particles (Howard *et al*., 2003). For example, increased concentration and impaired clearance of TRL during insulin resistance facilitates the CETP-mediated exchange between CE in HDL/LDL and TG in TRL. This exchange produces CE-depleted and TG-enriched particles which are better substrates for HL. The resulting particles are smaller, denser, atherogenic-prone LDL and smaller, denser HDL which are thought to be rapidly cleared from the circulation. At the same time, the acquisition of more CE by TRL, particularly the remnants, makes them more atherogenic as a result of the increased cholesterol load per particle (Mangat *et al*., 2011).

1.3.3.2 The proposed direct effect of TRL on increased CVD risk

In addition to this indirect influence of TRL metabolism on atherogenic dyslipidemia, significant evidence also supports a direct involvement of elevated TG in the pathogenesis of CVD. Specifically, it has been demonstrated that intestinal-derived TG-rich remnant lipoproteins can

penetrate the arterial tissue and become entrapped within the subendothelial space (Mamo and Wheeler, 1994; Proctor and Mamo, 2003 and 2004), where they can contribute to the formation of foam cells, a hallmark feature of early atherosclerosis (Yu and Mamo, 1997; Tomono *et al*., 1994; Batt *et al*., 2004). Furthermore, the postprandial phase is associated with increased inflammation which in turns exacerbates the development of atherosclerosis. It has been proposed that postprandial remnant lipoproteins themselves can activate circulating monocytes, upregulate the expression of endothelial cellular adhesion molecules and facilitate adhesion and migration of inflammatory cells into the subendothelial space, thus inducing macrophage lipid loading and eventually foam cell formation (Klop *et al*., 2012). In addition, it has been demonstrated that the increased production and arterial retention of cholesterol-rich intestinal-derived remnants in insulin resistance states can contribute to accelerated CVD progression (Mangat *et al*., 2012). Because humans are in the postprandial state for most of the day, their arteries are thus exposed to this postprandial plasma most of the time. Therefore it has been proposed that postprandial TG values would correlate better with prognosis than fasting values and this has been shown in recent epidemiological studies (discussed in the following Section).

1.3.3.3 Evidence for the role of non-fasting triglyceridemia on increased CVD risk

Adjustment for HDL-C usually attenuates the association between hypertriglyceridemia and CVD risk because plasma TG levels are inversely correlated with HDL-cholesterol (HDL-C). Thus, the causal association between plasma TG and CVD progression remains uncertain (Goldberg *et al*., 2011). However, recent reports from large population-based studies suggest that non-fasting TG independently predict CVD risk. In the Women's Health Study cohort, non-fasting (as opposed to fasting) TG levels maintained a strong independent association with

cardiovascular events after adjusting for measures of insulin resistance and total and HDL cholesterol (Bansal *et al*., 2007). Similarly, in the Copenhagen cohort study, non-fasting TG was also shown to significantly predict future vascular events in both men and women after a multifactorial analysis (Nordestgaard *et al*., 2007). In both studies, the greatest non-fasting TG concentrations were associated with a highest risk.

Elevated TG concentrations during insulin resistance and MetS have numerous negative effects that can contribute to the progression of CVD but can be positively influenced by diet and lifestyle interventions. The fatty acid composition in the diet has been shown to affect TRL production and metabolism. Findings from pre-clinical studies suggest that the predominant naturally occurring TFA, VA, can reduce fasting and post-prandial hypertriglyceridemia during established symptoms of MetS. However, whether VA would retain its lipid-lowering benefits when supplemented in a background diet that includes dairy-derived SFA is unknown. One of the objectives of my thesis was to investigate the effects of VA on hepatic *versus* intestinal TG secretion in a rat model of the MetS, by using an approach that involves *in vivo* LPL inhibition in both fasting and non-fasting conditions.

1.4 Integrated Role of Intestine, Liver and Adipose Tissue in Lipid Metabolism and Metabolic Syndrome

As discussed above, the liver and the intestine play complementary and coordinated roles in regulating lipoprotein metabolism through the assembly and secretion of TRL. Both organs are also responsible for delivering dietary and endogenously synthesized lipids to peripheral tissues such as the skeletal muscle and adipose tissue during fasting and fed conditions. This transition from the fasted to the fed state is tightly regulated by insulin, the action of which is blunted during conditions of

obesity-induced insulin resistance. A brief review of how insulin modulates the physiological transition from the fasted to the fasted state and how these regulatory effects of insulin become blunted during obesity and insulin resistance is presented in the following Section.

1.4.1 The physiological role of insulin (transition from the fed to the fasted state)

Upon ingestion of carbohydrate, insulin is released from the β cells of the pancreas and activates its receptors on muscle, adipose and liver cells. This results in the modulation of gene expression and protein activity that turns on a variety of enzymatic processes facilitating the transport and storage of nutrients into the cell.

In skeletal muscle and adipose tissue cells, insulin increases the glucose transport by stimulating the translocation of the transporter GLUT4 from intracellular sites to the plasma membrane. Skeletal muscle is the major consumer of postprandial glucose accounting for up to 75% of the insulin-stimulated glucose utilization (disposal) while adipose tissue uptake accounts for only a small fraction (Klip and Paquet, 1990). Insulin also promotes the synthesis of glycogen in these tissues which is used locally as an immediate reserve of glucose during fasting conditions.

Glucose uptake in the liver is independent of insulin and is mediated by the GLUT2 glucose transporter. After a meal, high levels of glucose reach the liver via the portal vein. Insulin promotes glycogen synthesis, glycolysis and glucose oxidation by increasing the expression of glucokinase which converts glucose into glucose-6-phosphate (an intermediary product that cannot escape the cell). The quick removal of glucose facilitated by this enzyme is thus the driving force for more hepatic glucose uptake (Vidal-Alabró *et al*., 2012). Insulin also acts in the liver by diminishing the expression of genes required for gluconeogenesis, mainly phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-

phosphatase through the Akt/FoxO1 pathway **(**Brown and Goldstein, 2008). The net result is decreased hepatic glucose output. Conversely, the drop of insulin during the fasted state increases hepatic gluconeogenesis and promotes glycogenolysis.

From the lipid centric view, insulin is central to regulating lipid metabolism postprandially by promoting lipid synthesis and inhibiting lipolysis. In the liver, adipose tissue and presumably the intestine, insulin promotes *de novo* lipogenesis by activating the transcription factor sterol regulatory element-binding protein 1-c (SREBP1-c). This in turn increases the transcription of genes involved in the synthesis and uptake of fatty acids and TG (Chen *et al.,* 2004). Lipogenic enzymes such as ACC and FAS, which use substrates from glucose metabolism, are transcriptionally up-regulated by insulin (Foufelle *et al*., 1996).

In adipose tissue, insulin reduces the activity of hormone sensitive lipase (HSL), the rate-limiting enzyme in adipose tissue lipolysis, resulting in reduced release of FFA into the circulation. In addition to increasing lipid synthesis and esterification, insulin also up-regulates the activity of LPL in adipose tissue while down-regulating its activity in other tissues such as the skeletal muscle (Picard *et al.,* 1999). These tissue-specific differences thus determine the partitioning of lipids between storage and oxidation during the fasted to fed transition. The overall effect of insulin stimulation in adipose tissue is the significant increase in its lipid storage capacity.

1.4.2 The role of adipose tissue in obesity and metabolic syndrome

In the face of chronic overnutrition and physical inactivity that characterize modern lifestyle patterns, excessive expansion of adipose tissue is highly involved in the development of MetS. From an evolutionary point of view, the human body still possesses its ability to respond to alterations in nutrient deprivation and excess through adipocyte proliferation (hyperplasia) and expansion (hypertrophy). However, this adipose tissue

remodeling capacity is pathologically accelerated during obesity (Sun *et al*., 2011).

It has been proposed that pathological expansion of adipose tissue is characterized by a rapid growth of fat pads through enlargement of existing fat cells and a higher degree of macrophage infiltration. Such pathological expansion is associated with a state of chronic grade inflammation that ultimately results in the development of insulin resistance (Sun *et al*., 2011).

1.4.2.1 Anatomic and functional differences between fat depots: links to metabolic risk

The distribution of fat among specific regional depots appears to be more important than the total adipose mass for the developing obesity-associated abnormalities (Neeland *et al*., 2012). Subcutaneous adipose tissue (SAT) is located under the skin and has greater energy storage capacity than visceral adipose tissue (VAT). Intra-abdominal fat depots are associated with internal organs and in humans are classified as intra- and retroperitoneal. Intra-peritoneal depots are associated with digestive organs and include the omental (associated with the stomach) and mesenteric (associated with the intestine) depots. These visceral fat depots are characterized by the presence of lymphatic nodes where immunocytes (e.g neutrophils, T cells, lymphocytes, macrophages) accumulate contributing to the over-expression of immune-related proteins (Lee and Fried, 2010; Thomou *et al.,* 2010).

In addition to the inherent differences in secretory profiles between fat depots, the adverse metabolic effects of visceral obesity have also been associated with its anatomical location. For instance, it has been proposed that FFA and adipokines from VAT may directly reach the liver via the portal vein. In contrast, FFA released from SAT enter the systemic circulation where directed to the skeletal muscle can be oxidized.

Different adipose tissue depots have distinct endocrine and metabolic properties. Inflammatory cytokines that have been implicated in monocyte/macrophage chemotaxis and activation (e. g. monocyte chemoattractant proteins (MCP)-1 and colony-stimulating factor (CSF)-1) are expressed at higher levels in omental than SAT, presumably leading to the higher macrophage infiltration in this fat depot (Harman-Boehm, *et al*., 2007). This increased number of resident macrophages in VAT may contribute to the increased pro-inflammatory cytokines (e.g. interleukin (IL)-6, IL-8, tumor necrosis factor (TNF-α) and plasminogen activator inhibitor (PAI)-1) secreted from this fat depot (Lee and Fried, 2010).

The literature also documents that adipocytes in VAT are larger in size and less sensitive to the anti-lipolytic effects of insulin than those in SAT, resulting in a greater lipolytic capacity of visceral fat (Lee and Fried, 2010; Thomou *et al.,* 2010). Table 1-5 summarizes some of the most relevant physiological differences between fat depots.

One of the most potent therapeutic regulators of adipose tissue distribution and size are the insulin sensitizing drugs, thiazolidinediones (TZD) which act by activating the nuclear receptor, peroxisome proliferator receptor-γ in adipose tissue. It has been recently proposed that the lipid-lowering and anti-inflammatory effects of VA may be partially associated with its ability to activate PPAR-γ regulated pathways (Wang *et al*., 2012; Jaudszus *et al*., 2012). *However, whether dietary supplementation with VA can affect adipose tissue distribution and size is not known. This led to the rationale in this thesis for investigating the ability of VA to alleviate the putative stimulatory effects of dairy fat on ectopic (hepatic and intestinal) lipids and visceral adiposity. A more descriptive review of the most relevant concepts regarding PPAR regulation by fatty acids and clinical outcomes of their activation by synthetic ligands is presented in Section 1.6.2.2.*

Table 1-5 Physiological differences between fat depots (adapted from Lee and Fried, 2010; Thomou *et al.,* 2010).

Variable	**Subcutaneous (SAT)**	**Visceral (VAT)**
Fuel storage	Long-term/ protection against lipotoxicity	Short-term/rapid fatty acid release to the liver
Anatomic constrains	None	Restricted, especially during pregnancy
Tissue growth	Fat cell number	Fat cell size
Adipocyte size		Larger than SAT
Catecholamine-induced lipolysis	Higher expression of anti-lipolytic α-adrenergic receptors	Increased number of β-adrenergic receptors and blunted α-receptor-mediated decline in cAMP levels; thereby increased lipolysis than SAT
Insulin-induced lipolysis		Less responsive to anti-lipolytic effects of insulin
Adipogenic-related genes	Higher PPARγ and C/EBPα than VAT	
Lipogenic-related genes		ASP and C3a, SREBP-1c, ACC
Lipid uptake and lipid droplet-related genes	Higher FABP4 (aP2), CD36 and perilipin than VAT	
Macrophage infiltration		Higher than SAT
Cytokine and adipokine secretory profile	Higher leptin mRNA and secretion, adiponectin and IL-10 than VAT	Secrete more pro-inflammatory cytokines (IL-6, IL-8, TNF-α, PAI-I, MCP-1 and GM-CSF-1

cAMP, cyclic AMP; FABP4, fatty acid protein-4; ASP, Acylation-stimulating protein (promotes fat cells TG synthesis); C3a, complement factor C3a (post-translational modification leads to ASP generation)

1.4.3 The pathophysiology of insulin resistance in obesity and dysfunctional lipid homeostasis

The insulin resistant state is characterized by the inhibition of insulin signaling pathways (Aguirre *et al.,* 2000). Insulin resistance refers to a state in which normal circulating levels of insulin fail to produce its expected physiological effects. As a compensatory mechanism, insulin production is increased from the β cells of the pancreas. However, this compensatory hyperinsulinemia may still be insufficient to trigger adequate signaling in certain pathways, but may increase excessive effects in others. For instance, insulin resistant subjects typically present selective hepatic insulin resistance with impaired glucose homeostasis (through the Akt/FoxO1 pathway) but enhanced insulin-induced hepatic *de novo* lipogenesis (through the SREBP1-c pathway) (Brown and Goldstein, 2008).

Insulin resistance is the underlying metabolic abnormality of the MetS and visceral and/or ectopic fat storage are the core components of the syndrome (Carr *et al*., 2004; Neeland *et al*., 2012; Fabbrini *et al*., 2009). A potential explanation for the obesity-induced insulin resistance is the development of a state of lipotoxicity in insulin sensitive tissues such as the liver and skeletal muscle.

1.4.3.1 Increased ectopic lipid accumulation during obesity and insulin resistance

Chronic caloric surplus and subsequent adipose tissue hypertrophy results in the activation of an inflammatory response, which leads to the accumulation of macrophages within this tissue. In the obese state, macrophages produce a number of cytokines (e.g. TNF-α and IL-6) that can act locally in paracrine or autocrine manner to amplify the pro-inflammatory and insulin resistant state within the adipose tissue. Adipose

tissue can also function as an endocrine organ whereby cytokines travel to the liver and skeletal muscle resulting in local inflammation and insulin resistance within these organs (de Luca and Olefsky, 2006).

Hypertrophic adipocytes are also characterized by increased lipolysis during fasting. Some of these FFA are shunted to the liver and stored in lipid droplets while others will be oxidized in other tissues such as skeletal muscle. Furthermore, decreased ability of adipose tissue to appropriately store lipids (FFA) after a meal, as observed by McQuaid *et al*., 2011 in abdominally obese men, will result in lipid accumulation in non-adipose tissues. This lipid overload, when exceeding the storage and oxidative capacity of the cell, leads to the generation of lipid signaling intermediaries (e.g. diacylglycerol, long-chain fatty acyl CoA and ceramides). These lipid intermediaries activate a number of kinases such as protein kinase C, as well as inhibitory κB kinase β (IKKβ) and nuclear factor κB (NFκB), which are also activated by TNF-α and IL-6. This in turn results in the serine/threonine phosphorylation of the IRS-1 or Akt/protein kinase B (PKB) which inhibits insulin signaling and cause insulin resistance (Delarue and Magnan, 2007). Conversely, increased oxidation of excess fatty acids may lead to ER stress and increased production of reactive oxygen species (ROS), both of which may also lead to impaired insulin signaling (Hotamisligil *et al.,* 2010).

Ectopic lipid accumulation in the liver referred to as non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease and ranges in severity from simple hepatic steatosis with no inflammation, to steatohepatitis (NASH) which can progress to liver cirrhosis. NAFLD is strongly associated with obesity, T2DM and insulin resistance (Lazo and Clark, 2008). However, it is not clear whether insulin resistance is a prerequisite for the development of NAFLD or whether hepatic lipid accumulation precedes insulin resistance.

Physiologically, the development of hepatic steatosis is attributed to a mismatch between hepatic lipid uptake and synthesis and lipid export (decreased oxidation or apoB100 secretion). It has been reported in patients with NAFLD that around 60% of TG in the liver arises from circulating FFA (mainly from adipose lipolysis), 26% from de novo lipogenesis and 15% from intestinal-derived CM and their remnants. It was also observed that the lipogenic machinery, which should be repressed during fasting conditions, was already high in the fasted state and failed to further increase postprandially in subjects with NAFLD (Donnelly *et al*., 2005). Therefore, increased adipose tissue lipolysis, *de novo* lipogenesis and intestinal CM overproduction typically observed in the insulin resistant state can all contribute to increase ectopic lipid accumulation.

Currently, there is no specific drug therapy approved for the treatment of NAFLD (Naniwadekar, 2010). Lifestyle and dietary management, such as those recommended for the MetS, remain the best therapeutic option. Some bioactive fatty acids, such as omega-3 polyunsaturated fatty acids (PUFA) have been shown to have promising therapeutic effects for the treatment of NAFLD at a dose of 1-2 g per day (Capanni *et al.,* 2006; Zhu *et al*., 2008). Vaccenic acid has also been shown to significantly decrease hepatic TG accumulation in a rat model of MetS. However, whether VA alleviates the progression of NAFLD during MetS is currently unknown. *This in turn led to the rationale in this thesis for investigating the effects of VA on body fat distribution and NAFLD progression.*

1.4.3.2 Effect of insulin resistance on lipoprotein production and metabolism

1.4.3.2.1 Overproduction of TRL during insulin resistance

Hepatic overproduction/secretion of VLDL is considered the primary cause for MetS-associated dyslipidemia (Ginsberg *et al*., 2006). Studies in both animal and humans models of insulin resistance and T2DM have revealed that in addition to hepatic VLDL, production of intestinally derived apoB48

containing lipoproteins is also significantly increased during these conditions (Duez *et al*., 2008a).

Insulin has been shown to have an acute direct inhibitory effect on the secretion of hepatic (Reviewed by Lewis and Steiner, 1996) and intestinal-derived lipoproteins (Federico *et al*., 2006) *in vitro* and *in vivo* studies; but this effect becomes blunted during chronic hyperinsulinemic and insulin-resistance. Evidence indicates that this lipoprotein overproduction in chronic hyperinsulinemia is associated with alterations in insulin signaling pathways in the liver and the intestine. For instance, acute studies in primary cultured hepatocytes (Taghibiglou *et al*., 2002) and enterocytes (Federico *et al*., 2006) from fructose-fed hyperinsulinemic hamsters have shown reduced insulin receptor and Akt phosphorylation and increased activation of protein tyrosine phosphatase-1B signaling. Although the underlying mechanisms by which insulin directly suppresses CM production are not fully understood, it appears that insulin inhibits the assembly and secretion of hepatic VLDL by increasing posttranslational degradation of apoB and reducing the expression of MTP (Theriault *et al*., 1992; Lin *et al*., 1995).

Experimental evidence has shown that insulin also inhibits hepatic and intestinal TRL production indirectly by suppressing circulating FFA (Lewis *et al*., 2004; Duez *et al*., 2008a; Pavlic *et al*., 2010). This regulatory effect of insulin is blunted with impaired insulin responsiveness. Indeed, increased circulating FFA levels and increased FFA flux to the liver, as observed in insulin resistant states, stimulates hepatic VLDL overproduction (Lewis, 1997; Zhang *et al*., 2004; Siri *et al*., 2001; Taghibiglou *et al.,* 2000). Most recently, it has been demonstrated in individuals with T2DM that intestinal-derived CM production is also resistant to the normal acute suppressive effect of insulin (Nogueira *et al*., 2012). Additionally, increased flux of FFA to the intestine in insulin resistance may also contribute to the overproduction of CM during these

conditions. How and to what extent these non-dietary sources of FFA contribute the CM overproduction in insulin resistance is still unknown.

Increased *de novo* lipogenesis in insulin resistance may also provide additional substrate for TRL synthesis by increasing fatty acid/TG pool size. Direct measurement of *de novo* lipogenesis *in vivo* in humans has revealed that although newly formed fatty acids are a quantitatively minor source of VLDL-TG (8%) (Barrows and Parks, 2006), its contribution to VLDL production is significantly increased (up to 23-30%) during obesity and insulin resistance (Donnelly *et al.,* 2005). Although not documented in humans yet, increased intracellular lipids due to enhanced *de novo* lipogenesis in enterocytes was associated with increased intracellular apoB48 and CM oversecretion in the fructose-fed hamster (Haidari *et al*., 2002).

In obesity and insulin resistance, increased inflammation is associated with elevated circulating levels of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α). TNF-α was shown to stimulate the overproduction of intestinal apoB48-containing lipoproteins by impairing the insulin signaling cascade in the intestine (Qin, *et al*., 2007).

The production of TRL can be modulated by diet and the fatty acid composition in the diet has been shown to play a key role in this modulation. Findings from pre-clinical studies suggest that VA can reduce CM secretion in a rat model of MetS. Despite the proposed mechanism of action of VA on activating PPAR regulated pathways, putative benefits of VA to insulin resistance and energy use and storage have not been clear in early studies. Therefore, one of the objectives of this thesis was to investigate whether VA can reduce or neutralize the putative adverse effects of SFA on parameters of MetS. Specifically, we investigated whether VA can favorable modulate adipose tissue distribution, reduce

ectopic lipid accumulation and associated lipid secretion from the liver and the intestine.

1.4.3.2.2 Impaired clearance of TRL during insulin resistance

Although increased CM and VLDL secretion is an important mechanism leading to dyslipidemia in insulin resistance, impaired clearance of these particles is also involved (Taskinen *et al*., 2011). Postprandial LPL activity is likely to be an important factor in the modulation of plasma TG levels after a meal. A reduction in adipose tissue LPL activity has been observed in obese insulin-resistant animal models (Picard *et al*., 2002) and humans (Ong and kern, 1989). Increased CM particles, as seen in insulin resistance, may compete with VLDL for removal from the circulation. In addition, hepatic uptake of TRL remnants can also be altered due to reduced expression of LDLR (Ginsberg *et al*., 2006).

As discussed throughout this thesis, a dysfunctional lipid metabolism that characterizes the pathophysiology of insulin resistance and MetS contributes to accelerated CVD progression. In order to develop preventive and ameliorative treatments of disease progression, animal models that mimic human disease processes have been developed (Russell and Proctor, 2006). The JCR:LA-cp rat model has been extensively characterized and shown to spontaneously develop symptoms associated with the MetS and the pre-diabetic state typically observed in humans. The following Section summarizes some of the most relevant characteristics of this animal model that makes it suitable for the type of dietary interventions conducted in this thesis.

1.5 The JCR:LA-*cp* Rat, a Model of Insulin Resistance and Metabolic Syndrome

Rats are typically resistant to the development of atherosclerosis. Lipid metabolism of the normal rat (as in mice) is primarily based on HDL, rather than on LDL, as in humans, possibly contributing to resistance to

atherogenesis. Even dietary cholesterol contents as high as 10% w/w are not usually sufficient to create significant hypercholesterolemia. However, a few genetically modified strains of rats that mimic important aspects of abnormal metabolism and CVD seen in humans have been developed (Reviewed extensively by Russell and Proctor, 2006).

The JCR: LA-*cp* strain of rats is one of a number of strains incorporating the autosomal recessive *cp* gene first isolated by Koletsky (Koletsky, 1973). The corpulent (*cp*) trait presents a stop codon in the extracellular domain of the leptin receptor (ObR) leading to a complete absence of the ObR in the plasma membrane of the *cp/cp* animals. The JCR:LA-*cp* strain, if homozygous (*cp*/*cp*), spontaneously develop symptoms associated with the MetS and the pre-diabetic state typically observed in humans including obesity, insulin resistance and dyslipidemia (Brindley and Russell, 2002). In contrast to other rat models of insulin resistance (e.g. Fatty Zucker rat, Zucker Diabetic Fatty rats, Sand rat), the JCR:LA-*cp* rat spontaneously develops ischemic lesions of the heart as well as early intimal atherogenesis commonly seen in human coronary arteries (Russell and Amy, 1986ab). Therefore, the JCR:LA-*cp* rat provides an excellent model for insulin resistance, MetS and progression to CVD. Rats that are heterozygous (*+/cp*) or homozygous normal (+/+) are lean and not distinguishable from their parent strain in any respect.

1.5.1 JCR:LA-*cp* rat as a model of insulin resistance

Rats of the JCR:LA-*cp* strain are detectably obese at three weeks of age. A modest hyperinsulinemia is present at four weeks of age, which develops rapidly (Brindley and Russell, 2002) as a consequence of the absence of leptin inhibition of insulin release (Emilsson *et al*., 1997).

The JCR:LA-*cp* rat presents a normal basal turnover of glucose but a profound insulin insensitivity in peripheral tissues (Russell *et al*., 1994). Despite this profound insulin resistance, the *cp/cp* rat is able to maintain

relative euglycemia, even in the postprandial state, albeit at the expense of very high plasma insulin levels (Russell, 1999). This response resembles the hyperinsulinemia observed in humans following an analogous meal challenge. Despite the high rate of insulin secretion, the *cp/cp* rats do not develop pancreatic β cell failure and the resultant development of insulin-dependent diabetes.

1.5.2 JCR:LA-*cp* rat as a model of fasting and postprandial lipemia

The JCR:LA-*cp* rat presents with classic hypertriglyceridemia and hepatic VLDL over-secretion that is consistent with observations in humans with MetS. Under normal dietary conditions (chow diet), hyperlipidemia is essentially confined to TRL fractions, while elevations of cholesterol are generally marginal compared with lean counterparts. The observed hypersecretion of VLDL in the *cp/cp* rat is thought to be a response of the liver to the large amounts of glucose absorbed by the hyperphagic animals (Russell and Proctor, 2006). In addition, the profound peripheral insulin resistance in the JCR:LA-*cp* rat leads to a diversion of glucose to hepatic TG synthesis and subsequent hepatic VLDL over-secretion. This appears to develop in response to the upregulation of lipogenic factors, including SREBP-1 (Elam *et al*., 2006).

Most recently, the *cp/cp* rat has been shown to have significant postprandial lipemia, as measured by oral fat challenge experiments and assessment of plasma apoB48 kinetics (Vine *et al*. 2007). The over-production of intestinal-derived nascent CM has also been demonstrated by using lymph cannulation procedures (Hassanali *et al*., 2008; Wang *et al*., 2009). Thus, the insulin-resistant/hyperinsulinemic state in the *cp/cp* rat is associated with enhanced circulating levels of both hepatic and intestinal-derived lipid particles. This hyperlipidemic profile similar to that of pre-diabetic humans offers a valuable opportunity to study putative lipid-lowering dietary treatments.

1.5.3 Use of the JCR:LA-*cp* rat model in nutritionally-balanced studies

The JCR:LA-*cp* rat phenotype of MetS has been extensively characterized together with the type of nutritional studies presented in this thesis. For instance, previous studies by our laboratory have demonstrated that the *cp*/*cp* phenotype is highly responsive to dietary treatment with bioactive fatty acids including n3-PUFA and VA (Hassanali *et al*., 2008; Wang *et al*., 2008 and 2009). Due to the spontaneous development of the MetS in this animal model, no additional dietary manipulation is required in comparison with excessively high fat diets needed for animal models of diet-induced obesity (DIO). This enables accurate control of fatty acid content in all our synthesized diets while the phenotype spontaneously develops. In addition, in this thesis we have conducted intervention study designs that require the phenotype to be well established prior to dietary intervention. *Therefore, the JCR:LA-cp rat is an excellent animal model for this type of dietary intervention approach.*

1.6 Regulation of Lipid Metabolism and Insulin Action by Dietary Fatty Acids

1.6.1 Effect of fatty acids on TRL metabolism

It is well documented that both the amount and type of dietary fat present in a meal can influence postprandial lipid metabolism. In acute studies testing the effect of a single fatty meal, SFA have been shown to cause the most pronounced lipemia, followed by monounsaturated fatty acids (MUFA), while n-3 PUFA ameliorate this response (Lopez Miranda, *et al*., 2007). Notably, dietary habits need to be sustained in the long term in order to observe health benefits. An olive meal (36% MUFA) was shown to lead to the formation of a reduced number of similar-sized TRL particles compared to a butter meal (35% SFA) in healthy volunteers, perhaps suggesting a more efficient metabolism of these particles only after

chronic intake of olive oil (Perez-Martinez *et al*., 2011). In contrast, chronic dietary supplementation of n-3 PUFA (4-6 wk) has been shown to efficiency decrease postprandial lipemia mainly due to decreased production of TRL in hypertriglyceridemic men (Harris and Muzio, 1993; Westphal *et al*., 2000).

The mechanism by which PUFA ameliorate the postprandial TG response seems to be attributable to a diminished production of lipoprotein particles. Levy *et al*. (2006) showed that fish oil feeding in the Sand rat resulted in decreased CM assembly and apoB48 synthesis, as assessed in cultured jejunal explants incubated with either [14C]-oleic acid or [35S]-methionine, respectively. In this study, reduced secretion of apoB48 was shown to be mediated by post-translational degradation of the newly synthesized apoB48 and occurred subsequently to limited lipid availability (TG, PL and CE). This reduced TG synthesis was associated with decreased activity of MGAT and DGAT. Notably, animals fed the fish oil diet also had reduced body weight gain. Therefore, decreased intestinal lipid availability by fish oil may also be secondary to decreased fatty acid delivery to the intestine or increased β oxidation. Additionally, it has also been reported that fish oil feeding may attenuate postprandial lipemia by inducing a transient lipid accumulation in the enterocytes, which may result in a delayed TG efflux from the intestine into the circulation (Larsen *et al*., 2003). *It is plausible that the same mechanism of action could explain the ability of VA to decrease lymphatic CM secretion in the JCR:LA-cp rat (Wang et al., 2009).*

Dietary habits represented by diets rich in SFA and poor in PUFA have been reported in patients with NASH (Musso *et al.,* 2003). Relative to body mass index-matched healthy controls, NASH patients were observed to have a higher postprandial TG response but reduced number of postprandial apoB48 and apoB100 particles. This may suggest impaired secretion of CM and/or VLDL particles during NASH. Indeed, a reduced

synthesis rate of hepatic apoB100 has been reported in NASH patients (Charlton *et al*., 2002). Different fatty acids (including SFA) have been shown to inhibit apoB100 secretion by different pathways. Caviglia *et al.,* (2011) demonstrated that exposure to high doses of oleic acid or palmitic acid *in vitro and in vivo* induced ER stress and inhibited apoB100 secretion, with palmitic acid having a more potent effect because it also increased ceramide production. In contrast, docosahexaenic acid was shown to be the more potent inhibitor of apoB100 acting via stimulation of autophagy.

1.6.2 Regulation by transcription factors

1.6.2.1 Sterol regulatory element binding proteins

Dietary PUFA are well established as negative regulators of *de novo* lipogenesis via SREBP. The SREBP-1 isoform is more active driving fatty acid synthesis. Thus, SREBP-1 but not SREBP-2, which drives cholesterol synthesis, is subjected to selective feedback inhibition by PUFA. This is because transcription of SREBP-1 is activated by the nuclear liver X receptor (LXR), which antagonized by PUFA (Ou *et al*., 2001). In addition, fatty acids including oleic acid and PUFA, have been shown to suppress the release of the mature transcriptionally active form of SREBP-1 by inhibiting the proteolytic degradation of insulin-induced gene (INSIG). In contrast, SFA are unable to suppress the proteolytic cleavage activation of SREBP-1 (Lee *et al*., 2008). Further, PUFA also have the ability to reduce SREBP-1 mRNA levels attributed to the repression of gene transcription rate and mRNA stability (Xu *et al*., 2001).

1.6.2.2 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) constitute a family of nuclear receptors acting as transcription factors upon activation by natural or synthetic ligands. Fatty acids including both SFA and MUFA act as

ligands for the various PPAR isoforms, although PUFA tend to be preferred. Given that VA has been recently proposed to act as a natural ligand for PPAR, the focus of this section is to describe the molecular mechanisms of PPAR activation and the downstream physiological effects associated with this activation

1.6.2.2.1 Activation of PPARs and associated physiological effects

Binding of an agonist to the PPAR ligand binding domain (LBD), induces a conformational change in the transactivation domain (activation function 2 or AF2). This initiates the recruitment of co-activators (e.g. cAMP response element-binding protein, PPAR gamma cofactor-1α) and release of co-repressors. Upon ligand activation, PPAR heterodimerizes with RXR, another nuclear receptor required for PPAR activity. The PPAR/RXR complex binds to PPAR response elements (PPRE) located in the promoter region of target genes via a DNA binding domain (DBD), and alters coactivator/corepressor dynamics to induce transcription. For instance, the DNA binding of the adipogenic transcription factor CCAAT/ enhancer binding protein (C/EBPα) is spatially and temporally associated with PPARγ binding, and the two proteins cooperate in inducing the adipogenic gene transcription program in adipose tissue (Astapova and Leff, 2010).

Ligand-dependent activation of PPARs can also negatively regulate gene expression by inhibiting the activity of transcription factors. For instance, PPARγ elicits anti-inflammatory regulations by transrepression of transcription factors such as NFκB and activator protein 1 (AP-1); thus inhibiting the transcription of pro-inflammatory cytokines such as IL-2 and TNF-α (Ricote et al. 1998).

There are three current members of the PPAR family: PPARα, PPARδ and PPARγ. Each receptor modulates pathways involved in lipid and glucose metabolism and inflammation, which makes them physiologically

and clinically relevant (Bensinger and Tontonoz, 2008). PPARα is highly expressed in the liver, heart and skeletal muscle and targets genes involved in fatty uptake, binding and activation, mitochondrial β oxidation, and lipoprotein metabolism (Duval *et al*., 2007). PPARα is pharmacologically activated by fibrate drugs to lower circulating TG and raise HDL (Barter and Rye, 2008). PPARδ is broadly expressed and its pharmacological activation was shown to improve blood lipid profile and hepatic TG accumulation and potentially increase fatty acid oxidation in skeletal muscle in obese men (Risérus *et al*., 2008). PPARγ has two isoforms, -γ1 and –γ2. While PPARγ1 is widely expressed in tissues such as the intestine, brain, vascular cells and immune cells, PPARγ-2 is mainly expressed in adipose tissue. Activation of PPARγ in adipose tissue appears to be the mechanism of action for the insulin sensitizing effects of thiazolidinediones (TZD). *One of the objectives of this thesis was to investigate the ability of VA to reduce the risk of developing ectopic (hepatic and intestinal) and visceral fat and the MetS when supplemented on a background diet containing dairy derived-SFA. Assuming that VA also acts as a PPARγ ligand in adipose tissue, we have hypothesized that VA supplementation will favorably alter adipose tissue distribution and that this will be associated with its ability to attenuate symptoms of MetS including NAFLD progression. A brief review on the metabolic functions and clinical outcomes of PPARγ activation provided below.*

1.6.2.2.2 Metabolic functions and clinical outcomes of PPARγ activation

TZD are chemically synthesized compounds that have been widely used in clinical practice to treat patients with two components of MetS: dyslipidemia and insulin resistance. The insulin-sensitizing TZD family (e.g., pioglitazone, rosiglitazone) is a class of potent PPARγ activators, which improve whole-body insulin sensitivity and inflammatory regulation.

Mechanistically, PPARγ activation by TZD has been shown to promote adipogenesis of subcutaneous fat, stimulating the differentiation of preadipocytes into adipocytes. The resulting greater number of smaller adipocytes possess an increased lipid storage capacity (Adams *et al*., 1997; Festuccia *et al*., 2009). The remodeling effect of PPARγ activation in SAT is accompanied by enhanced anabolic capacity (e.g. increased fatty acid uptake and esterification) in this fat depot (Laplante *et al*., 2006; Festuccia *et al*., 2009). In contrast, visceral adipocytes also become smaller after PPARγ activation which is thought to be mediated by increased lipolysis, fatty acid oxidation and thermogenesis in this tissue, rather than cell recruitment and differentiation (Laplante *et al*., 2006). Furthermore, PPARγ activation by TZD also results in the improvement of adipokine secretory profiles with increased secretion of adiponectin and decreased secretion of pro-inflammatory cytokines (IL-6, PAI-1 and TNF-α) (Sharma and Staels, 2007).

The resulting metabolic effects of PPARγ activation by TZD include enhanced insulin-sensitivity, improved glycemic control, decreased plasma FFA, TG and inflammatory markers and increased adiponectin and HDL-C. Indeed, TZD are among the most effective drugs to reduce plasma FFA concentrations.

1.6.2.2.3 Activation of PPARs by natural ligands

A wide variety of lipids including fatty acyl-CoA species, eicosanoids (prostaglandins and leukotrienes), oxidized fatty acids, and oxidized phospholipids have also been implicated in PPAR activation (Delerive *et al*., 2000; Forman *et al*., 1997; Kliewer *et al*., 1997; Krey *et al*., 1997). This has led to suggestions that dietary fatty acids and their derivatives can modulate lipid homeostasis by activating PPARs, which in turn act as physiological sensors of lipid levels in cells.

It has been proposed that endogenously synthesized rather than dietary-derived ligands are physiologically more relevant PPAR regulators. A

phospholipid with a palmitate and oleate moiety was identified to be a strong ligand for PPARα. It was also demonstrated that the ability of this moiety to activate PPARα downstream targets was dependent on FAS and Cept1 (choline ethanolamine phosphotransferase-1, required for phosphatidylcholine synthesis) (Chakravarthy, *et al.,* 2009). These findings also suggested that different intracellular pools of lipid signaling molecules may determine the activation PPAR regulated pathways. *During the course of my thesis, complementary studies in our group as well as from others have discovered that VA can act as a ligand for PPARs in cell culture studies (Wang et al., 2012; Jaudszus et al., 2012). However, whether this ability of VA to activate PPAR downstream pathways is determined by its processing into lipid signaling molecules is not known.*

1.6.3 Role of dietary lipids in MetS associated inflammation

The pro-inflammatory effects of SFA are well documented while n-3 PUFA are known to counteract or alleviate these effects. Because dietary fatty acids are transported by TRL, it has been proposed that the composition of these particles may play a key role in the proinflammatory process. It has been shown that TRL isolated after a meal are more proinflammatory when enriched in SFA rather than MUFA or PUFA (Williams *et al*., 2004). Further, incubation of smooth muscle cells with VLDL rich in palmitic acid isolated from dyslipidemic subjects was shown to significantly increase the secretion of proinflammatory cytokines (Ståhlman *et al.,* 2012).

The literature also documents that SFA, specifically palmitic acid may alter critical mechanisms impacting insulin signaling via stimulating inflammatory signaling (Kien, 2009). For instance, SFA but not *cis*-unsaturated fatty acids promote inflammatory activation of macrophages in part mediated by binding of the toll-like receptor 4 (TLR4). This in turn results in the activation of the inflammatory signaling cascades that induce the production of pro-inflammatory cytokines resulting in the attenuation of

insulin action (Shi *et al*. 2006). In contrast, activation of the G protein-coupled receptor 120 (GPR120) by n3-PUFA has been shown to be the mechanism responsible for their anti-inflammatory and insulin sensitizing effects (Oh *et al*., 2010). Figure 1-8 summarizes the regulation of signaling pathways by SFA and n-3 PUFA involved in the development of insulin resistance.

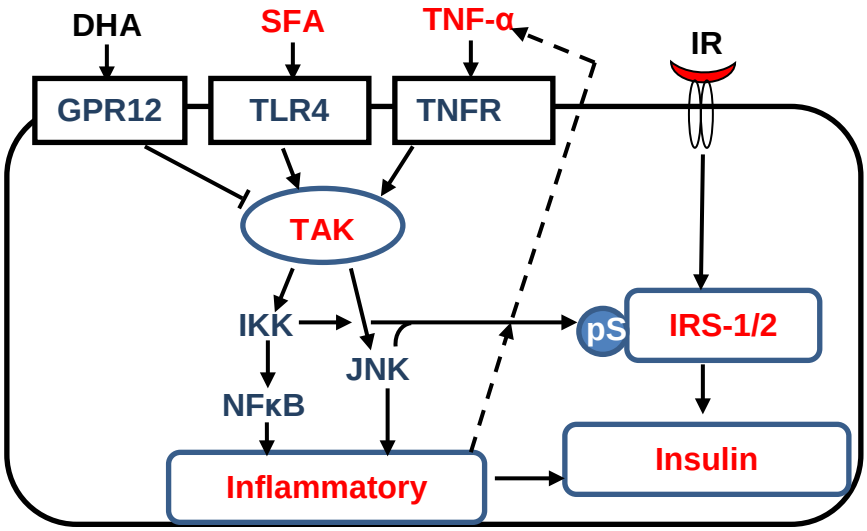


Figure 1-8 Regulation of inflammatory signaling pathways by SFA and n-3 PUFA that modulate insulin action *(Adapted from Oh et al., 2010; Osborn and Olefsky, 2012). Activation of TLR4 and the TNF receptors leads to activation of the IKK and JNK kinases, leading to serine phosphorylation of the IRS and increased transcription of inflammatory pathways, which contribute to insulin resistance. In contrast, activation of the GPR120 by the n3-PUFA DHA, inhibits TAK activation with subsequent inhibition of the inflammatory pathway. IKK, IκB-kinase; JNK, Jun N-terminal kinases; DHA, docosahexaenoic acid; TAK, transforming growth factor-β-activated kinase-1.*

Considering the recent evidence for the health benefits of VA, one of the objectives of my thesis was to investigate whether enriching dairy fat with VA could increase the health value of dairy-derived SFA, specifically during conditions of MetS. Because dairy fat consists of a complex food

matrix (including SFA, MUFA and CLA), it is difficult to discriminate the effects of VA from other bioactive fatty acids. *Therefore, the approach in this thesis was to add purified synthetic VA into dairy fat allowing to carefully control for the fatty acid composition of experimental diets.*

1.7 Proposed mechanisms of action for the health benefits of VA

1.7.1 Activation of PPARs by VA

Accumulating evidence from pre-clinical studies suggest that VA (either in the natural or purified form) has lipid-lowering and anti-inflammatory effects during conditions of dyslipidemia and/or MetS (Lock *et al*., 2005; Bauchart *et al*., 2007; Basset *et al*., 2010; Blewett *et al*., 2009; Ruth *et al*., 2010; Tyburczy *et al*., 2009; Wang *et al*., 2008; Wang *et al*., 2009). Furthermore, it was demonstrated recently that VA is a partial agonist to PPAR-α and –γ *in vitro* acting with similar affinity PPAR agonists commonly used for the treatment of dyslipidemia and type-2 diabetes. In that study, VA was shown to suppress cardiomyocyte hypertrophy in a PPAR-α and –γ dependent manner (Wang *et al*., 2012). Thus, this has provided a potential mechanistic explanation of how VA may exert its hypolipidemic, anti-inflammatory and cardio-protective effects.

VA was shown to ameliorate the atherosclerotic development relative to the iTFA, elaidic acid (EA) in the LDLr$^{-/-}$ mice. These protective effects of VA versus EA on atherosclerosis were independent of changes in plasma cholesterol or TG (Bassett *et al*., 2010). This has led to the proposal that TFA may be stimulating or ameliorating (in the case of VA) atherosclerosis in a manner independent on cholesterol (Ganguly and Pierce, 2012).

The opposing inflammatory response towards iTFA *versus* rTFA may provide an alternative mechanism. iTFA consumption has been associated with elevated systemic inflammatory responses, including high levels of TNF-α and its receptors, IL-6, MCP-1 and C-reactive protein (Mozaffarian and Willett, 2007). These pro-inflammatory effects of iTFA

are likely to account for their adverse effects on CVD (e.g. inflammation and endothelial dysfunction). Indeed, Iwata *et al*., (2011), investigated the effects of iTFA (EA and linoelaidic acid) *versus* VA on NFκB activation and nitric oxide production (NO) in human endothelial cells. It was found that iTFA but not VA, stimulated NFκB activation (as measured by IL-6 levels, phosphorylation of IκBα and superoxide production), impaired endothelial insulin signaling and NO production. These effects of iTFA on endothelial dysfunction were also observed by incubation of cells with palmitic acid. Furthermore, VA was shown to normalize inflammatory markers (e.g. IL-2 and TNFα) in mesenteric lymphatic nodes and splenocytes in a rat model of chronic inflammatory state, the JCR:LA-*cp* rat.

The underlying mechanism of action for these anti-inflammatory effects of VA is not known but it could be associated with its ability to activate PPARγ-dependent anti-inflammatory pathways. Indeed, it has been recently reported that VA was able to reduce IL-2 and TNF-α expressing T-helper cells of human peripheral blood mononuclear cells in a PPARγ dependent manner. It was concluded that this effect of VA was independent on CLA since no bioconversion was observed in these cells (Jaudszus *et al*., 2012).

1.7.2 Incorporation of VA into cellular membranes

Fatty acids can also regulate cellular activities by altering membrane composition and consequently, affecting membrane biophysical properties. Indeed, Niu *et al*., (2005) demonstrated that iTFA-derived PL had higher cholesterol membrane affinity than their *cis* analogues. They also found that PL membranes containing iTFA exhibited a higher acyl chain packing order which was associated with reduced membrane receptor activation. The impact of membrane incorporation of SFA, iTFA and VA on cellular activities has been investigated recently. Fournier *et al*., (2012) showed that EA, palmitic acid and VA were all efficiently incorporated in macrophage membranes. They also found that EA and

palmitic acid but not VA, reduced ABCA-1 mediated cholesterol efflux and that this effect was stronger in cholesterol loaded cells. It was concluded that this opposing effect of EA *versus* VA may be associated with decreased membrane fluidity thereby reducing ATPase activity and the function of the transporter.

Another mechanism of action by which most bioactive long chain PUFA (LCPUFA) have been shown to act is by replacing or interfering with the synthesis/incorporation of arachidonic acid (AA) into membrane PL. This has important biological implications since these AA-containing PL have been shown to function as precursors of signaling molecules (called endocannabinoids) known to control food intake and metabolism. *Given the ability of fatty acids to influence cellular activities by modulating membrane PL composition, one of the latter objectives of this thesis was to investigate whether VA alone or in combination with CLA could alter the concentration of tissue endocannabinoids.*

1.8 Regulation of Endocannabinoids by Dietary Fatty Acids in Health and Disease

Endocannabinoids (EC) are lipid mediators endogenously synthesized from essential fatty acids available from dietary sources. EC exert most of their functions by binding and activating two G protein coupled receptors, cannabinoid receptor 1 (CB1) and 2 (CB2) that are expressed in the brain and peripheral tissues (e. g. liver, adipose tissue, skeletal muscle and the gastrointestinal tract) at various levels depending on the tissue and conditions. Two of the best characterized EC, the *N*-acylethanolamine *N*-arachidonoylethanolamide (Anandamide) and the monoacylglycerol 2-arachidonoyl glycerol (2-AG), are metabolic derivatives of the n-6 arachidonic acid (AA; C20:4) in PL. They are also found at various levels depending on the tissue and conditions.

EC are chronically up-regulated in animal models of obesity (both genetically and/or diet induced) (Lo Verme *et al*., 2009; Izzo *et al*., 2009 and 2010) or human obesity (Matias *et al.,* 2006). The endocannabinoid system is involved in central energy balance regulation (Cota *et al*., 2003; Matias *et al*., 2008a) and its excessive activity leads to increased food intake, weight gain and associated dyslipidemia and insulin resistance. Selective CB1 receptor antagonists have shown to reverse these effects (Di Marzo, 2008b).

The dysregulation of EC during obesity is partially attributed to the alterations in the expression or activity of their biosynthetic and degradative enzymes. Additionally, increased availability of AA in PL due to increased dietary intake of linoleic acid has been causally associated in the development of obesity (Alvheim *et al.,* 2012). Conversely, n3-LCPUFA have been shown to decrease EC concentrations presumably by reducing the amount of their PL biosynthetic precursors. *In this thesis we have proposed that VA may also have similar regulatory effects on tissue EC.* Therefore, a brief review of these concepts will be discussed in the following Section.

1.8.1 Endocannabinoid biosynthesis and regulation

EC are produced enzymatically from cell membrane PL-fatty acid precursors. EC are thought not to be stored but generated on ‘demand’ in response to physiological or pathological stimuli. It is proposed that once the ‘on demand’ stimulus initiates their release, EC are taken up by cells via a putative membrane transporter (which may be also involved in their release). Then, EC present in the extracellular space are accumulated by neurons and other cells by facilitated diffusion where they act as local pro-homeostatic factors. However, the mechanism that regulates their release and uptake into membranes is not well understood (Pacher *et al*., 2006; Alger and Kim, 2011).

The action of EC on CB receptors and other receptors is terminated intracellularly by specific lipases. The dominant enzyme responsible for inactivation of anandamide is fatty acid amide hydrolase (FAAH), while monoacylglycerol lipase (MAGL) is the principal 2-AG hydrolase. Collectively, the CB receptors, endocannabinoids and their biosynthetic or degrading enzymes constitute the endocannabinoid system (ECS).

Figure 1-9 shows the schematic representation of endocannabinoid biosynthesis and degradation.

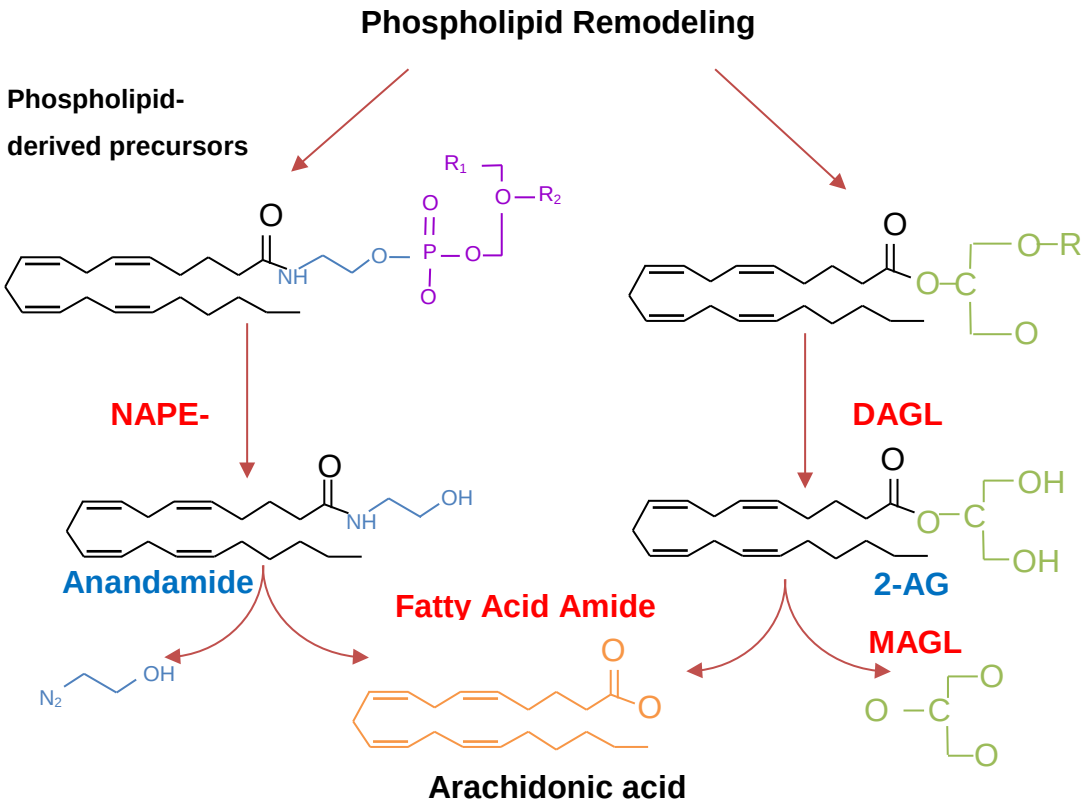


Figure 1-9 Schematic representation of endocannabinoid biosynthesis and degradation. A calcium-dependent acyl transferase catalyzes the formation of N-arachydonoyl-phosphatidylethanolamine (PE) from AA and PE. Anandamide is then synthesized from membrane N-acyl-PE (NAPE) precursors through different non-specific lipolytic pathways. 2-AG is generated from DG by diacylglycerol lipase (DAGL) selective for the sn-1

position (Bisogno et al., 2005). DG can be generated from phosphatidylinositol by a specific phospholipase-C (PLC) or phosphatidic acid by phosphatidic acid phosphohydrolase. The inactivation of anandamide and 2-AG occurs intracellularly by several lipases. FAAH, a membrane bound hydrolase, is the primary enzyme hydrolyzing anandamide but can also inactivate 2-AG, while MAGL is the principal 2-AG hydrolase.

1.8.2 Endocannabinoid analogues and their receptors

Oleoylethanolamine (OEA) and palmitoylethanolamine (PEA) are other *N*-acylethanolamines (NAEs) also produced and hydrolyzed by the same enzymes regulating anandamide synthesis and degradation. These NAEs, which only differ from anandamide by their acyl chain, have also been shown to act as important signaling molecules (Borelli and Izzo, 2009; Godlewski *et al*., 2009). However, unlike anandamide and 2-AG, OEA and PEA do not activate CB receptors and therefore cannot be considered as EC. Rather, they have shown to activate the nuclear receptor PPAR and the G protein coupled receptor GPR119. For instance, OEA has been shown to induce satiety (Fu *et al*., 2003) while PEA exerts anti-inflammatory effects (Lo Verme *et al*., 2005) through activation of PPARα. OEA is also considered one of the most potent ligands for the GPR119 which is only weakly activated by PEA (Godlewski *et al*., 2009). This receptor is highly expressed on L cells of the gastrointestinal tract, where it regulates the release of anti-diabetic peptide glucagon-like peptide-1 upon activation by OEA (Lauferr *et al*., 2009).

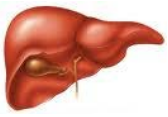
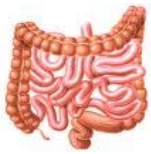
1.8.3 Physiological actions of CB receptor activation linking to obesity and MetS

It has been proposed that an overactive ECS is likely to have a causal role in obesity acting through multiple organ systems. For example, the

intestinal ECS is also involved in energy balance regulation where anandamide is proposed to act as a 'hunger signal', being up-regulated under fasting in normal healthy conditions (Izzo *et al*., 2010). It has also been shown that the intestinal ECS has a greater capacity to transmit this orexigenic signal in animal models of obesity. For instance, intestinal EC are up-regulated in mice fed a high fat diet and also in fasted and re-fed obese Zucker rats (Izzo *et al.,* 2010). Intriguingly, the intestinal EC, via CB1 or CB2 receptor activation, mediates protective effects in animal models of inflammatory bowel disease (IBD) (Di Marzo and Izzo, 2006; Storr *et al*., 2008 and 2009). This is consistent with the known effects of Cannabis in the treatment of enteric inflammatory conditions including IBD (Lal *et al*., 2011). Consequently, there is also growing interest in the development of therapeutic strategies targeted at blocking local endogenous degradation of EC (mainly via inhibition of FAAH), and thus indirectly activating the protective pathways of the ECS. Interestingly, the FAAH inhibitor, arachidonoyl-serotonin (AA-5-HT), was recently shown to be endogenously present in the ileum and jejunum of the gastrointestinal tract (Verhoeckx *et al.,* 2011).

In the liver, hepatic CB1 receptor increases *de novo* lipogenesis (Osei-Hyiaman, *et al*., 2005) and contributes to the diet-induced steatosis and associated insulin resistance and dyslipidemia. Interestingly, while liver-specific CB receptor knockout mice develop a similar degree of obesity as that of wild type mice on a high fat diet, they display similar metabolic improvements as those of global knockout mice (Osei-Hyiaman, *et al*., 2008). These studies led to the proposal that peripheral CB1 receptors could be selectively targeted for the treatment of fatty liver, insulin resistance and associated dyslipidemia. Table 1-6 summarizes the effects of high fat diet feeding on the expression of CB receptors and EC and the physiological actions of CB receptor activation in different tissues.

Table 1- 6 The physiological actions of CB receptor activation in different tissues (Adaptad from Matias *et al.,* 2008b).

Organ	Effect of CB_1 or CB_2 activation	Effects of HFD	Potential metabolic consequences of HFD
Hypothalamus 	↑ Food intake	↑ Anandamide and 2-AG	↑ Body weight ↑ Waist circumference
Liver 	↑ Lipogenesis ↓ AMPK activity (increased ACC)	↑ Anandamide and 2-AG ↑ CB_1 receptor	↑ Dyslipidemia ↑ Dyslipoproteinemia ↑ Steatosis
Small Intestine 	↓ Satiety ↑ Food assimilation ↑ Activation of NO and prostaglandins ↑ Relaxation of the lower oesophageal sphincter ↓ Gastric acid secretion ↓ Intestinal motility ↓ Inflammation	↓ Anandamide	↓ Ghrelin? ↓ Food intake? ↑ Inflammation?
Visceral Adipose Tissue 	↑ Lipogenesis (FAS) ↑ PPARγ expression ↑ Glucose uptake ↓ AMPK activity (increased ACC) ↓ Adiponectin	↑ Anandamide and 2-AG	↑ Energy storage ↑ Dyslipidemia ↑ Insulin resistance ↑ Waist circumference
Muscle 	↓ Glucose uptake ↓ Glucose oxidation	↑ Anandamide and 2-AG ↑ CB_1 receptor	↑ Insulin resistance ↓ Energy expenditure

AMPK: adenosine monophosphate activated protein kinase; NO: nitric oxide production.

1.8.4 Regulation of endocannabinoids by dietary fatty acids as an alternative strategy to favourably modulate the endocannabinoid system during obesity and MetS

Pharmacological blockage of CB1 receptor is proven to be effective in treating obesity and associated metabolic abnormalities in clinical trials

(Despres *et al.,* 2005). However, the highly selective CB1 receptor antagonist Rimonabant (SR141716, Acomplia), was withdrawn from the Eurpoean market in 2008 because of psychiatric side effects, including suicidal tendencies (Christensen *et al*., 2007). Therefore, the development of CB1 antagonists that do no cross the blood –brain barrier has been proposed as an alternative approach to reduce overactivity of the ECS (Di Marzo and Després, 2009). Another possible therapeutic strategy is the inhibition of EC biosynthesis by either modulating biosynthetic or degradative enzymatic pathways or by using nutritional interventions that target the biosynthetic precursor AA (Di Marzo and Després, 2009).

Tissue PL concentrations of AA are dependent upon the competition between dietary intakes of n-6 and n-3 PUFAs. Accordingly, dietary supplementation with the n3-LCPUFA, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) has been shown to modulate the composition of PL that act as EC precursors and consequently reduce tissue EC levels in animal models (Berger *et al*., 2001; Watanabe *et al*., 2003; Batteta *et al*., 2009; D' Asti *et al.*, 2010; Di Marzo *et al*., 2010). In contrast, Alvheim *et al.,* (2012) demonstrated that excessive dietary intake of linoleic acid (LA), the precursor of AA, induces EC hyperactivity and has an underlying causal role in the development of obesity in the C57BL/6 mice. This study also showed that the adipogenic effect of LA can be prevented by replacing 1% of energy from LA with EPA and DHA leading to a reduction of the AA-PL pool and thus normalizing the EC tone. Furthermore, levels of the anorectic lipid OEA and the orexigenic lipid anandamide in the intestine have also been found to be opposed-regulated through the remodeling of their precursors upon food deprivation (Petersen *et al*., 2006).

Most recently, it has been shown that tissue incorporation of LCPUFAs not only affects the synthesis of EC but can also stimulate the synthesis of specific n-3 PUFA-derived conjugates with serotonin, a neurotransmitter

present in high concentrations in the intestine. Notably, fish oil feeding increased the formation of DHA-serotonin (DHA-5-HT) and EPA-serotonin (EPA-5-HT) in jejunal tissue of mice, both of which were able to inhibit FAAH activity *in vitro* (Verhoeckx *et al.,* 2011).

CLA is also metabolized into LCPUFA by the same pathway as LA (Sébédio *et al*., 1997; Banni *et al.,* 1999; Banni *et al*., 2001a). It can be desaturated and elongated into conjugated diene AA (CD 20:4). Notably, VA follows the same metabolic pathways of CLA and therefore can further contribute to the production of CD 20:4 (Banni *et al*., 2001b). Thus, it is likely that CLA and/or its precursor VA may modulate the composition of PL-derived precursor of EC by competing with LA for desaturation and/or elongation. Indeed, a recent study in hypercholesterolemic subjects has shown that consumption of cheese naturally enriched in VA and CLA can significantly reduce plasma anandamide concentrations (Pintus *et al*., 2012). However, whether this effect of the enriched cheese is associated with the high content of VA, CLA, their combination or the low SFA content in the dairy product, is not known.

1.9 Working Hypothesis and General Objectives of the Thesis

The review of the literature presented in this Chapter led us to the following working hypothesis and three main objectives for this thesis:

The overall general working hypothesis for this thesis is:

That VA can synergistically enhance the putative hypolipidemic effects of CLA, and that VA can independently reduce potential detrimental health effects commonly associated with-dairy derived SFA during MetS. Specifically, we hypothesized that VA can favourably modulate integrative physiological pathways of intestinal and hepatic lipid homeostasis, whole body energy utilization and storage and that these effects are convergent with regulation of lipid signaling pathways of the endocannabinoid system.

General Objective 1. To evaluate the synergistic effect of dietary supplementation with purified forms of ruminant *trans* fatty acids, *cis*-9, *trans*-11 CLA and VA on impaired lipid metabolism in a rat model of the MetS, the JCR:LA-*cp* rat.

General Objective 2. To investigate whether enriching dairy fat with synthetic VA (fortification) can favourably modulate energy use and storage and intestinal and hepatic lipid metabolism during conditions of insulin resistance and MetS.

General Objective 3. To explore the independent regulatory effect of VA on lipid-derived signaling molecules (endocannabinoids) known to control energy storage and utilization by acting in peripheral tissues and the brain.

These general objectives are explained in further detail with specific hypotheses and objectives in Chapter 2.

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Chapter 2 Rationale: Objectives and Hypotheses

2.1 Rationale and introduction to research hypothesis

Trans-11 vaccenic acid (VA) is the most abundant ruminant derived *trans* fatty acid in the food chain; however, in addition to being acknowledged as an alternative source of CLA, there remains limited information regarding its own bioactivity. Studies using VA/CLA enriched butter in different animal models of CVD were the first to suggest that unlike iTFA, VA may actually improve CVD risk factors (Lock *et al*., 2005; Bassett *et al*; 2010; Bauchart *et al*., 2007). Previous studies from our laboratory as well as from others (using purified forms of VA), have suggested a novel bioactivity for this fatty acid, in particular its hypolipidemic and anti-inflammatory modulating properties (Wang *et al*., 2008; Wang *et al*., 2008; Bassett *et al*., 2010; Tyburczy *et al*., 2009; Herrera-Meza *et al*., 2013; Blewett et al., 2009). However, *whether VA per se provides an additional benefit when found in VA/CLA enriched products remains to be elucidated.* Consistent with this rationale, the first objective of my thesis was to investigate whether VA can synergistically enhance the putative hypolipidemic effects of CLA.

Given the growing evidence for the health effects of VA, one logical approach would be to produce VA enriched animal-derived foods that could provide an additional health benefit. However, dairy products are also the predominant source of SFA in the diet, which according to current dietary recommendations (Lichtenstein *et al*., 2006; Health Canada Dietary Recommendation Guidelines), may be sufficient reason to limit their consumption. Whether VA could independently reduce or *'neutralize'* putative adverse effects commonly associated with dairy-derived SFA is currently unknown and therefore, we aimed to investigate novel bioactive properties of VA on a background diet resembling that typically found in the food chain.

During the course of my thesis, complementary studies in our group as well as from others, revealed that VA can act as a ligand for PPARα and γ in cell culture studies (Wang *et al*., 2012; Jaudszus *et al*., 2012), which has provided a potential mechanistic explanation on how VA may improve blood lipids and the pro-inflammatory state during conditions of MetS. Despite the proposed mechanism of action, putative benefits of VA to insulin resistance and/or energy use/storage were not determined. This rationale led me to investigate whether VA could favourably modulate whole body energy utilization, storage and/or intestinal and hepatic lipid homeostasis when supplemented on a background diet containing dairy-derived fat.

To explore alternative hypotheses related to our current understanding of the mechanisms of action for VA, we further investigated a potential regulatory effect on convergent lipid signaling pathways. The endocannaninoid system has been shown to play a predominant role in the control of energy balance and lipid homestasis. The ability of long chain fatty acids to alter membrane phospholipid composition has led to propose their use as modulators of the endocannabinoid system (Calder, 2006; Banni and Di Marzo, 2010). Indeed, it has been shown recently that bioactive long chain fatty acids derived from diet are incorporated into plasma membrane phospholipids and potentially replace or interfere with the synthesis of PL-derived lipid signaling molecules, including endocannabinoids (Berger *et al*., 2001; D'Asti *et al.*, 2010; Watanabe *et al*., 2003; Alvheim *et al.,* 2012). However, the incorporation of VA into membrane phospholipids and potential effects on endocannabinoid pathways remains unknown.

Notably, a recent clinical trial in hypercholesterolemic subjects provided the first evidence that a dairy product naturally enriched in VA and CLA significantly reduces plasma concentrations of the endocannabinoid anandamide after three weeks of intervention (Pintus *et al*., 2012).

However, the direct effect of VA on endocannabinoid modulation was not able to be determined in this study. Indeed, this question provides the premise for one of the final objectives of my thesis, as described below.

The overall general working hypothesis for this thesis is:

That VA can synergistically enhance the putative hypolipidemic effects of CLA and that VA can independently reduce potential detrimental health effects commonly associated with-dairy derived SFA during MetS. Specifically, we hypothesized that VA can favourably modulate integrative physiological pathways of intestinal and hepatic lipid homeostasis, whole body energy utilization and storage and that these effects are convergent with regulation of lipid signaling pathways of the endocannabinoid system.

The primary objectives of this study were to investigate the ability of naturally occurring *trans* VA to enhance putative hypolipidemic effects of CLA. Additionally, we investigated whether VA can reduce *ectopic* (hepatic and intestinal) lipids and *visceral adiposity* and *dysfunctional intestinal and hepatic lipid metabolism during MetS,* when supplemented on a background diet containing dairy-derived SFA. We also explored novel regulatory effects of VA on lipid signaling pathways of the endocannabinoid system convergent with these metabolic benefits. The general approach for this thesis was to use a rat model of MetS and to apply physiological and targeted lipidomic analyses to answer these questions.

In order to test the working hypothesis, the following general and specific objectives were proposed for thesis.

2.2 Thesis Objectives and Hypothesis

Chapter 3: Increased hypolipidemic benefits of conjugated linoleic acid in combination with vaccenic acid in the JCR:LA *cp* rat

General Objective 1. To evaluate the synergistic effect of dietary supplementation with purified forms of ruminant *trans* fatty acids, *cis*-9, *trans*-11 CLA and VA on impaired lipid metabolism in a rat model of the MetS, the JCR:LA-*cp* rat.

Hypothesis: Dietary supplementation with both *trans* fatty acids (VA+CLA) that are naturally present in ruminant-derived fat, provide an additional lipid-lowering effect in comparison to supplementation with CLA alone.

Specific objectives

1 *(i)* To determine if adding VA to CLA in the diet favorably affects body weight/composition or caloric intake compared to supplementation with CLA alone.

Hypothesis: Supplementation with both VA and CLA increases the putative beneficial effects of CLA on body composition without altering body weight or food intake.

Expected Outcome: After supplementation with either CLA alone or in combination with VA (VA+CLA) for 16 weeks, it is expected that the VA+CLA diet will have a greater effect on improving body composition, particularly hepatic TG accumulation, relative to diet supplemented with CLA only.

1 *(ii)* To assess the increased health benefit of adding VA to a diet supplemented with CLA on plasma dyslipidemia and insulin resistance.

Hypothesis: Adding VA to a diet supplemented with CLA further increases putative lipid-lowering benefits compared to a diet supplemented with CLA alone.

Expected Outcome: It is expected that supplementation with VA will increase the purported hypolipidemic effects of supplementation with CLA alone in the JCR:LA-cp rat.

The results from these series of experiments are presented in Chapter 3. Data from this Chapter have been published (see Jacome-Sosa *et al*., 2010).

Chapter 4 Diets enriched in *trans*-11 vaccenic acid attenuate ectopic (hepatic and intestinal) lipid storage and increase energy utilization in the JCR:LA-*cp* rat.

General Objective 2. To investigate whether enriching dairy fat with synthetic VA (fortification) can favourably modulate energy use and storage and intestinal and hepatic lipid metabolism during conditions of insulin resistance and MetS.

Hypothesis: Fortifying a diet containing a typical 'dairy-like' fatty acid profile with VA will increase its nutritional value on parameters of MetS including visceral adiposity, insulin resistance, NAFLD progression and lipid over-secretion from the liver and the intestine.

Specific objectives

2 *(i)* To assess whether fortification with VA can favourably attenuate putative stimulatory effects of dairy-derived SFA on visceral fat accumulation.

Hypothesis: Fortification of dairy fat with VA ameliorates putative fat accretion effects of dairy-derived SFA.

Expected Outcome: It is expected that adding synthetic VA to dairy fat will ameliorate total fat mass and ectopic (hepatic and intestinal) lipid accumulation and that these effects are partially associated with increased energy utilization.

2 *(ii)* To determine whether fortifying dairy fat with VA alleviates the putative stimulatory effects of SFA on insulin resistance and NAFLD progression.

Hypothesis: Fortifying dairy fat with VA can attenuate putative SFA-induced exacerbation of insulin resistance and NAFLD.

Expected Outcome: Adding VA to dairy fat will be able to mitigate/attenuate exacerbated insulin resistance and NAFLD progression during MetS.

2 *(iii)* To investigate whether VA will retain its lipid-lowering effects on hepatic and intestinal TG secretion when supplemented on a background diet that includes SFA from dairy fat.

Hypothesis: Fortifying dairy fat with VA mitigates putative stimulatory effects of dairy-derived SFA on hepatic and intestinal TG secretion.

Expected Outcome: Fortification of dairy fat with VA can reduce hepatic (fasting) and intestinal (post-prandial) TG secretion relative to a diet containing dairy fat without VA.

2 *(iv)* To validate the PPAR mechanism of action for the lipid-lowering effects of VA in the liver and the intestine when supplemented on a background diet that includes SFA from dairy fat.

Hypothesis: VA exerts its lipid-lowering effects by regulating hepatic and intestinal genes involved in lipid synthesis and/or PPAR-dependent oxidative pathways.

Expected Outcome: It is expected that VA will reduce the mRNA expression of genes involved in lipid synthesis and increase the expression of PPAR-downstream targets in the liver and the intestine.

The results from these series of experiments are presented in Chapter 4 and have been submitted for publication.

Chapter 5: Metabolic effects of *trans*-11 vaccenic acid are associated with its ability to exert tissue specific regulation of endocannabinoids in the JCR:LA-*cp* rat

General Objective 3. To explore the independent regulatory effect of VA on lipid-derived signaling molecules (endocannabinoids) known to control energy storage and utilization by acting in peripheral tissues and the brain.

Hypothesis: The metabolic benefits of VA are convergent with its ability to regulate tissue EC concentrations in the JCR:LA-*cp* rat.

Specific objectives

3 *(i)* To investigate the independent effects of VA and CLA or their combination on regulating the concentration of EC in peripheral tissues and the brain.

Hypothesis: Dietary supplementation with VA alone or in combination with CLA can favourably regulate the concentrations of tissue EC.

Expected Outcome: It is expected that VA alone or in combination with CLA can regulate tissue EC concentrations converging with its metabolic effects.

3 *(ii)* To investigate whether dietary supplementation with VA alone or in combination with CLA can directly influence tissue concentrations of EC by altering the composition of their membrane PL precursors.

Hypothesis: VA and/or CLA are efficiently incorporated into tissue PL thereby interfering with the synthesis of PL-derived EC.

Expected Outcome: Incorporation of VA and/or CLA in membrane PL will result in a concomitant decrease of EC biosynthetic precursors.

The results from these series of experiments are presented in Chapter 5.

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Chapter 3 Increased hypolipidemic benefits of conjugated linoleic acid in combination with vaccenic acid in the JCR:LA-*cp* rat

*The research described in this Chapter has been published as a manuscript (Please see **Appendix 'A'**).*

3.1 Introduction

Conjugated linoleic acid (CLA) refers to diverse positional and geometrical isomers of linoleic acid and its numerous health related effects have been extensively investigated. CLA was first described as a potent anti-carcinogenic component and more recently has been associated with improving dyslipidemia, insulin sensitivity and the pro-inflammatory state related to obesity and the metabolic syndrome (Wahle *et al*., 2004; Bhattacharya *et al*., 2006). However, some animal studies (specifically those using mouse models), in addition to a handful of clinical trials, have indicated that the major isomers found in CLA mixtures (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) are responsible for different physiological effects (Taylor and Zahradka, 2004; Terpstra, 2004; Park and Pariza, 2007; Risérus *et al*., 2002).

CLA is found naturally in ruminant-derived lipids and *cis*-9, *trans*-11 CLA is the major natural isoform, accounting for about 80-90% of the total CLA isomers (Mendis *et al*., 2008). *Trans*-11 vaccenic acid (VA) is the precursor to endogenous synthesis of the *cis*-9, *trans*-11 CLA isomer in rats (Santora *et al*., 2000; Corl *et al*., 2003) and humans (Turpeinen *et al*., 2002), and is the predominant isomer of the total *trans* fatty acids found in ruminant-derived fats such as dairy and meat products. We have shown previously that unlike industrially produced *trans* fatty acids, VA has lipid-lowering properties associated with reduced hepatic lipogenesis and chylomicron secretion in the obese and insulin resistant JCR:LA-*cp* rat (Wang *et al*., 2009). Interestingly, our observations indicate that VA has

neutral effects under normolipidemic conditions and induces hypotriglyceridemic effects under conditions of dyslipidemia (Wang *et al*., 2008). We also observed that VA supplementation for 16 weeks had a greater potential to influence lipoprotein metabolism (Wang *et al*., 2009) compared to a shorter term feeding (Wang *et al*., 2008). These findings are supported by several clinical (Tholstrup *et al*., 2006; Motard-Belanger *et al*., 2008; Chardigny *et al*., 2008) and animal studies (Lock *et al*., 2005; Roy *et al*., 2007; Bauchart *et al*., 2007; Tyburczy *et al*., 2009; Bassett *et al*., 2010) showing that dietary *trans* fats derived from ruminants have either neutral or beneficial effects on cardiovascular disease risk factors compared to industrially produced *trans* fats.

As a result of the increasing evidence associating CLA and more recently VA with health benefits, there has been a growing interest to increase the concentrations of these natural *trans* fats in meat and dairy products (Bauman *et al*., 2000; Lock and Bauman 2004; Jones *et al*., 2005; Cruz-Hernandez *et al*., 2007). Interestingly, both VA and CLA (*cis*-9, *trans*-11) can account for more than 15% of the total fat in naturally enhanced dairy products (Lock and Bauman, 2004) which could provide an additional health value to animal-derived fats. Consequently, in this study we hypothesized that chronic supplementation with both CLA and VA would enhance the lipid lowering effects to improve whole body lipid metabolism. Therefore, the aim of this study was to evaluate the effect of dietary supplementation with purified forms of both *cis*-9, *trans*-11 CLA and VA on impaired lipid metabolism in an established animal model of the metabolic syndrome, the JCR:LA-*cp* rat.

3.2 Research Design and Methods

3.2.1 Animals and diets

All experimental procedures were approved by the University of Alberta Animal Ethics Committee and conducted in accordance with the Canadian

Council on Animal Care. Twenty four male obese JCR:LA-*cp* rats (*cp/cp*) were raised in our established breeding colony at the University of Alberta as previously described (Vine *et al*., 2007). At 3 wk of age, rats were transferred from the isolated breeding colony areas to an individually ventilated caging environment (TecniplastTM, Exton PA, USA) and had access to a standard rat chow diet (5001, PMI Nutrition International) (see ***Figure 3-1***).

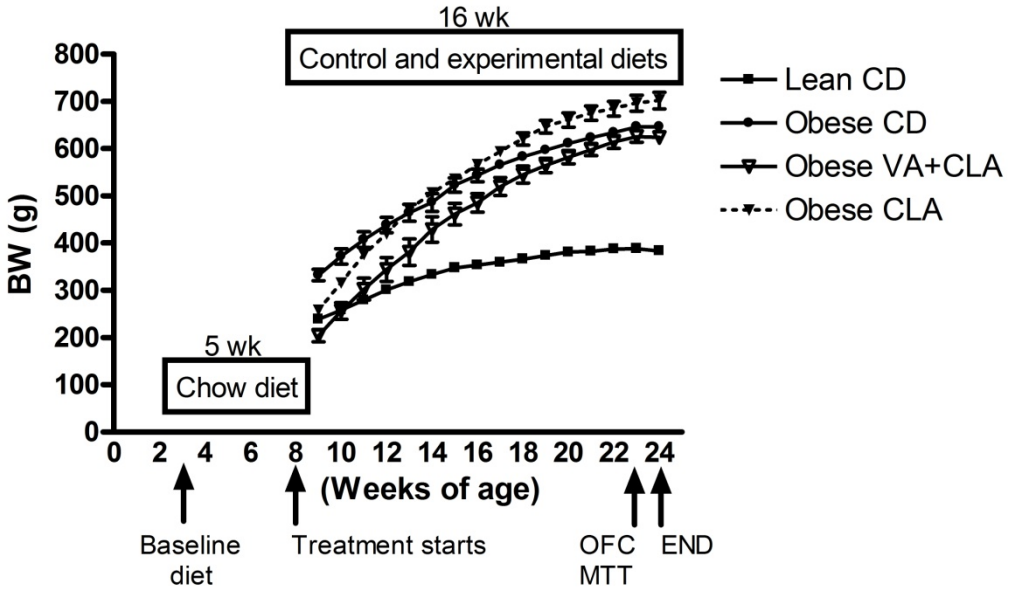

Figure 3-1 Changes in body weight throughout the study and schematic representation of the experimental design. Rats (n=8) were fed a standard chow diet prior to the study (from 3-8 wk of age). Then, control and experimental diets were provided for 16 wk. An oral fat challenge test (OFC) and a meal tolerance test (MTT) were conducted on different rats (n=4 in each test).

At 8 wk of age, rats (n = 8) were randomized and assigned to one of three diets for 16 wk (control and experimental diets) and had free access to food and water. Age and weight matched lean littermates (n = 8) were fed the control diet to mimic cholesterol/high fat feeding under normolipidemic conditions. Food intake and body weight (BW) were monitored weekly throughout the study. At 23 wk of age, a meal tolerance test (MTT) as previously described (Russell *et al*., 1987), was performed in four randomly chosen rats from control and treatment groups in order to determine plasma glucose and insulin concentrations after a meal. We also performed an oral fat challenge test (OFC), as previously described (Vine *et al*., 2007) in four additional rats from each group. At the end of the treatment period (24 wk of age), rats were fasted overnight and anesthetized using isofluorane anesthesia. Plasma was sampled from the left ventricle and heart, liver and fat pads were excised, weighted and immediately frozen at -80°C until analysis. Adipose fatty acid composition was measured from total triglyceride on the epidydimal fat pad as previously described (Wang *et al*., 2008).

Three iso-caloric diets were prepared with a constant polyunsaturated to saturated fatty acid ratio (P:S) of 0.4. A control diet (CD) was supplemented (w/w) with 1% cholesterol and contained 42% of energy from carbohydrate, 23.7% from protein and 34.3% from fat. Experimental diets were prepared by adjusting the lipid composition of the CD to provide 1.0% w/w of *cis*-9, *trans*-11 CLA alone (CLA) or both 1% of VA and 1% w/w of *cis*-9, *trans*-11 CLA (VA+CLA). Semi-purified *cis*-9, *trans*-11 CLA (G-c9t11 80:20) containing 59.8% of *cis*-9, *trans*-11 CLA and 14.4% of *trans*-10, *cis*-12 CLA was kindly provided by Lipid Nutrition. The amount of CLA and VA (1% w/w) was chosen based on previous studies allowing for metabolic sufficiency while maintaining a normal dietary fatty acid proportion (Wang *et al*., 2008; Wang *et al*., 2009; Tholstrup *et al*., 2006; Raff *et al*., 2006). Purified VA was synthesized by a chemical alkali

isomerisation from linoleic acid-rich vegetable oil (Swain, 1949). The diet mixture was extruded into pellets, dried at RT and stored at 4°C. Fatty acid composition of the three diets was confirmed by gas chromatograph analysis (Cruz-Hernandez *et al*., 2007) of the fat blend samples (***Table 3-1***).

3.2.2 Plasma biochemical components

The concentration of biochemical parameters in fasting plasma from lean and obese groups were assessed using commercially available enzymatic colorimetric assays. Triglyceride (TG) (Wako Pure Chemical Industries, catalog no. 998-40391, 0.01 mmol/L minimum), total cholesterol (TC) (Wako Pure Chemical Industries, catalog no. 993-00404, 0.002 mmol/L minimum), LDL cholesterol (LDL-C) (Wako Pure Chemical Industries, catalog no. 993-00404, 0.03-10.4 mmol/L) and nonesterified fatty acids (NEFA) (HR Series NEFA-HR, catalog no. 999-34691, Wako Diagnostics) were measured using direct colorimetric chemical enzymatic reactions. Plasma glucose was measured as per the glucose oxidase method (Diagnostic Chemical, catalog no. 220-32, 0.03-33.3 mmol/L) and plasma insulin was determined using commercially available enzymatic immunoassays for rodents (Ultrasensitive rat insulin ELISA, Mercodia, catalog no. 80-INSRTU-E01, 0.03-1.0 pmol/L). Samples were analyzed in triplicate using assay kits from a single lot and performed in a single batch.

Table 3-1 Fatty acid composition (% of total fatty acids) of control and experimental diets

Fatty acid	Control diet (CD)	CLA diet	VA+CLA diet
C16:0	9.1	8.5	9.1
C18:0	47.3	46.9	44.3
18:1 *t*-11 (VA)	ND	ND	5.6
18:1 *c*-9 (OA)	17.3	11.4	10.5
18:1 *c*-11	ND	0.5	0.5
C18:2 n6 (LA)	23.3	23.4	20.4
C18:3 n3 (ALA)	1.6	1.7	1.6
CLA *c*-9, *t*-11	0	5.2	3.9
CLA *t*-10, *c*-12	ND	1.1	0.8
other CLA	ND	0.3	0.3
Summary			
∑ total SFA[2]	57.2	56.3	54.2
∑ C12:0, C14:0, C16:0[3]	9.1	8.5	9.1
∑ *cis* MUFA[4]	17.4	12.0	11.1
∑ PUFA[5]	25.0	25.1	22.0
∑ n-6 PUFA	23.4	23.4	22.0
∑ n-3 PUFA	1.6	1.7	1.6
P:S ratio[6]	0.4	0.4	0.4
∑ CLA	0.0	6.6	5.0

[1.] No detectable
[2] Sum of all saturated fatty acids
[3] Sum of lauric, myristic and palmitic acids
[4] *cis* MUFA, sum of all monounsaturated excepting *trans* fatty acids
[5] Sum of all polyunsaturated fatty acids excepting CLA
[6] Ratio of polyunsaturated to saturated fatty acids

3.2.3 Tissue homogenization and hepatic TG

Liver and adipose tissue samples (0.5 g) were homogenized in 200 µL lysis buffer [PBS (pH 7.4) with 1.5% TritonX-100 and 1% protease inhibitor cocktail (Sigma)] and hepatic TG levels were determined by a commercially available enzymatic colorimetric assay (Wako Pure Chemical Industries, catalog no. 998-40391, 0.01 mmol/L minimum), using an aliquot of the whole homogenate and adjusting by the protein concentration of the homogenate. The remainder of the homogenate was centrifuged at 700 g for 15 min and the supernatant was collected and stored at -80C for western blot and citrate synthase activity analysis.

3.2.4 Hepatic and adipose tissue citrate synthase activity

Citrate synthase activity in liver and adipose tissue samples was determined using a commercially available kit from Sigma (catalog no. CS0720). The coefficient of variation of this assay in our laboratory is < 10%. The citrate synthase activity was expressed as μmol/min/g protein.

3.2.5 Relative protein abundance of lipogenic enzymes

Hepatic acetyl-CoA carboxylase-1 (ACC-1) and fatty acid synthase (FAS) were determined by western blot analysis. Briefly, proteins were separated by SDS-PAGE electrophoresis on 3–8% Tris-acetate polyacrylamide gels (InVitrogen), transferred to a polyvinylidene fluoride membrane and incubated with an anti-ACC-1 rabbit polyclonal (catalog no. 3662, Cell Signaling Technology®), anti-FAS mouse monoclonal (catalog no. sc-55580, Santa Cruz Biotechnology, Inc.) and anti-β-actin mouse polyclonal (1:5000; catalogue no. ab8226; abcam®, St Louis, MO, USA) antibodies. Detection was achieved using an anti-rabbit and anti-mouse secondary antibodies and the ECL advance detection system (Amersham Biosciences). ACC-1 and FAS relative abundance were normalized based on the respective β-actin protein mass (internal control).

3.2.6 Statistical analysis

Statistical analysis was performed using the Graph pad Prism software, version 4.0. Data was tested for normal distribution and one-way ANOVA followed by Tukey post-hoc tests were used to identify differences among both lean and obese controls and treatment groups (CLA and VA+CLA). Post-prandial glucose and insulin metabolism as well as post-prandial TG response were assessed by area under the curve (AUC) analysis. Fasting concentrations of these parameters were further subtracted from the total AUC to yield the incremental area under the curve (iAUC). Results are

expressed as means ± SEM and the level of significance was set at $P<0.05$.

3.3 Results

3.3.1 Food intake, body weight and body composition

Obese rats fed the combination of VA+CLA showed increased food intake compared to those obese rats fed either the CD or CLA diet. Paradoxically, the VA+CLA fed rats showed reduced body weight ($P<0.001$) compared to the CLA group (***Table 3-2***). Despite the higher body weight of rats fed the CLA diet as compared to obese control, no difference was observed in absolute and relative heart weights or fat pad deposition ($P>0.05$), as measured by the amount of absolute and relative perirenal and inguinal fat pad weights compared with the obese rats fed the CD (***Table 3-2***). In contrast, feeding either the CLA or the VA+CLA diet resulted in a lower absolute liver weight by 15% and 26%, respectively, and both diets reduced the ratio of liver weight to total body weight by 22% as compared to obese rats fed the CD ($P<0.001$).

Table 3-2 Food intake, body weight and body composition of rats in dietary groups

	Dietary groups			
	Lean control	Obese control	Obese CLA	Obese VA+CLA
Food intake (g/day)	19.7±0.3^{c}	32.4±0.5^{b}	31.4±0.6^{b}	36.1±0.6***^{a}
BW/16 wk (g)	384±8.2^{c}	646±9.3^{b}	702±17.9^{a}*	624±9.5^{b}
Heart (g)	0.89±0.0^{b}	1.19±0.0^{a}	1.26±0.0^{a}	1.23±0.0^{a}
weight, %BW	0.23±0.0^{a}	0.18±0.0^{b}	0.18±0.0^{b}	0.20±0.0^{b}
Liver (g)	9.1±0.4^{d}	23.1±0.7^{a}	19.7±0.6***^{b}	17.2±0.3***^{c}
weight, %BW	2.4±0.1^{c}	3.6±0.1^{a}	2.8±0.1***^{b}	2.8±0.0***^{b}
Perirenal AT[1] (g)	1.3±0.1^{c}	7.4±0.4^{ab}	8.6±0.8^{a}	6.7±0.4^{b}
weight, %BW	0.33±0.0^{b}	1.2±0.1^{a}	1.2±0.1^{a}	1.1±0.1^{a}
Inguinal AT (g)	1.4±0.2^{b}	17.9±1.1^{a}	18.5±1.0^{a}	15.7±1.0^{a}
weight, %BW	0.36±0.0^{b}	2.8±0.2^{a}	2.6±0.1^{a}	2.5±0.1^{a}

Values are means ± SEM, n=8. Means in the same row with different letters are significantly different; *P<0.05, **P<0.01, ***P<0.001.

AT[1]= Adipose tissue

3.3.2 Citrate synthase activity in liver and adipose tissue

The activity of citrate synthase in liver and adipose tissue is shown in ***Figure 3-2***. There was a higher (P<0.001) citrate synthase activity in liver and inguinal adipose tissue after feeding either CLA or VA+CLA diets as compared to lean and obese rats fed the CD. Interestingly, the citrate synthase activity in liver and adipose tissue did not differ between lean and obese rats fed the CD (P>0.05).

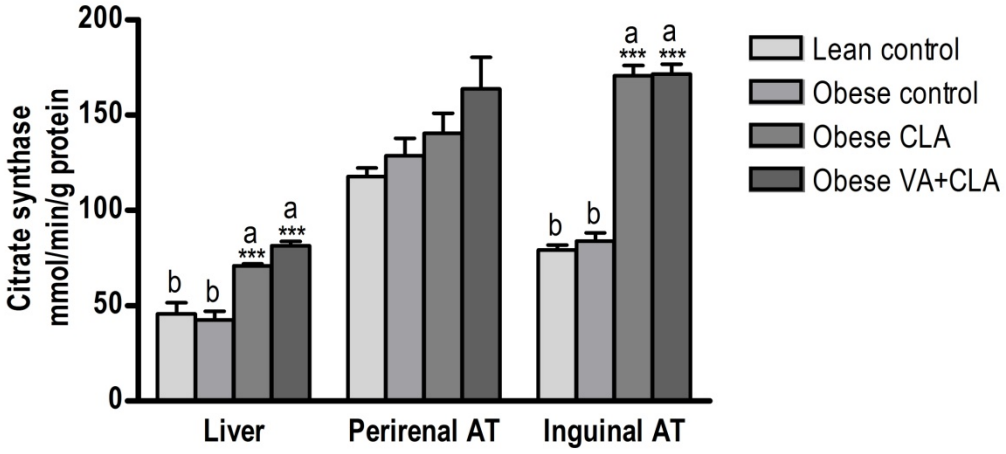

Figure 3-2 Citrate synthase activity in liver, perirenal and inguinal adipose tissue**.** Values are mean ± SEM, n=8. Means with different symbol are significantly different as compared to obese control; *P<0.05, **P<0.01, ***P<0.001. Means with different letters are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA). AT, adipose tissue.

3.3.3 Fatty acid profile in epididymal adipose tissue triglyceride

The fatty acid composition in adipose tissue triglyceride was shown to directly reflect the dietary fatty acid composition as shown in ***Table 3-3***. Obese rats fed with the CLA and VA+CLA diets had a markedly increased proportion of the *cis*-9, *trans*-11 CLA isomer relative to the obese control group. The content of this isomer, expressed as a percentage of total fatty acids was different between the treated groups. The CLA diet showed the greatest incorporation of the cis-9, trans-11 CLA isomer (70 fold greater than control group) compared to the VA+CLA diet, which was only 46 fold higher than the obese control group. Rats fed VA+CLA diet showed a greater incorporation of *trans*-11 18:1 (VA) compared to CLA and obese control rats ($p < 0.001$). Interestingly, rats fed the VA+CLA diet had lower proportions of linoleic acid (18:2 n6), α-linolenic acid (18:3 n3) and arachidonic acid (20:4 n6).

3.3.4 Fasting plasma lipid, glucose and insulin concentrations

As shown in ***Table 3-4***, fasting plasma TG, TC and LDL-C were significantly lower in obese rats fed either the CLA or the VA+CLA diet, as compared to the CD. However, feeding the VA+CLA diet further reduced plasma FFA concentration relative to the CLA fed rats and lowered TG and LDL-C concentrations not different from lean rats fed the CD ($P>0.05$). The CLA or VA+CLA diet lowered fasting insulin to that comparable of lean rats ($P>0.05$) and reduced total insulin concentration (AUC) after the MTT ($P<0.05$). There was no statistical difference between groups for either glucose metabolism (fasting or iAUC) or the relative change (iAUC) in insulin.

Table 3-3 Fatty acid composition (% of total fatty acids) of triglyceride in epididymal adipose tissue

	Dietary groups			
	Lean control	Obese control	Obese CLA	Obese VA+CLA
Fatty acid				
C18:0	13.9±0.71[a]	6.36±0.14[b]	5.47±0.07[b]	6.31±0.17[b]
C18:1 *t*-11 (VA)	0.05±0.01[b]	0.02±0.00[b]	0.08±0.00[b]	1.72±0.06***[a]
C18:1 *c*-9	28.4±0.14[d]	37.9±0.56[a]	32.7±0.32***[c]	35.2±0.49**[b]
C18:2 n6	35.76±0.22[a]	20.51±0.24[b]	20.15±0.25[b]	15.06±0.52***[c]
C18:3 n3	1.2±0.03[a]	0.91±0.02[b]	0.96±0.02[b]	0.47±0.06***[c]
CLA *c*-9, *t*-11	0.05±0.01[c]	0.04±0.01[c]	2.85±0.04***[a]	1.91±0.09***[b]
C20:4 n6	0.45±0.01[a]	0.33±0.25[b]	0.32±0.01[b]	0.22±0.0**[c]

Values are mean ±SEM, n=8. Means in the same row with different symbol are significantly different as compared to obese control; *P<0.05, **P<0.01, ***P<0.001. Means in the same row with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA). Long chain n3 PUFA were not detectable.

Table 3-4 Fasting plasma lipid concentrations, glucose and insulin AUC after MTT and hepatic TG

	Dietary groups			
	Lean control	Obese control	Obese CLA	Obese VA+CLA
TG (mmol/L)	0.47±0.0^{c}	3.28±0.4^{a}	2.05±0.3*^{b}	1.4±0.1***^{bc}
FFA (mmol/L)	0.20±0.02^{c}	0.48±0.02^{ab}	0.57±0.03^{a}	0.42±0.05^{b}
TC (mmol/L)	2.26±0.0^{c}	6.18±0.4^{a}	4.22±0.2***^{b}	4.92±0.2**^{b}
LDL-C (mmol/L)	0.97±0.1^{c}	2.28±0.2^{a}	1.56±0.1**^{b}	1.2±0.1***^{bc}
Fasting glucose (mmol/L)	5.94±0.08	6.81±0.4	5.94±0.36	6.75±0.72
Glucose iAUC (mmol/L.h)	45.19±4.5	52.22±7.8	70.25±14.7	42.7±33.05
Fasting insulin (µIU/L)	73.41±27.4^{b}	629.2±165.3^{a}	216±13.6*^{b}	372.8±98.24^{ab}
Insulin AUC (µIU/L.h)	8739±1566 ^{c}	40437±4384 ^{a}	24548±3465*^{b}	23001±2744*^{b}
Insulin iAUC (µIU/L.h)	4452±1464	11805±7603	11585±3885	4451±3015
Liver TG (mmol/g protein)	2.2±0.3^{d}	21.2±1.0^{a}	16.6±1.1*^{b}	12.1±1.3***^{c}

Plasma lipid and liver TG values are mean ±SEM, n=8. Insulin AUC is mean ±SEM, n=4. Means in the same raw with different symbol are significantly different as compared to obese control; *P<0.05, **P<0.01, ***P<0.001. Means in the same raw with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA).

3.3.5 Post-prandial plasma TG response

Obese rats had a higher post-prandial plasma TG response (iAUC) compared to lean rats following an OFC (***Figure 3-3***). AUC analysis showed an improved total TG concentration (P<0.05) over the 10-h post-prandial period in rats fed either the CLA or VA+CLA diet (35 ± 5 and 38 ± 8 mmol/L.h, respectively), compared to obese rats fed the CD (62 ± 5 mmol/L.h). However, the post-prandial iAUC for TG was not different between obese control and obese treatment groups (CLA and VA+CLA).

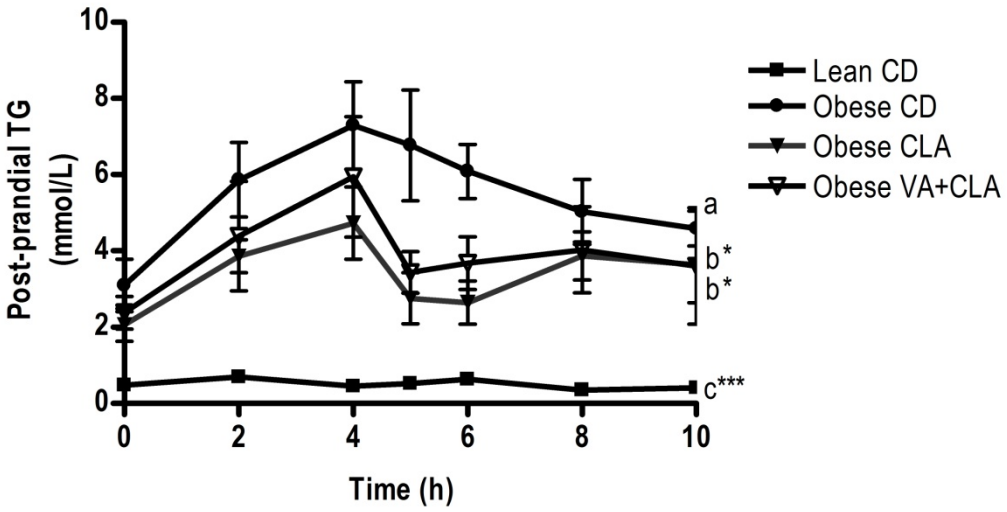

Figure 3-3 Post-prandial triglyceride response following an oral fat challenge. Values are mean ± SEM, n=4. AUC differ relative to obese rats fed the CD; [b]P<0.05, [c]P<0.001. iAUC differ relative to obese rats fed the CD; ***P<0.001.

3.3.6 Liver TG concentration and relative abundance of hepatic lipogenic enzymes

Liver TG concentration was higher in obese control rats compared to lean control rats. However, feeding the CLA diet resulted in a liver triglyceride concentration that was 22% lower than the obese rats fed the CD (***Table 3-4***). Interestingly, the VA+CLA diet further lowered liver TG concentration by 43% and 27% as compared to obese control and CLA groups, respectively. In addition, CLA and VA+CLA diets resulted in significantly lower hepatic ACC-1 protein abundance relative to obese control (34% and 38%, respectively) and was normalized to concentrations similar to lean rats fed the CD ($P>0.05$) (***Figure 3-4A, B***). The relative abundance of hepatic FAS protein did not differ between the obese groups ($P>0.05$) (***Figure 3-4A, C***).

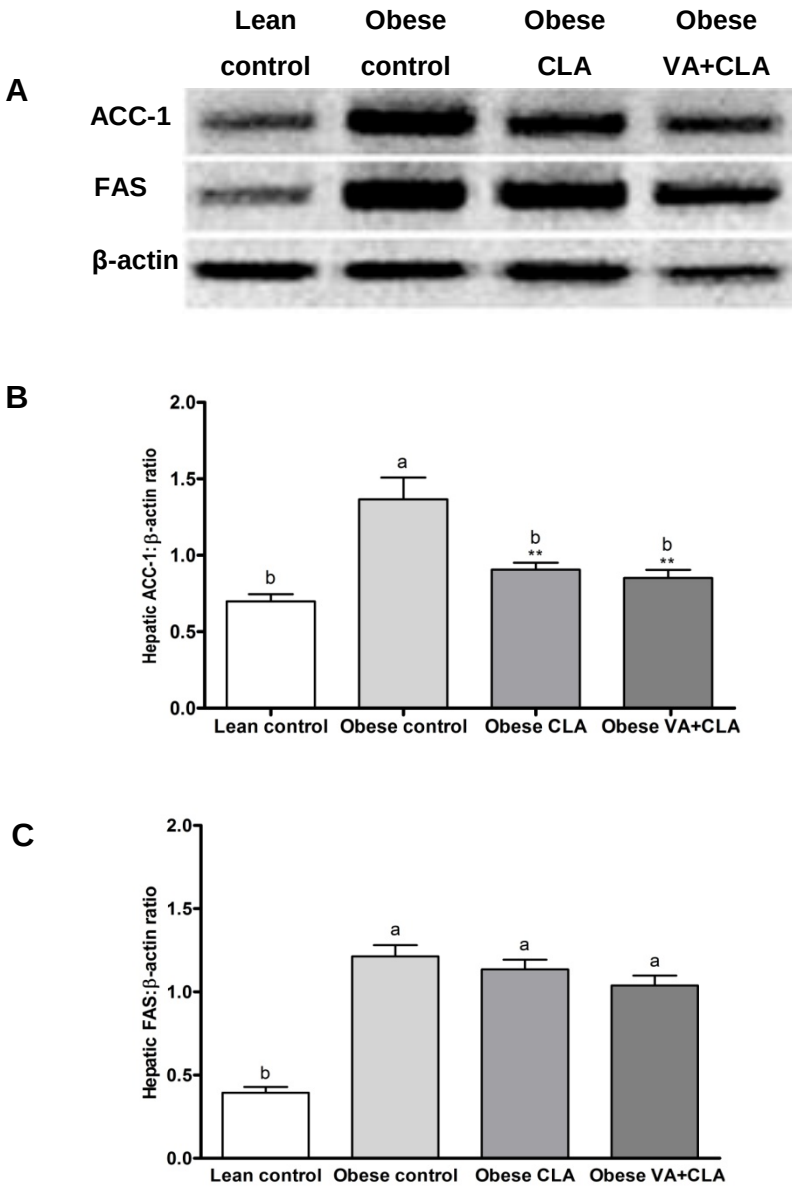

Figure 3-4 Effects of CLA and VA+CLA on hepatic protein abundance of lipogenic enzymes. Western blots of hepatic lipogenic enzymes (A), and relative abundance of ACC-1 (B) and FAS (C). Values are mean ± SEM, n=8. Means with different symbol are significantly different as compared to obese rats fed the CD; *P<0.05, **P<0.01, ***P<0.001. Means with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA).

3.4 Discussion

3.4.1 Combination of VA+CLA increases food intake and decreases liver weight

Dyslipidemia and insulin resistance are common features associated with cardiovascular disease and the metabolic syndrome. The homozygous obese (*cp/cp*), JCR:LA-*cp* rat, has a complete absence of the leptin receptor. As a consequence, the JCR:LA-*cp* rat spontaneously develops hyperphagia and obesity, associated dyslipidemia, insulin resistance, and macro-and micro-vascular dysfunction (Russell and Proctor, 2006; Elam *et al*., 2001; Russell *et al*., 2007). In the present study, we used this unique animal model to evaluate hypolipidemic effects of *cis*-9, *trans*-11 CLA in combination with VA. Obese rats fed the CLA diet had a higher final body weight relative to obese control rats. However, this was not associated with an increase in fat deposition. *Cis*-9, *trans*-11 CLA has been observed to regulate metabolic pathways involved in fatty acid oxidation as well as energy production and thermogenesis (Choi *et al*., 2004; Ryder *et al*., 2001; Rodriguez *et al*., 2002 Choi *et al*., 2007); therefore we investigated whether combination VA+CLA might promote fatty acid oxidation.

3.4.2 CLA and VA+CLA increase the enzymatic activity of a marker of mitochondrial oxidative capacity in liver and adipose tissue

Peroxisome proliferator-activated receptor (PPAR)-agonists, such as thiazolidenediones (TZDs), are effective drugs for the treatment of type 2 diabetes by inducing adipogenesis as well as increasing the uptake and metabolism of free fatty acids in adipose tissue. Increased mitochondrial oxidative capacity of white adipose tissue has been observed after treatment with TZD (Bogacka *et al*., 2005; Rong *et al*., 2007). CLA is a natural PPAR ligand (Moya-Camarena *et al*., 1999; Wargent *et al*., 2005; Brown *et al*., 2003) and has been observed to promote fatty acid oxidation

(Choi *et al*., 2007) or TG synthesis in adipose tissue contributing to lower circulating FFA and TG concentrations (Roche *et al.,* 2002). We observed a significant increase in citrate synthase activity in liver and inguinal adipose tissue following supplementation with either CLA or VA+CLA diet but not in perirenal adipose tissue. Interestingly, citrate synthase activity was not different between lean and obese control rats. It has been reported that insulin resistance is associated with an increase in muscle mitochondrial content and oxidative capacity (Turner et al., 2007; Hancock et al., 2008; Hoeks *et al*., 2008). Similarly, it has been demonstrated that mitochondrial biogenesis increases during adipose tissue differentiation (Wilson-Fritch *et al*., 2003). Therefore, it can be proposed that JCR:LA-*cp* rats maintain similar mitochondrial fatty acid oxidation relative to lean rats but during increased dietary lipid consumption, it is insufficient to prevent TG deposition. Treatment with CLA or VA+CLA may stimulate mitochondrial fatty acid oxidation and in turn this may contribute to improvements in liver and adipose tissue metabolism in the JCR:LA-*cp* rat.

3.4.3 CLA and VA are similarly incorporated in adipose tissue triglyceride

It is well established that the fatty acid composition of adipose tissue is dependent on dietary intake. However, endogenous synthesis of fatty acids, fatty acid transport and inter-conversion processes (elongation and desaturation) are also significant contributing factors to the composition of adipose tissue (Hodson *et al*., 2008). As expected, supplementation with *cis*-9, *trans*-11 CLA (CLA diet) and VA (VA+CLA diet) increased the proportion of these fatty acids in adipose tissue from obese rats. We also wish to note that the endogenous synthesis of *cis*-9, *trans*-11 CLA from VA may have also occurred in this study. In both humans and animals, the conversion of dietary VA to CLA has been reported to be at a rate of approximately 12-19% (Santora e*t al*., 2000; Turpeinen *et al*., 2002). We

also note that the dietary ratio of VA:CLA in this study was ~1.5:1, respectively (***Table 3-1***) and that the resultant incorporation of these fatty acids into adipose tissue was found to be 1:1 following supplementation (***Table 3-3***). While we cannot infer a rate of conversion from VA to CLA *per se* from this data, it would support that previously published.

3.4.4 Combined dietary VA+CLA has a greater effect to reduce dyslipidemia and hepatic steatosis

One of the most striking effects of the combined treatment (VA+CLA) in the present study was the reduction in hepatic TG concentration. The improvement in fasting lipid parameters by VA+CLA diet (i.e. TG and LDL-C) suggest an additional benefit with the combination diet and is consistent with previous observations that dietary VA has lipid lowering properties independent from CLA (Wang *et al*., 2009). We also wish to note that both diets (CLA and VA+CLA) consistently contained 0.15 w/w of the *trans*-10, *cis*-12 CLA isomer, which is also known for its hypolipidemic properties.

ACC-1 and FAS are two key lipogenic enzymes involved in the synthesis of fatty acids and subsequent TG synthesis. TG is then either stored as lipid droplets within the hepatocyte, secreted into the blood compartment as VLDL or hydrolyzed via oxidation (Postic and Girard, 2008). It is plausible that reduced hepatic lipogenesis may contribute (at least in part) to reduced hepatic TG in rats fed either the CLA or VA+CLA diet, which is supported by a lower hepatic ACC-1 protein abundance relative to obese rats fed the CD. We have reported previously that VA may act in part via ACC-1 and FAS pathways resulting in reduced VLDL secretion to decrease circulating concentrations of plasma TG and LDL-C (Wang *et al*., 2009). However, in conditions of insulin resistance, hepatic steatosis is thought to be caused by an increased free fatty acid flux from adipose tissue into the liver (Lewis *et al*., 2002). As discussed above, rats fed the

combined treatment (VA+CLA) showed lower circulating FFA concentrations compared to rats fed the CLA diet alone. We propose that reduced FFA may also contribute to a further decrease in hepatic TG. In addition, activation of ACC is regulated by phosphorylation/dephosphorylation (Abu-Elheiga *et al*., 2000) and thus, it may be possible that CLA and VA+CLA diets may differently regulate post-translational modifications of ACC-1.

In conclusion, results in this study confirm hypolipidemic effects of chronic supplementation with *cis*-9, *trans*-11 CLA alone or in combination with *trans*-11 VA in the dyslipidemic and insulin resistant JCR:LA-*cp* rat. Our data also support the hypothesis that a dietary formulation enriched with both CLA and VA may further enhance their hypolipidemic properties, particularly during conditions of hypertriglyceridemia, hypercholesterolemia and/or hepatic steatosis.

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Chapter 4 Diets enriched in *trans*-11 vaccenic acid attenuate ectopic (hepatic and intestinal) lipid storage and increase energy utilization in the JCR:LA-*cp* rat.

This chapter is presented in the format of manuscript that has been prepared for publication.

4.1 Introduction

Trans-11 vaccenic acid (VA) is the predominant *trans* fatty acid (TFA) in the food chain accounting for up to 70% of total TFA in ruminant derived fats (Lock and Bauman, 2004; Cruz Hernandez *et al*., 2007). It is produced naturally by bacterial bio-hydrogenation of dietary unsaturated fatty acids in ruminant animals and is found in products such as beef, lamb and dairy. VA is also the precursor to endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in animals and humans (Palmquist *et al*., 2005). Thus, VA can be endogenously converted to CLA with purported health benefits in animal models and humans (Banni *et al*., 2001; Turpeinen *et al*., 2002). However, in addition to being acknowledged as a potential source of CLA, there remains a void of information regarding the independent bioactivity of VA *per se*.

Studies using VA-enriched butter in different animal models of cardiovascular disease (CVD) were the first to provide insights into its lipid lowering effects (Lock *et al*., 2005; Bauchart *et al*., 2007; Bassett *et al*., 2010). There is now accumulating evidence that suggests dietary supplementation of purified synthetic VA can improve fasting and post-prandial dyslipidemia and the pro-inflammatory state in animal models of CVD and the metabolic syndrome (MetS) (Wang *et al*., 2008; Wang *et al*., 2008; Blewett *et al*., 2009; Ruth *et al*., 2010; Tyburczy *et al*., 2009). Most recently, it has been demonstrated that synthetic VA is a partial agonist to peroxisome proliferator activated receptors (PPAR-α and –γ) *in vitro,*

acting with similar affinity to PPAR agonists commonly used for the treatment of dyslipidemia and type-2 diabetes (Wang *et al*., 2012). Despite this proposed mechanism of action, putative benefits of VA to insulin resistance and energy use and storage have not been clear in these early studies.

Dairy products are the predominant (natural) source of VA in the food chain, yet conversely are also characterized by a high proportion of select saturated fatty acids (SFA) including; myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid; as well as a high proportion of the monounsaturated fatty acid (MUFA), oleic acid (C18:1). While contentious, SFA have historically been associated with negative effects on health. For instance, high SFA intake is associated with insulin resistance and post-prandial lipemia in normolipidemic patients with non-alcoholic steatohepatitis (NASH) (Musso *et al*., 2003). The literature also documents that replacing SFA (specifically palmitic acid) with polyunsaturated fatty acids (PUFA) (Mozaffarian *et al*., 2010) or low-fat dairy products (Ohlsson, 2010) lowers LDL/HDL cholesterol and total/HDL cholesterol ratios. Given the recent evidence for the health effects of VA, the logical approach would be to produce VA enriched animal-derived foods that could provide an additional health benefit. However, literature so far has only documented effects of purified synthetic VA on a vegetable oil background diet (Wang *et al*., 2008; Wang *et al*., 2008; Blewett *et al*., 2009; Ruth *et al*., 2010; Tyburczy *et al*., 2009). In this study we investigated whether supplementation of dairy fat with synthetic VA (fortification) could increase the putative health value of dairy-derived SFA specifically during conditions of MetS. Additionally, we used a systemic approach to determine the effects of VA-supplemented dairy fat on energy utilization and metabolic abnormalities in a rodent model of NAFLD and the MetS, the JCR:LA-cp rat.

4.2 Materials and methods

4.2.1 Animals and diets

Rats of the JCR:LA-*cp* strain that are homozygous for the corpulent trait (*cp/cp*) have a complete absence of the leptin receptor in the plasma membrane and spontaneously develop symptoms associated with the MetS and the pre-diabetic state typically observed in humans; including obesity, insulin resistance and dyslipidemia (Russell and Proctor, 2006). Heterozygous rats (+/?) from the same strain are lean and metabolically normal and were used as controls. Male JCR:LA-*cp* rats were raised in our established breeding colony at the University of Alberta as previously described (Vine *et al*., 2007). At 8 weeks (wk) of age, rats were randomized and assigned to either control diet (CD) or CD supplemented with VA for 8 wk and had free access to food and water. Age and weight matched lean littermates were fed the CD to represent normal metabolic conditions. Food consumption and body weight were monitored weekly throughout the study. For the purpose of this study, the term MetS will be used to define rats of the JCR:LA-*cp* strain fed the CD while those rats fed the CD supplemented with VA will be defined as MetS+VA. All tests were done on the same batch of animals except for the *in vivo* hepatic and intestinal TG secretion experiments, which were performed on a separate group of animals.

The CD was prepared by adding 1% w/w cholesterol and 15% w/w of fat to an 85% basal mix diet (Harlan laboratories, TD.06206) and contained 42% of energy from carbohydrate, 23.7% of energy from protein and 34.3% of energy from fat. The fat portion of the diet was adjusted by using predominantly SFA from dairy fat (***Supp. Table 4-1-Appendix 'B'***), thus reflecting the typical composition of a western diet. The VA diet was prepared by adjusting the fatty acid composition (replacing oleic acid by VA) of the CD to provide 1.0% w/w of VA. Both diets had a constant PUFA

to SFA ratio of 0.4 and a constant n6 to n3 PUFA ratio of 8. Purified VA was synthesized by chemical alkali isomerization from linoleic acid-rich vegetable oil (Swain, 1949). Fatty acid composition of diets was confirmed by gas chromatograph analysis (Cruz Hernandez *et al*., 2007) of the fat blend samples (***Supp. Table 4-2- Appendix 'B'***). Animal care and experimental procedures were conducted in accordance with the Canadian Council on Animal care and approved by the University of Alberta Animal Care and Use Committee - Livestock.

4.2.2 Body composition analysis

Body composition was assessed non-invasively at baseline and every subsequent wk by NMR for small animals (Minispec LF90 Body Composition Analyzer, Bruker, Germany). Body weight (BW) was assessed prior to measurements to calculate fat mass percentage. After sacrifice, visceral and subcutaneous adipose tissue depots were excised and weighed to determine body fat distribution.

4.2.3 Adipocyte size of visceral and inguinal depots

Sections (5 µm) were prepared from paraffin-embedded fat pads of MetS and MetS+VA rats (n=5) and subjected to hematoxylin and eosin staining using standard procedures. Histology sections were viewed at 10X magnification and images obtained with a Westover Digital AMID-1 [D] microscope. The cell area (μm^2) of 130 adipocytes per rat was measured with the open-source image analysis software (ImageJ, National Institutes of Health, http://rsbweb.nih.gov/ij).

4.2.4 Assessment of energy expenditure by indirect calorimetry

Oxygen consumption (VO_2) and respiratory exchange ratio (RER) were measured by indirect calorimetry in rats at the end of the study. Rats were fed *ad libitum* the control and experimental diets and were maintained

under 12 hr light and dark cycles beginning at 6:00 pm and 6:00 am, respectively. Rats were acclimatized for 24 hr before recording and measurements of VO_2 were recorded every 14 min over 24 hr using the Oxymax System (Columbus Instruments, Columbus, OH). To adjust for metabolic body size, VO_2 was then expressed in mL/h/kg of body weight$^{0.75}$. The RER equals volumes of CO_2 released by volumes of O_2 consumed.

4.2.5 Meal tolerance test (MTT) and the post-prandial response of insulin and glucose

Insulin and glucose concentrations were measured in conscious, unrestrained rats after consuming a standard test meal (Lab diet 5001, PMI Nutrition International, Brentwood, MO) in order to mimic an oral glucose tolerance test in humans as described previously (Russell *et al*., 1987). Briefly, 0.5 mL of blood was taken from the tip of the tail as t=0 min after an overnight fasting. Rats (n=6) were then given 5 g of the test meal. Timing was started when 50% of the test meal had been consumed and two additional samples of blood were taken at 30 and 60 minutes following the initial consumption of the food pellet. The homeostasis model assessment-insulin resistance (HOMA-IR) index was used as an estimate of insulin resistance. The HOMA-IR index was calculated as fasting insulin (µU/mL) x fasting glucose concentrations (mg/dL)/405 (Levy *et al*., 1998).

4.2.6 Liver histology

Sections (5 µm) were prepared from paraffin-embedded liver portions and subjected to hematoxylin and eosin staining using standard procedures. Slides from lean, MetS and MetS+VA rats (n=5-6) were blindly scored for steatosis, hepatocellular ballooning and portal and lobular inflammation by an independent veterinary pathologist as previously described (Brunt *et al*., 1999). The NAFLD activity score was determined by adding the scores

for steatosis, ballooning and lobular inflammation and was used to assess the progression of NAFLD (Kleiner *et al*., 2005; Brunt, 2007).

4.2.7 Tissue homogenization

Adipose tissue, intestine and liver samples were harvested after an overnight fast (16hr). Samples of the small intestine were washed with PBS and a two centimeter section from the middle of jejunum was cut. Samples (0.25 g) were homogenized in 500 µL of homogenization buffer (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 1% protease inhibitor cocktail (P8340, Sigma Aldrich). Protein concentration of the homogenate was measured colorimetrically with a BCA^{TM} Protein Assay Kit (Pierce, Rockford, IL, USA).

4.2.8 Quantification of phosphorylated hormone sensitive lipase (pHSL) protein

Phosphorylation of HSL Ser^{660} was detected in adipose tissue homogenates by Western blotting. Proteins were separated by SDS-PAGE electrophoresis on 3–8% Tris-acetate polyacrylamide gels (InVitrogen), transferred to a polyvinylidene fluoride membrane and incubated with an anti-pHSL (Ser 660) rabbit polyclonal (1:2000; catalogue no. 4126; Cell Signaling, Technology®) antibody. Detection was achieved using an anti-rabbit secondary antibody and the ECL advance detection system (Amersham Biosciences). Equal loading of total HSL was confirmed using an anti-HSL rabbit polyclonal (1:1000; catalogue no. 4107; Cell Signaling, Technology®) antibody. Results are expressed as a ratio of pHSL to total HSL protein.

4.2.9 Quantification of intestinal and hepatic lipids

The mass of intestinal and hepatic TG and cholesteryl esters (CE) was determined by gas chromatography as described previously (Sahoo *et al*.,

2004) in tissues collected after an overnight fast. Briefly, lipids from tissue homogenate (0.5 mg protein) were extracted by the Folch method (Folch *et al*., 1957) in the presence of tridecanoylglycerol as the internal standard. Extracted lipid samples were derivatized with Sylon BFT (Supelco, Bellefonte, PA) and analyzed by gas chromatography (Agilent Technologies, 6890 Series equipped with a flame ionization detector; Palo Alto, CA). The concentration of each lipid class was adjusted by the protein concentration of the tissue homogenate.

4.2.10 Hepatic and intestinal gene expression

An array of target genes (n=44) involved in lipid synthesis, oxidation and transport were measured by performing real time-PCR using a quantitative 'high-throughput' method (Deminice *et al*., 2011) in tissues harvested following an overnight fast.

Total RNA was isolated from frozen segments of both the liver and intestine using TRIzol® (Invitrogen, Canada) as described in the manufacturer's protocol and reversed transcribed into cDNA. Forty-eight gene assays (44 target genes + 4 housekeeping genes) and cDNA samples were loaded into separate wells on a 48.48 format Chip gene expressionassay (Fluidigm). Target gene expression was detected by qPCR (40 amplification cycles) using the Fluidigm Biomark system. Relative mRNA expression for each target gene was normalized to the housekeeping genes, β-actin (Actb) and 36b4 (Arbp) and quantified using the comparative cycle threshold (Ct) method. Data was expressed as a fold change in mRNA expression relative to MetS control rats $^{(2\text{-}\Delta\Delta Ct)}$. All assays were performed in triplicate.

4.2.11 Assessment of hepatic and intestinal TG secretion *in vivo*

Hepatic and intestinal TG secretion *in vivo* was conducted in a separate group of rats. Hydrolysis of plasma lipoprotein TG was inhibited by an

intraperitoneal injection of poloxamer-407 (P-407) in fasted rats (14 hr overnight fast). P-407 is a non-ionic detergent that inhibits mainly lipoprotein lipase, reducing the enzymatic activity by 95% three hours following administration (Johnston, 2004). For the hepatic TG secretion study, blood samples were collected from the tip of the tail and plasma obtained before (0 hr) and 1, 2, 3 and 4 hr after the P-407 injection. An aliquot of plasma was frozen for assessing TG by a commercially available colorimetric method (Wako Chemicals USA, Inc.).

In the case of the intestinal TG secretion study, following the P-407 injection (30 minutes), rats were oral gavaged with 10 uCi [^{3}H] triolein in 500 µL of olive oil. Blood samples were then collected and plasma obtained at 0 h (before P-407 administration) and 1, 2, 3 and 4 hr after the oral gavage. An aliquot of plasma was frozen for assessing radioactivity by liquid scintillation counting as described below.

4.2.12 Plasma lipid extraction and radioactivity counting

Total lipids were extracted from plasma samples by the Folch method and separated by TLC using heptane/isopropyl ether/acetic acid (60:40:4, by vol) to separate neutral lipids, as described previously (Lehner and Vance, 1999). The lipid classes were visualized by exposure to iodine vapor, the bands were scraped and the associated radioactivity was determined by liquid scintillation counting. Radioactive counts in TG were expressed as dpm per milliliter of plasma.

4.2.13 Plasma biochemical assays

The concentrations of select biochemical variables in fasting plasma from lean and MetS groups were assessed using commercially available enzymatic colorimetric assays. TG (Wako Chemicals USA, Inc.) were measured using direct colorimetric chemical enzymatic reactions. Plasma insulin (Insulin (Rat) Ultrasensitive EIA, AIPCO Immunoassays, 80

INSRTU-E01) was determined using commercially available enzymatic immunoassays for rodents and plasma glucose was measured as per the glucose oxidase/peroxidase method (Genzyme Diagnostics P.E.I. Inc.). Samples were analyzed in triplicate using assay kits from a single lot and performed in one batch.

4.2.14 Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test when experiments included more than two groups (Graph Pad Prism 5.0, USA). Changes in plasma TG concentrations over time (*in vivo* secretion studies) were analyzed via two-way ANOVA (variables: treatment and time) (SAS 9.2, USA). Two-way ANOVA and two-tailed, unpaired Student *t* tests also followed by Tukey's post hoc test, were used as indicated. Post-prandial glucose and insulin metabolism were assessed by area under the curve (AUC) analysis. Fasting concentrations of these parameters were further subtracted from the total AUC to yield the incremental area under the curve (iAUC). The level of significance was set at $P<0.05$.

4.3 Results

4.3.1 VA decreases total body fat mass and favorably alters fat distribution and adipocyte size

In the present study, enrichment of dairy fat with VA did not affect food intake or body weight relative to CD in MetS rats (***Supp. Figure 4-1-Appendix 'B'***). However, body composition analysis by NMR revealed that MetS+VA rats had a modest but significant reduction in total body fat mass (6%, $P<0.01$) relative to control MetS rats after 4 wk of feeding (***Figure 4-1A***). Furthermore, VA supplementation resulted in changes in body fat distribution as assessed by dissection of truncal fat depots during

sacrifice (***Figure 4-1B***). MetS+VA rats had lower mesenteric (17%, P<0.001) but higher inguinal fat mass (29%, P<0.001) relative to control MetS rats. In addition to this selective adipose re-distribution effect, VA reduced adipocyte size in both visceral and inguinal fat pads by 44 and 37%, respectively relative to CD (P<0.001, ***Figure 4-2***), which is reminiscent of PPARγ-induced adipose differentiation and improved function (Johnson *et al*., 2007). Interestingly, total white adipose tissue (WAT) was not different (P>0.05) between MetS groups suggesting that the reduction in total fat mass by VA may be associated with decreased ectopic fat accumulation. Indeed, VA was also observed to reduce liver weight (13.2%, P<0.001) as compared to CD. It is important to note that we did not quantify the lipid mass in extra hepatic organs such as pancreas, heart and muscle. However, the lower total fat mass observed in VA-fed rats could not be atributed to decreased hepatic lipid storage only. Therefore, for the purpose of this thesis we have defined the decrease in total fat mass as “reduced ectopic lipid accumulation”.

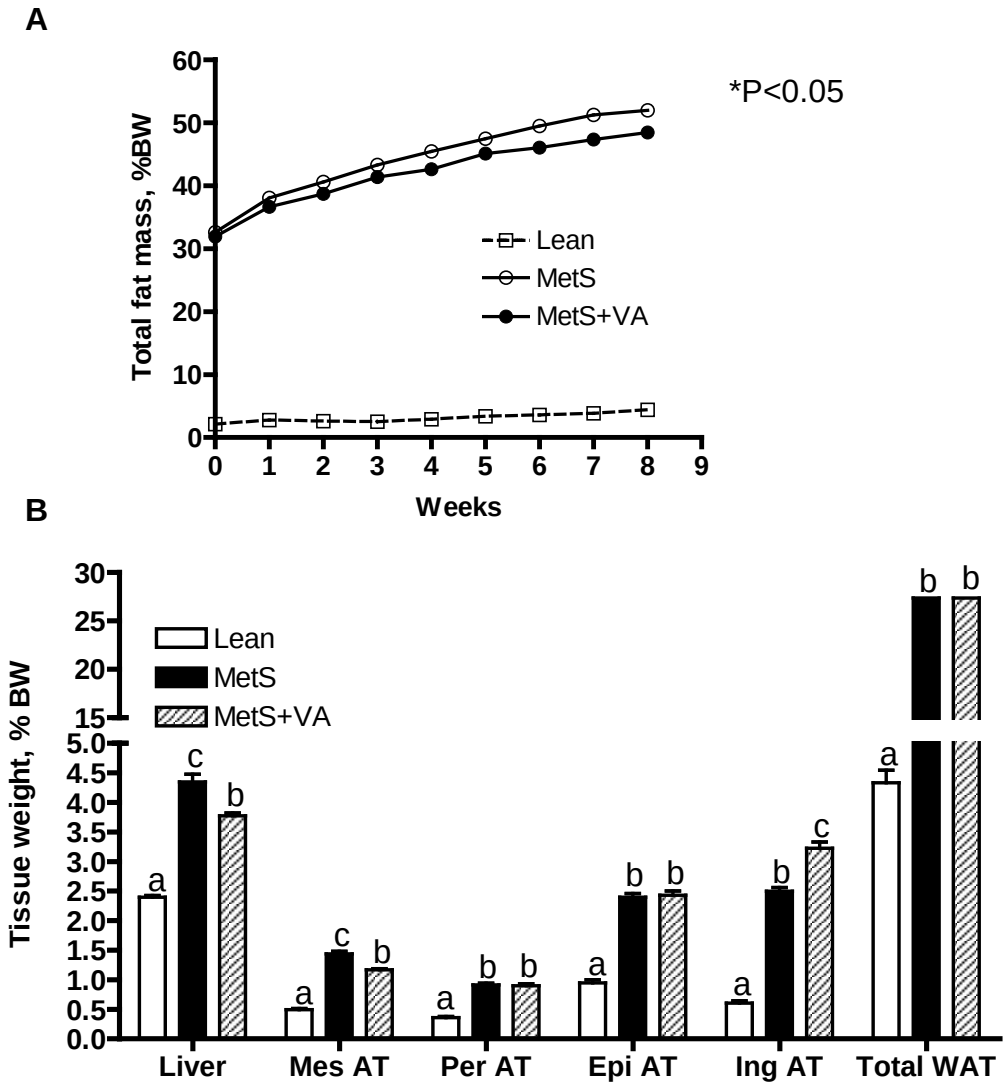


Figure 4-1 Body composition of lean (open squares), MetS (open circles) and MetS+VA rats (black circles); A) total fat mass assessed by NMR (% BW) throughout the study and B) liver and adipose tissue weight assessed during sacrifice. Values are means, with standard errors (SEM) represented by vertical bars (n=8). Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test. Mes, mesenteric; Per, perirenal; Ing, inguinal; WAT, white adipose tissue.

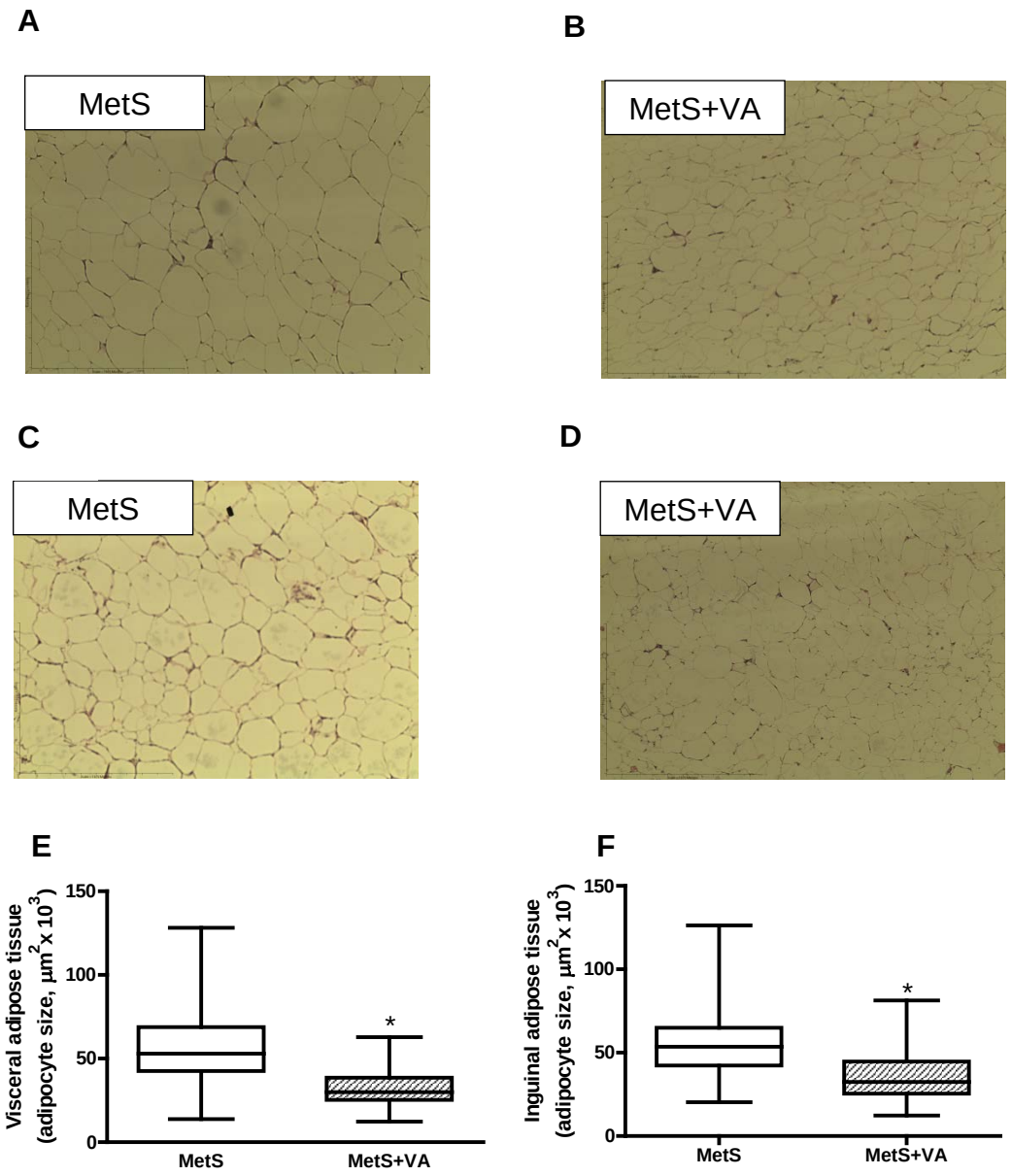


Figure 4-2 Representative images of visceral (Panel A and B) and inguinal (Panel C and D) adipocytes (magnification 10X) and the respective adipocyte size distributions (Panel E and F) of MetS and MetS+VA rats (n=5). Mean adipocyte size values (μm^2) of MetS+VA group differ relative to those of control MetS rats (*P<0.001) as assessed by two-tailed, unpaired Student *t* test and a non-parametric test (Mann-Whitney test). In (E) and (F), error bars denote maximum and minimum of size distribution.

4.3.2 VA decreases the active form of HSL in adipose tissue

The protein expression of pHSL, the active form of the rate-limiting enzyme in adipose tissue lipolysis, was significantly reduced (25%; P<0.01) in the adipose of MetS+VA rats relative to control MetS rats (***Figure 4-3***).

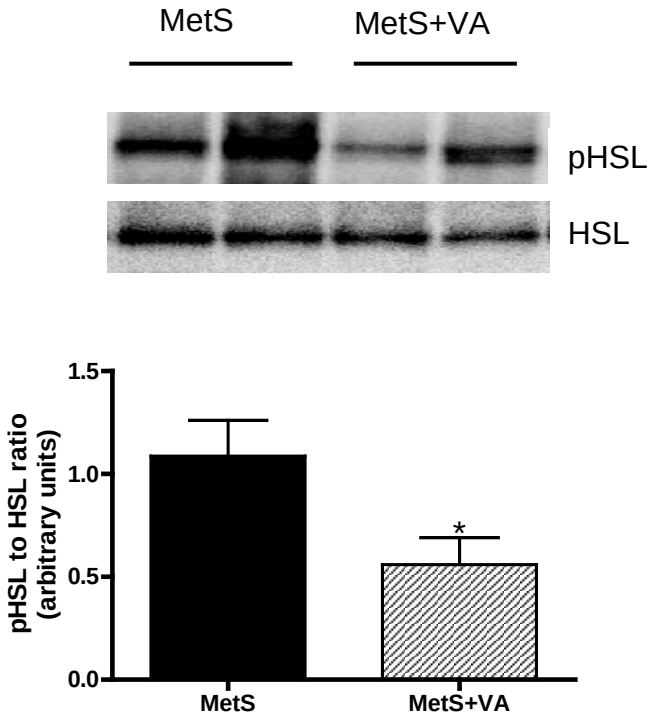


Figure 4-3 Protein abundance of the active form of HSL (pHSL) was assessed via immunoblot analysis. pHSL protein was measured in the adipose tissue of MetS and MetS+VA groups and expressed as a ratio of pHSL to total HSL. A representative blot and graph, presented as a measure of arbitrary units, are shown. Values are means, with standard errors (SEM) represented by vertical bars (n=5). Mean values of the MetS+VA group were significantly different from those of the control MetS group (*P<0.05) as assessed two-tailed, unpaired Student *t* tests.

4.3.3 Enrichment of dairy fat with VA increases energy expenditure

Total energy expenditure was measured by indirect calorimetry at the end of the study to determine whether decreased total fat mass by VA was associated with increased metabolic rate. Results confirmed increased total energy expenditure in MetS+VA rats relative to control MetS rats, as measured by oxygen consumption (***Figure 4-4***). This modest increase in oxygen consumption cannot be explained by lean mass which did not differ between MetS and MetS+VA rats (***Supp. Figure 4-2-Appendix 'B'***).

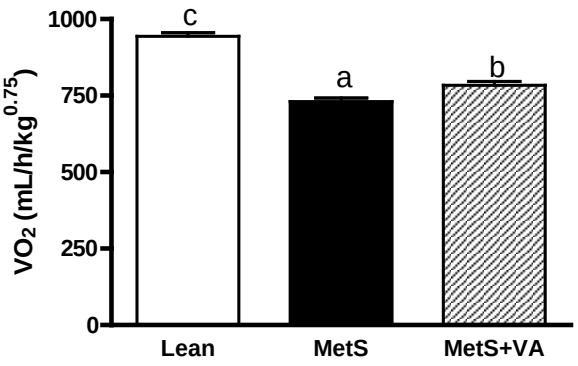
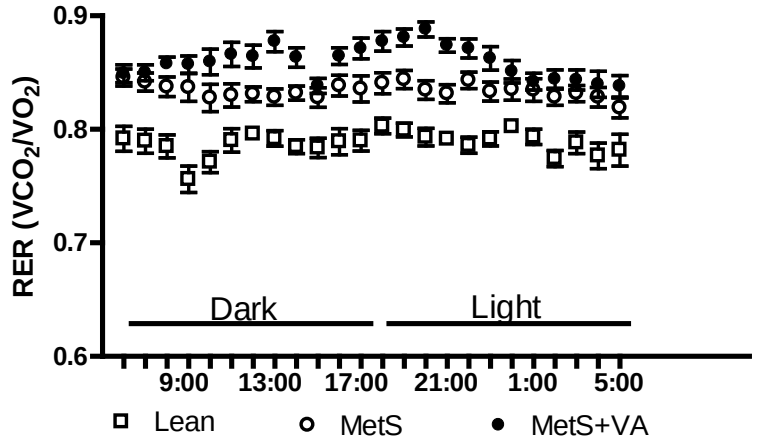


Figure 4-4 Oxygen consumption of lean (open squares), MetS (open circles) and MetS+VA rats (black circles). Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test.

Interestingly, increased metabolic rate in MetS+VA rats was concomitant with higher RER (***Figure 4-5***), indicating increased whole body glucose utilization over lipid as a substrate for oxidation for energy production. It is noteworthy that the indirect calorimetric analysis was performed in rats fed *ad-libitum,* suggesting increased fuel availability (mainly glucose) in the hyperphagic JCR:LA-*cp* rats. Since glucose oxidation is positively associated with glucose disposal rates (Galgani *et al*., 2008a), an increase in RER in MetS+VA rats relative to control MetS rats may be explained by an improvement in glucose uptake capacity.

A



B

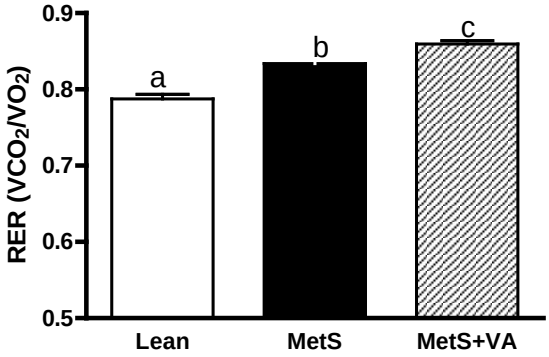


Figure 4-5 RER (Panel A) of lean (open squares), MetS (open circles) and MetS+VA rats (black circles) and means ±SEM over a 24 hr period (Panel B), n=8. Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test.

4.3.4 VA decreases fasting insulin and improves glucose response after a meal

Previous studies using the JCR:LA-*cp* rat have shown no effect of VA on insulin or glucose metabolism (Wang *et al*., 2008; Wang *et al*., 2009). In the present study, enrichment of dairy fat with VA in MetS rats resulted in a significant reduction in fasting insulin concentrations relative to CD ($P<0.001$) (***Figure 4-6A***). This was consistent with lower total insulin concentrations after the MTT ($P<0.05$), as determined by the analysis of total area under the curve (AUC) (***Figure 4-6B***).

Post-prandial incremental changes in plasma insulin concentrations (iAUC, $P>0.05$) (***Figure 4-6C***) or total glucose concentrations (AUC, $P>0.05$) (***Figure 4-7B***) did not differ between MetS or MetS+VA rats. However, MetS+VA rats had lower post-prandial glucose response relative to control MetS rats (iAUC, $P<0.05$) (***Figure 4-7A and 4-7C***). Notably, MetS+VA rats showed a lower HOMA-IR index relative to control MetS rats (***Figure 4-7D***) suggesting improved whole body insulin resistance.

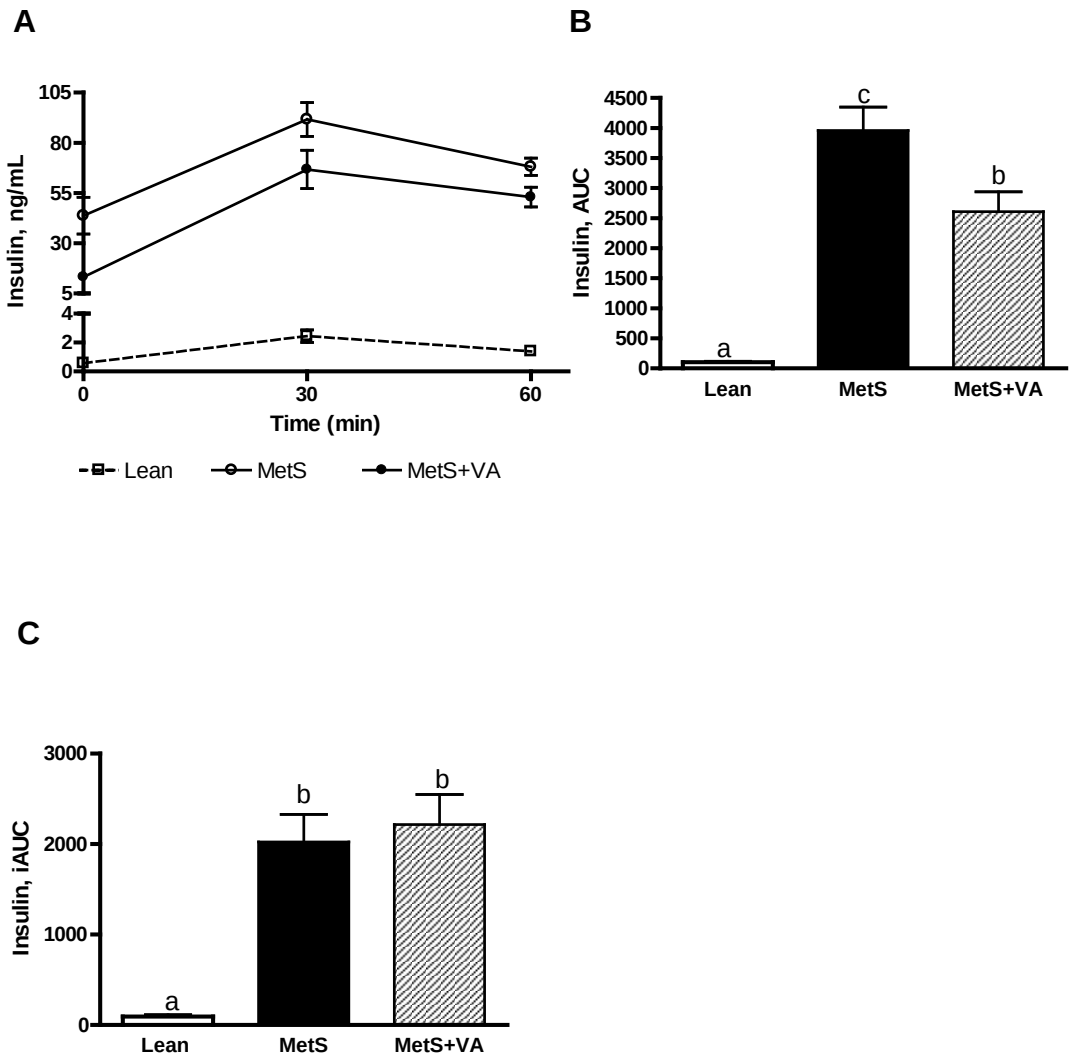


Figure 4-6 Post-prandial insulin (Panel A) response after a MTT of lean (open squares), MetS (open circles) and MetS+VA rats (black circles) and the corresponding AUC (Panel B) and iAUC (Panel C). MetS+VA rats had lower Bfasting insulin concentrations (Panel A; $p<0.001$). Values are means, with standard errors (SEM) represented by vertical bars (n=5). Means without a common letter differ ($P<0.05$) as assessed by one-way ANOVA followed by Tukey's post hoc test.

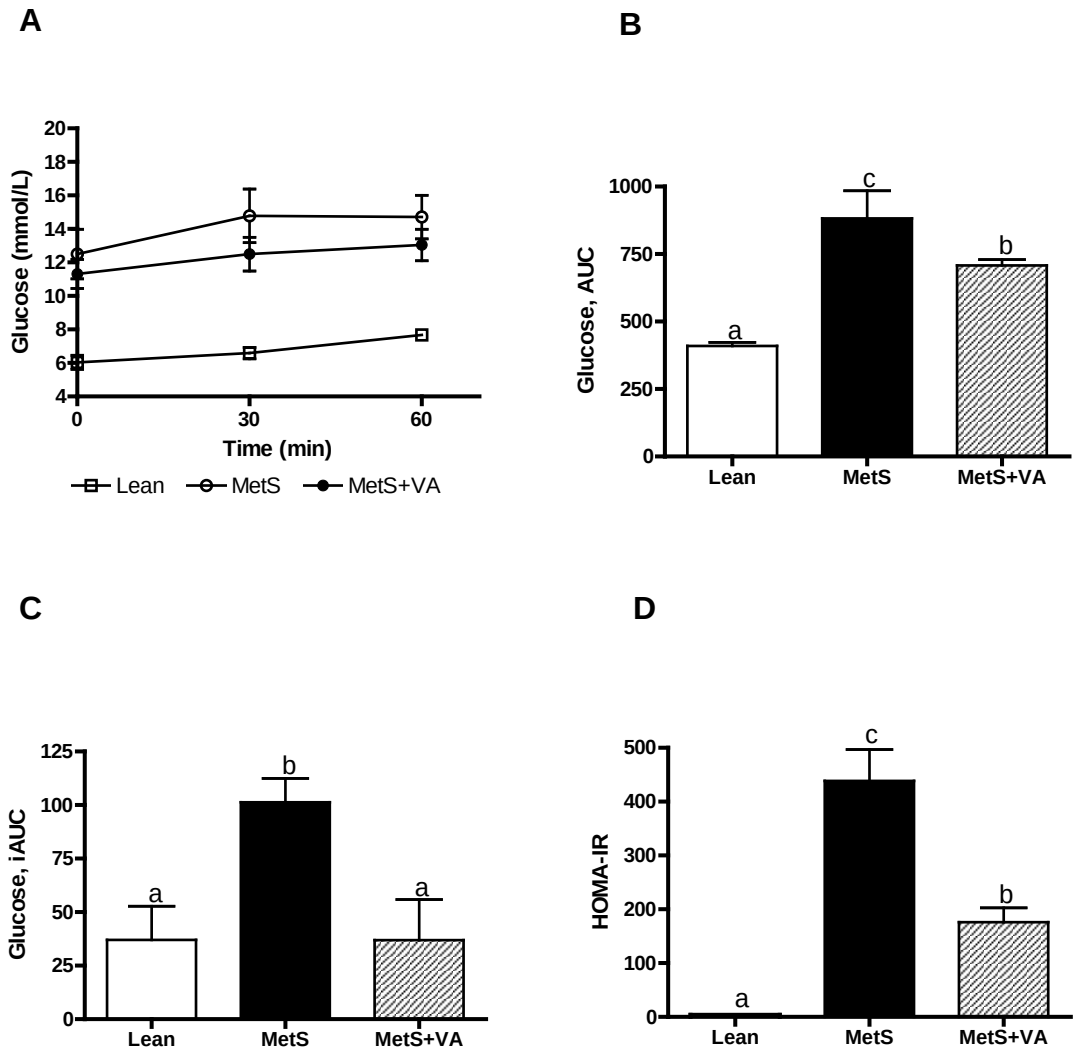


Figure 4-7 Post-prandial glucose (Panel A) response after a MTT of lean (open squares), MetS (open circles) and MetS+VA rats (black circles) and the corresponding AUC (Panel B) and iAUC (Panel C). The HOMA-IR index (Panel D) was calculated as fasting insulin (µU/mL) x fasting glucose concentrations (mg/dL)/405. Values are means, with standard errors (SEM) represented by vertical bars (n=5). Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test.

4.3.5 VA attenuates the progression of NAFLD

Enrichment of dairy fat with VA prevented the hepatic accumulation of lipids, an effect that was clearly evident in the hematoxylin and eosin stained histological sections (***Figure 4-8A***). Histopathological analysis revealed that MetS+VA rats had decreased steatosis (42%, P<0.01) and NAFLD activity scores (34%, P<0.01) as compared to control MetS rats (***Figures 4-8B and 4-8C***). There was no significant difference (P>0.05) in inflammation or hepatocellular ballooning between MetS and MetS+VA rats (Data not shown).

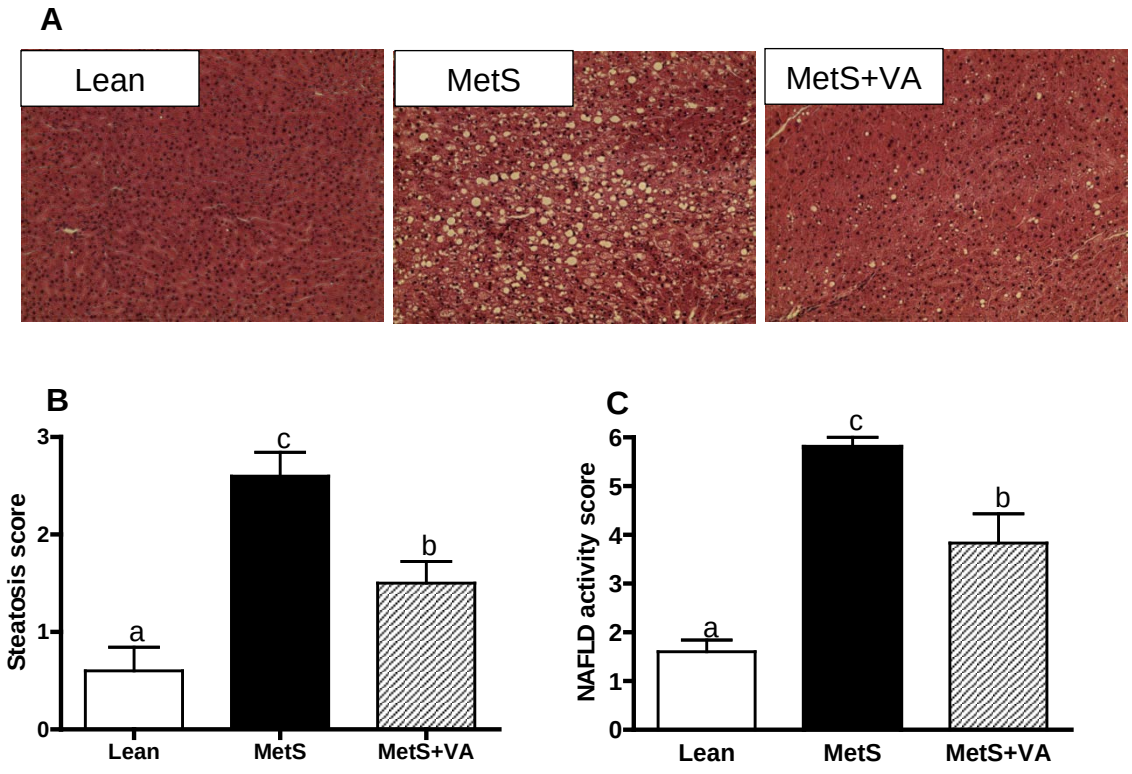


Figure 4-8 Progression of NAFLD in lean, MetS and MetS+VA rats. Liver sections were stained with H&E and histopathologically graded for B) steatosis and C) NAFLD activity scores; A) Original magnification 20 X. Values are means ±SEM, n=5. Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test.

4.3.6 VA affects intestinal and hepatic lipid deposition, but has a unique opposing effect on the regulation of mRNA expression for select genes

The effect of VA on reducing lipid accumulation in peripheral tissues was confirmed by quantitative analysis of intestinal and hepatic lipids. Results revealed that VA decreased intestinal TG to the level of lean rats ($P>0.05$) and by 43% ($P<0.001$) relative to control MetS rats. Hepatic TG and CE were also decreased in MetS+VA rats (68 and 76.5%, respectively, $P<0.001$) relative to control MetS rats (***Table 1***).

Table 4-1 Intestinal and hepatic lipid composition

	Lean	MetS	MetS+VA
Intestinal TG (µg/g protein)	93.2 ± 16.2^{a}	193.2 ± 43.8^{b}	109.7 ± 7.7^{a}
Intestinal CE (µg/g protein)	15.2 ± 0.8	12.6 ± 1.4	16.2 ± 1.1
Hepatic TG (µg/g protein)	47.5 ± 4.0^{a}	385.8 ± 44.3^{c}	123.3 ± 24.8^{b}
Hepatic CE (µg/g protein)	29.0 ± 3.7^{a}	145.3 ± 23.1^{b}	34.1 ± 2.1^{a}

Values are means ± SEM, n=8. Means without a common letter differ ($P<0.05$). Tissues from lean, MetS and Met+VA were collected after an overnight fasting (16 hr); TG, triglyceride; CE, cholesteryl ester.

Gene expression analysis demonstrated opposing transcriptional regulation by VA in the intestine and the liver (***Figure 4-9A and 4-9B***). VA was observed to down-regulate the expression of genes involved in *de novo* lipogenesis, SREBP1 and its downstream target FAS in the intestine but not in the liver, relative to CD ($P<0.05$). The mRNA expression of genes involved in intestinal/hepatic fatty acid oxidation and transport were not affected by the dietary treatment (***Supp. Table 4-3 and 4-4- Appendix 'B'***), except UCP2, which was decreased in the intestine and I-FABP which was increased in the liver of MetS+VA relative to control MetS rats. With regard to the mRNA expression of genes involved in TG synthesis and secretion, VA was observed to up-regulate DGAT2 in the liver relative to CD ($P<0.05$). Interestingly, VA down-regulated the mRNA expression of apoA1, the main apolipoprotein constituent of HDL in the intestine while up-regulating its expression in the liver as compared to CD ($P<0.05$). Conversely, the expression of SRB1, the receptor that mediates HDL-dependent cholesterol efflux was higher in the liver and the intestine of MetS+VA relative to control MetS rats ($P<0.05$).

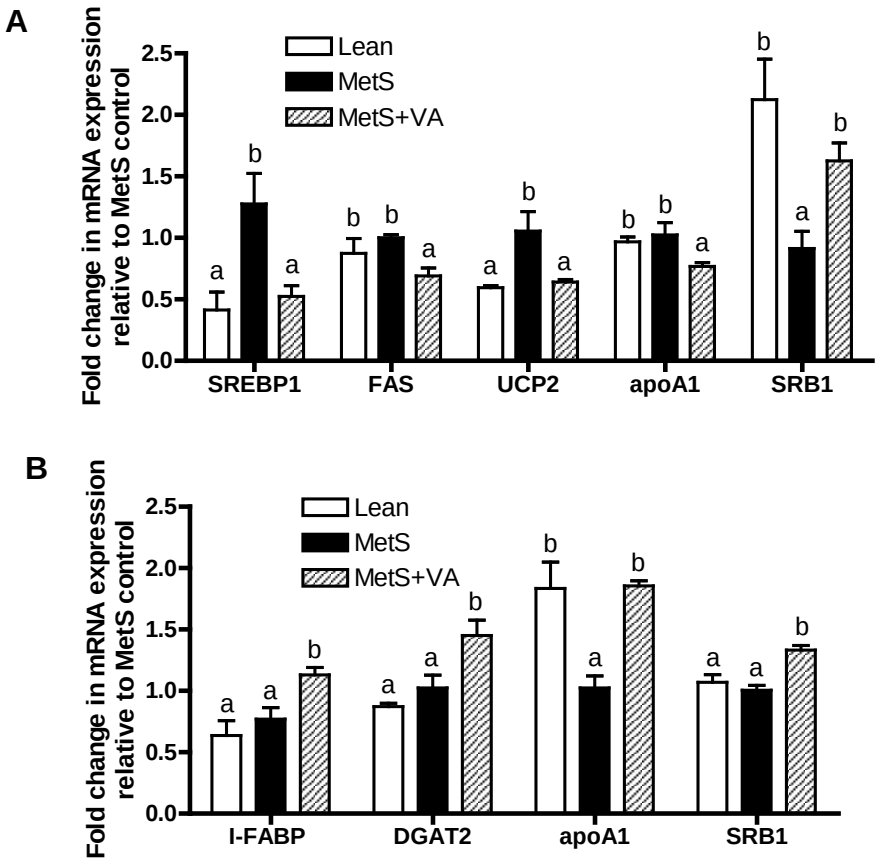


Figure 4-9 Intestinal (Panel A) and hepatic (Panel B) mRNA expression of genes involved in lipid synthesis, oxidation and transport that were regulated by VA. Values are means ±SEM, n=5. Means without a common letter differ (P<0.05) as assessed by one-way ANOVA followed by Tukey's post hoc test.

4.3.7 Enrichment of dairy fat with VA significantly reduces hepatic and intestinal TG secretion

Results from the functional secretion studies revealed that MetS+VA rats had a decreased hepatic (27% lower) and intestinal (39% lower) TG secretion relative to control MetS rats at time 4 hr post-injection of the lipase inhibitor P-407 (P<0.05). Interestingly, MetS+VA rats had similar (P>0.05) intestinal TG secretion relative to lean rats (***Figure 410A and 4-10B***).

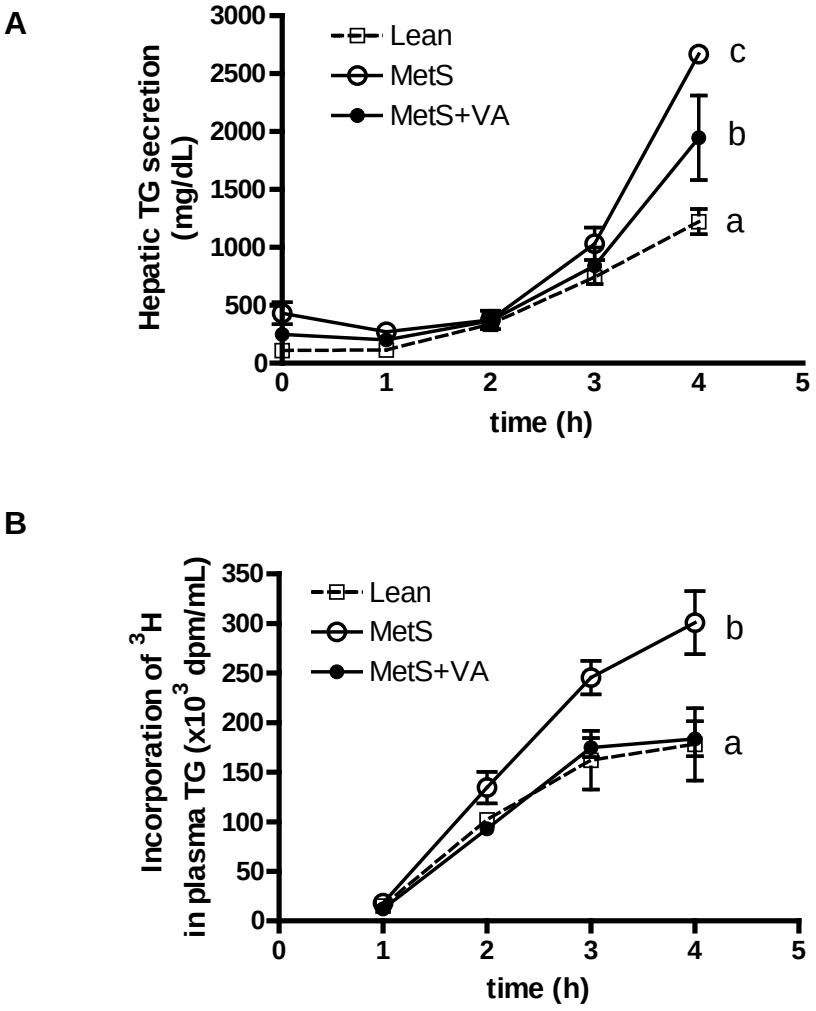


Figure 4-10 TG secretion after injection of the lipase inhibitor (P-407). Differences between groups in the TG secretion *in vivo* study were analyzed using two-way ANOVA followed by Tukey's post hoc test. A) Hepatic TG secretion was assessed during fasting conditions. Means of MetS (open circles) *versus* MetS+VA (black circles) were significantly different at 4 hr after P-407 injection ($P<0.05$); B) Intestinal TG secretion was assessed after an oral gavage of [^{3}H] triolein in olive oil following lipase inhibition. Mean values of MetS and MetS+VA rats differ at 4 hr after gavage ($P<0.05$). Means of MetS+VA *versus* lean rats (open squares) did not differ ($p>0.05$). Values in the TG secretion *in vivo* study are means ±SEM, n=3. Means without a common letter differ ($P<0.05$).

4.4 Discussion

4.4.1 Improved insulin sensitivity by VA may be associated with body fat re-distribution and decreased ectopic fat accumulation

The lipid centric hypothesis of obesity proposes that insulin resistance is causally related to peripheral ectopic fat accumulation and subsequent lipid toxicity (Galgani *et al*., 2008b). Longstanding evidence also suggests that dietary SFA can cause insulin resistance via pleiotropic mechanisms (Reviewed by Kien, 2009; Sacks and Katan, 2002). However, results from the present study demonstrate that VA can favorably attenuate dairy derived SFA-induced whole body insulin resistance in the JCR:LA-*cp* rat (***Figure 4-6 and Figure 4-7***). Improved whole body insulin resistance by VA was concomitant with an increase in RER (***Figure 4-5***), suggesting an improved (whole body) capacity to match fuel oxidation to fuel availability, a condition that is blunted in insulin resistant and type 2 diabetic subjects (Galgani *et al*., 2008a; Galgani *et al*., 2008b). Furthermore, enrichment of dairy fat with VA also decreased the active form of HSL in adipose tissue (***Figure 4-3***), a known effect of insulin action (Langin *et al*., 1996). Therefore, it is plausible that an increase in insulin sensitivity by VA prevents activation and subsequent lipolytic activity of HSL. We hypothesize that these insulin-sensitizing effects of VA may be associated with its potential to bind and activate PPARγ regulated pathways in adipose tissue. Indeed, we have recently demonstrated that VA has similar affinity to PPARγ relative to the insulin sensitizer pioglitazone (Wang *et al*., 2012), a thiazolidinedione class drug that exerts its effects via PPARγ activated pathways in adipose tissue. Consistent with findings from this study, Mohankumar *et al*., (2012) have recently shown that VA reduces adipocyte size in *fa/fa* Zucker rats. Therefore, it is plausible that activation of PPARγ by VA preferentially in inguinal adipose tissue may ameliorate adipose lipid storage capacity, thereby partitioning lipids from other insulin sensitive tissues (such as the liver).

4.4.2 Enrichment of dairy fat with VA decreases hepatic and intestinal lipid accumulation and results in opposing transcriptional control in the liver and the intestine

VA has recently been shown to reduce fasting and post-prandial triglyceridemia and to lower hepatic TG (Wang *et al*., 2008; Wang *et al*., 2009; Chaper 3). The lipid lowering properties of VA have been recently attributed to its potential in functionally activating both PPARα and PPARγ regulated pathways (Wang *et al*., 2012); however, putative benefits to insulin resistance and mechanisms of energy use and storage have not been clear. In this study we have shown for the first time the beneficial effects of VA on the progression of NAFLD. Specifically, we have demonstrated that enrichment of dairy fat with VA alleviates the putative stimulatory effects of SFA on hepatic steatosis and NAFLD progression (***Figure 4-8***). Considering that free fatty acids derived from adipose tissue are the main contributors to excessive hepatic lipid accumulation (Donnelly *et al*., 2005), it is plausible that the action of VA in adipose tissue via PPARγ may alleviate NAFLD under these dietary conditions.

Hepatic steatosis can also occur as an imbalance between lipid availability (lipid synthesis) and disposal (oxidation of fatty acids or VLDL secretion) (Ferré *et al*., 2010). We hypothesized that VA may further exert its effects by regulating endogenous synthesis and/or oxidative pathways. However, gene expression analysis revealed that reduced hepatic lipid accumulation by VA could not be explained at the transcriptional level (***Figure 4-9, Supp. Table 4-4- Appendix 'B'***). With the exception of the upregulation of hepatic PPARα targets, apoA1 and SRB1 (Jay and Ren, 2007; Duval *et al*., 2007), our data suggests that reduced hepatic lipid accumulation by VA is independent of PPARα-mediated lipid oxidation. Interestingly, previous findings have demonstrated lower protein expression of hepatic lipogenic enzymes and upregulated hepatic citrate synthase activity in JCR:LA-*cp* rats fed VA (Wang *et al*., 2008) or VA/CLA (Chapter 3)

supplemented diets. We therefore propose that VA may regulate *de novo* lipid synthesis and fatty acid turnover at a functional level but this may result in a compensatory transcriptional response by the liver.

In contrast to our hepatic observations, decreased intestinal TG accumulation by VA can potentially be explained by a lower mRNA expression of SREBP-1 and its lipogenic target gene FAS (***Figure 4-9***). During insulin resistance, SREBP-1 is thought to remain insulin-responsive (Wu *et al*., 2012), and in the present study, VA improved whole body insulin resistance (decreased fasting insulin; ***Figure 4-6***) that may result in the reduced levels of intestinal SREBP-1 mRNA (***Figure 4-9***). Furthermore, VA also lowered intestinal mRNA expression of UCP2, a mitochondrial uncoupling protein that has also been shown to be induced by high-fat feeding (Schrauwen *et al*., 2001). Interestingly, in addition to its thermoregulatory uncoupling function, UCP2 has been demonstrated to attenuate oxidative damage (Deng *et al*., 2012; Collins *et al*., 2005) and its expression is up-regulated during conditions of oxidative stress (Echtay *et al*., 2002; Lee *et al*., 1999; Horimoto 2004). We speculate that normalization of intestinal UCP2 by VA to the level of healthy lean rats may be an adaptive response to lower high fat feeding-induced oxidative stress milieu.

4.4.3 Enrichment of dairy fat with VA significantly improves intestinal and hepatic TG secretion

VA has been shown to exert lipid-lowering effects without altering body fat distribution or insulin metabolism in previous studies (Wang *et al*., 2008; Wang *et al*., 2009; Tyburczy *et al*., 2009; Chaper 3). In this study we provide evidence that enrichment of dairy fat with VA improves insulin resistance and reduces TG secretion from both the liver and the intestine (***Figure 4-10***). Specifically, we have shown that VA normalizes intestinal TG secretion to the level of healthy lean control rats. These striking effects

of VA on intestinal lipid metabolism could possibly be due to decreased lipid synthesis (by the intestine) and/or intestinal TG accumulation. It is noteworthy that gene expression and intestinal lipid mass were measured in tissue collected after an overnight fast and this may not necessarily reflect the physiological status in response to a lipid load. However, cumulative evidence suggests that increased intestinal TG accumulation due to impaired intestinal *de novo* lipogenesis is associated with exacerbated lipoprotein synthesis resulting in post-prandial dyslipidemia (Zoltowska *et al*., 2003; Haidari *et al*., 2002; Federico *et al*., 2006). Therefore, the resulting TG peak in response to a meal is largely determined by the metabolic priming of the enterocyte during fasting conditions.

In conclusion, enrichment of dairy fat with VA alleviates exacerbated symptoms of the MetS and the pre-diabetic state in the JCR:LA-*cp* rat. Specifically, these results suggest that VA exerts pleiotropic effects on the intestinal and adipose lipid metabolism which may further beneficially remodel the liver during NAFLD. Collectively, findings from this study provide further insights into the beneficial effects of VA and suggest that enriching dairy products with VA (naturally or by fortification) may be used as an approach to increase health value of dairy-derived fats, especially to those individuals with MetS.

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Chapter 5 Metabolic effects of *trans*-11 vaccenic acid are associated with its ability to exert tissue specific regulation of endocannabinoids in the JCR:LA-*cp* rat

This chapter is presented in the format of manuscript that has been prepared for publication.

5.1 Introduction

Trans-11 18:1 (vaccenic acid or VA) is the most abundant ruminant-derived *trans* fatty acid (rTFA) in the food chain and has sparked major interest due to mandatory labeling of all *trans* fat on foods in North America. VA is also the precursor to endogenous synthesis of conjugated linoleic acid (CLA), the first rTFA to be recognized as having numerous health-related effects. Interestingly, a growing body of evidence from studies in animal models has suggested a novel bioactivity for VA independent of its conversion to CLA, specifically its ability to attenuate complications observed in the metabolic syndrome (MetS), including dyslipidemia, fatty liver disease and low-grade inflammation (Wang *et al*., 2008; Wang *et al*., 2009; Blewett *et al*., 2009; Chapter 4). It has been recently proposed that the lipid-lowering and anti-inflammatory effects of VA may be partially associated with its ability to ligand activate PPAR-γ regulated pathways (Wang *et al*., 2012; Jaudszus *et al*., 2012), particularly in adipose tissue (Chapter 4). In general, bioactive long chain fatty acids also act by modifying the composition of membrane phospholipids (PL) and potentially replace or interfere with the synthesis of PL-derived lipid signaling molecules including endocannabinoids (Calder, 2006; Banni and Di Marzo, 2010). However, the degree to which VA is incorporated into membrane phospholipids and potential effects on endocannabinoid pathways remains unknown.

Endocannabinoids (EC) are endogenous lipids that modulate appetite behavior and energy metabolism through activation of cananabinoid (CB) receptors in the brain and peripheral tissues. The most common EC include *N*-arachidonoylethanolamide (Anandamide, AEA) and 2-arachidonoylglycerol (2-AG), and these are derivatives of arachidonic acid (AA; C20:4, n-6) in PL and have been shown to be modulated in response to dietary polyunsaturated fatty acid (PUFA) intake (Berger *et al*., 2001; D'Asti *et al.*, 2010; Watanabe *et al*., 2003; Alvheim *et al.,* 2012). Given the pathophysiological role of an overactive endocannabinoid system (ECS) in obesity, hepatic steatosis and associated insulin resistance and dyslipidemia (Di Marzo *et al*., 2001; Osei-Hyiaman *et al*., 2005; Osei-Hyiaman *et al*., 2008), nutritional interventions that target the EC pathways and the biosynthetic precursor AA, could be used as potential therapeutic strategies.

A recent study in hypercholesterolemic subjects provided the first evidence that a dairy product naturally enriched with VA and CLA, decreases plasma concentrations of anandamide in a dose-dependent manner (Pintus *et al*., 2012). However, the direct effect of VA on EC biosynthesis was not able to be determined in this study. Furthermore, given the bioactive properties of VA to favourably modulate whole body energy metabolism (Chapters 3 and 4), we proposed to explore novel regulatory effects of VA on tissue EC as a potential mechanism of action for these metabolic effects. In order to address the specific role of dietary VA alone or in combination with CLA in EC metabolism we supplemented the diets of an established rodent model of MetS (the JCR:LA-*cp* rat) with these bioactive long chain fatty acids, and examined tissue concentrations of CB receptor ligands anandamide and 2-AG, the biosynthetic precursor AA in membrane PL, and the two bioactive non-CB receptor ligands and anandamide analogues, *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA).

5.2 Materials and Methods

5.2.1 Animals and diets

Rats of the JCR:LA-*cp* strain that are homozygous for the corpulent trait (*cp/cp*) have a complete absence of the leptin receptor in the plasma membrane and spontaneously develop symptoms associated with the MetS and the pre-diabetic state typically observed in humans; including obesity, insulin resistance and dyslipidemia (Russell and Proctor, 2006). Male JCR:LA-*cp* rats were raised in our established breeding colony at the University of Alberta as previously described (Vine *et al*., 2007). At 8 weeks (wk) of age, rats (n=5) were randomized and assigned to one of four diets (control and experimental diets) for 8 wk and had free access to food and water.

The control diet was prepared as described previously (Chapter 4). Experimental diets were prepared by adjusting the fatty acid composition (replacing oleic acid by either VA, CLA or VA+CLA) of the control diet to provide approximately 1% w/w of VA (VA), 1% w/w of *cis*-9, *trans*-11 CLA (CLA) or both 1% w/w of VA + 0.5% w/w of *cis*-9, *trans*-11 CLA (VA+CLA). Control and experimental diets were iso-caloric and had a constant PUFA to SFA ratio of 0.4 and a constant n6 to n3 PUFA ratio of 8. Purified VA was synthesized by chemical alkali isomerization from linoleic acid-rich vegetable oil (Swain, 1949). Semi-purified *cis*-9, *trans*-11 CLA (G-c9t11 80:20) containing 59.8% of *cis*-9, *trans*-11 CLA and 14.4% of *trans*-10, *cis*-12 CLA was kindly provided by Lipid Nutrition. Fatty acid composition of diets was confirmed by gas chromatograph analysis (Cruz-Hernandez *et al*., 2007) of the fat blend samples (***Table 5-1***). After sacrifice, samples of the hypothalamus, skeletal muscle, visceral adipose tissue (VAT), liver, and jejunal segments of the intestine were excised and snap frozen at -80°C until analysis. Animal care and experimental procedures were conducted in accordance with the Canadian Council on Animal care and

approved by the University of Alberta Animal Care and Use Committee-Livestock.

Table 5-1 Fatty acid composition of control and experimental diets[1]

Fatty acid (g/100 FAME*)	Dietary groups			
	Control	VA	CLA	VA+CLA
C12:0	1.0	1.2	1.2	2.1
C14:0	3.6	4.2	4.4	7.6
C14:1	0.3	0.4	0.4	0.7
C16:0	19.0	18.1	17.7	22.6
C16:1	1.2	1.0	1.0	1.1
C18:0	11.0	10.9	10.6	7.7
C18:1 *trans*-9	0.5	0.5	0.4	0.2
C18:1 *trans*-11 (VA)	1.3	8.8	1.0	8.8
C18:1 *cis*-9 (oleic acid)	35.6	27.9	26.3	17.3
C18:1 *cis*-11	1.9	1.4	1.3	0.8
C18:2 n6	13.7	13.9	13.6	12.7
C18:3 n3	1.7	1.7	1.7	1.7
*cis*9, *trans*11 CLA	0.2	0.3	8.8	4.1
Summaries				
∑SFA	37.5	37.6	37.3	45.2
∑C12:0, C14:0, C16:0	23.6	23.5	23.3	32.2
PUFA	15.6	15.8	15.4	14.4
*cis*MUFA	40.0	31.6	29.8	20.5
*trans*MUFA	4.2	11.4	3.0	10.7
n6	13.8	13.8	13.8	13.8
n3	1.7	1.7	1.7	1.7
n6:n3 ratio	7.9	7.9	7.9	7.9
PUFA:SFA ratio	0.4	0.4	0.4	0.3

[1]Values are expressed as percentage of fatty acids; *FAME, fatty acid methyl esters

5.2.2 Tissue lipid extraction

Tissues (0.2-0.3 g) were homogenized and total lipids extracted with chloroform/methanol (2:1, v/v) containing internal deuterated standards for anandamide (AEA), 2-AG, OEA and PEA to quantify for recovery efficiency ($[^2H]_8$-AEA, 20 ng/mL), ($[^2H]_5$-2-AG, 200 ng/mL), ($[^2H]_2$-OEA, 20 ng/mL) and ($[^2H]_4$-PEA, 20 ng/mL) (Cayman Chemical, MI, USA). This mixture was washed with 0.25 volume of 0.9% KCl according to the Folch procedure (Folch, 1957) to separate the phases. Samples were centrifuged and the lipid containing lower phase was transferred to clean tubes and evaporated to dryness under a stream of nitrogen at room temperature. After lipid extraction from tissue samples, lipid classes were separated by solid phase extraction using commercial silica cartridges Strata SI-1 (Phenomenex, Torrance, USA). Samples were reconstituted in 500 µL of chloroform, vortexed and loaded to the column following by washing with 10 mL of chloroform to elute neutral lipids. The fractions containing endocannabinoids were then eluted with 10 mL chloroform/methanol (9:1, v/v), evaporated to dryness under nitrogen and reconstituted in methanol until analysis by LC/MS. The fractions containing phospholipids (PL) were eluted with 10 mL methanol and stored at -20 ºC until further preparation for fatty acid analysis. Recovery of endocannabinoids in the chloroform/methanol (9:1, v/v) eluates was confirmed by LC/MS and estimated to be higher than 90%. Purity of the PL fraction was confirmed by thin layer chromatography (TLC) using heptane/isopropyl ether/acetic acid (60:40:4, by vol) as previously described (Lehner and Vance, 1999).

5.2.3 Analysis of endocannabinoids and ananadamide analogues

Samples were analyzed by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) using an Agilent 1200 series HPLC coupled to a 3200 QTRAP mass spectrometer (AB

SCIEX; Concord, ON, Canada). LC separation was performed through an Ascentis Express C18 column (7.5 cm x 2.1 mm, 2.7μm particle size) at a flow rate of 300 mL/min. Two mobile phases were used: A) methanol with 0.2% formic acid and B) 50 mM ammonium formate, pH 3. The gradient elution method started at 85% A from 0-0.1 min; then the mobile phase A linearly increased to 95% from 0.1-2 min and held for 2 additional min (2.1-4 min). Then, the mobile phase returned to 85% A and held at this composition for 6 min equilibrium time prior to the next injection. The mass spectrometer was operated in the multiple reaction monitoring (MRM) scan mode under positive ionization. Nitrogen was used as curtain gas, drying and nebulizing gas. Anandamide, 2-AG, OEA and PEA in their protonated forms $[M+H]^+$ were identified as peaks with the appropriate *m/z* values and quantified by comparison with its external synthetic standards ran under the same conditions. The MRM transitions monitored were as follows: AEA *m/z* 348→62; AEA-d8 *m/z* 356→62; 2-AG *m/z* 379→287; 2-AG-d5 *m/z* 384→287; PEA *m/z* 300→62; PEA-d4 *m/z* 304→62; OEA *m/z* 326→62; OEA-d2 *m/z* 328→62 or 310. The linear range for calibration curves were 5 to 500 ng/mL for AEA, PEA and OEA and 0.1 to 10 μg/mL for 2-AG. Since 2-AG and 1-AG undergo rapid isomerization (Zoerner *et al*., 2011), results for 2-AG were reported as the sum of the individual peaks of 2-AG + 1-AG.

5.2.4 Fatty acid analysis in PL

PL fractions were transesterified using 0.5 N methanolic base (metallic sodium in methanol), (Sigma-Aldrich) at 80°C for 15 min forming fatty acid methyl esters (FAME). The FAME were flushed with N_2 and stored at −35°C until analysis and fatty acids were separated by GC/FID (Varian 3900, Mississauga, ON) using a 100-m CP-Sil 88 fused-silica capillary column (100 m x 0.25 mm i.d. x 0.2 μm film thickness; Varian Inc., Mississauga, ON) as previously described (Cruz-Hernandez *et al*., 2007). The FAME were identified by comparison with retention times of

commercial GC reference FAME standards (FAME mix #463 and CLA FAME #UC-59M) from Nu-Chek Prep Inc.

A detailed description of the procedures used in these two Sections is included in ***Appendix 'C'.***

5.2.5 Measurement of fatty acid amide hydrolase (FAAH) protein expression in the jejunum

Proteins from jejunal mucosa homogenates were separated by SDS-PAGE electrophoresis on Tris-acetate polyacrylamide gels (3–8%, InVitrogen), transferred to a PVF membrane and incubated with anti-FAAH1 rabbit polyclonal (1:1000; catalogue no. 9179; Cell Signaling, Technology®) and anti-β-actin mouse polyclonal (1:5000; catalogue no. ab8226; Abcam®, St Louis, MO, USA) antibodies as previously described (Lu *et al*., 2011). Detection was achieved using anti-rabbit and anti-murine secondary antibodies and the ECL advance detection system (Amersham Biosciences). Results are expressed as a ratio of target protein:β-actin protein.

5.2.6 Measurement of pro-inflammatory genes in the jejunum

Total RNA was isolated from frozen segments of jejunal mucosa using TRIzol® (Invitrogen, Canada) as described in the manufacturer's protocol and reversed transcribed into complementary DNA using MultiScribe Reverse transcriptase (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). The expression of CB_1, FAAH, and the pro-inflammatory cytokines; tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) relative to the housekeeping gene *Actb* (β-actin) was assessed by quantitative real-time PCR, using the StepOne™ Plus Real-Time PCR system (Applied Biosystems) and StepOne™ Software (version 2). PCR contained complementary DNA template, 100 nM of commercially available, pre-mixed target-specific

primers and Taqman® FAM™-labelled probe (Applied Biosystems) for CB_1, FAAH, TNF-α, IL-1β and *Actb*. Thermal cycling conditions were as follows: 95°C for 20 s, followed by forty cycles of 95°C for 1 s and 60°C for 20 s. Relative mRNA expression for each target gene was normalized to *Actb* mRNA and quantified using the comparative cycle threshold (Ct) method. Data was expressed as ratio of target mRNA expression relative to β-actin. All assays were performed in triplicate.

5.2.7 Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons between dietary groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. The level of significance was set at $P<0.05$ (Graph Pad Prism 5.0, USA).

5.3 Results

5.3.1 Dietary supplementation with VA alone or in combination with CLA decreases the concentration of 2-AG and anandamide analogues (OEA and PEA) in the liver and VAT

Supplementation with VA, CLA or VA+CLA for 8 wk significantly reduced 2-AG concentrations in the liver (81%, $P<0.001$; 49%, $P<0.05$ and 71%, $P<0.001$, respectively) relative to control diet. Interestingly, VA or VA+CLA (but not CLA) also resulted in decreased ($P<0.001$) concentrations of OEA (to not detectable levels) and PEA (-57% and -56%, respectively) in this tissue as compared to control (***Figure 5-1***).

VA and VA+CLA (but not CLA) significantly ($P<0.05$) reduced the concentrations of 2-AG (-86% and -87%, respectively) and OEA (-59%, $P<0.05$ and -74%, $P<0.01$, respectively) in VAT as compared to control ***(Figure 5-2***).

In contrast, dietary supplementation with VA, CLA or VA+CLA did not affect EC concentrations (anandamide and 2-AG) in the hypothalamus (***Figure 5-3***) or skeletal muscle (***Figure 5-4***) relative to control diet (P>0.05). All dietary treatments (VA, CLA and VA+CLA) significantly reduced (P<0.01) the concentrations of OEA (-20%, -19% and -17%, respectively) while only VA reduced the concentration of PEA (21%, P<0.05) in the skeletal muscle relative to control ***(Figure 5-4).*** Collectively, results from this study suggest that there were no additive effects of combining VA with CLA on reducing EC concentrations in liver and VAT.

5.3.2 Supplementation with VA increases the concentration of anandamide and anandamide analogues (OEA and PEA) in the jejunum

Dietary supplementation with VA (but not CLA or VA+CLA) significantly increased jejunal concentrations of the EC anandamide (3.8 fold, P<0.05) and its analogues OEA (1.7 fold, P<0.05) and PEA (1.9 fold, P<0.01) (***Figure 5-5***). It is important to mention that dietary VA has not been reported to alter food intake in previous studies using different animal models (Wang *et al*., 2008; Wang *et al*., 2009; Mohankumar *et al*., 2012; Tyburczy *et a*l., 2009). Therefore, it is unlikely that increased anandamide concentrations in the jejunum (following VA treatment) would result in an appetite stimulatory effect as a result of CB receptor activation by anandamide in the intestine.

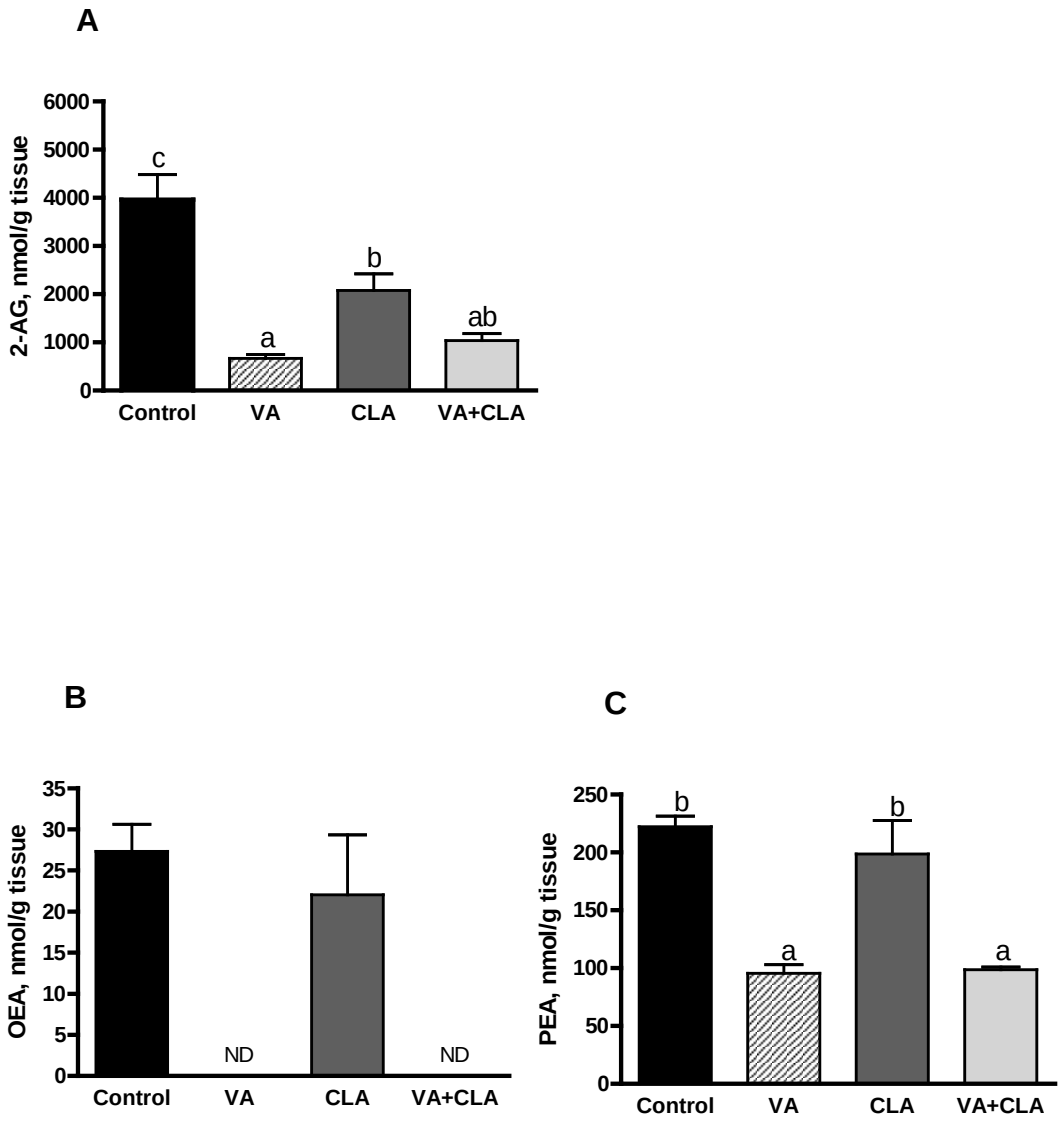

Figure 5-1 Endocannabinoid and *N*-acyl ethanolamide concentrations in the liver of JCR:LA-*cp* rats following dietary supplementation with VA, CLA or VA+CLA. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ ($p<0.05$). ND, not detectable.

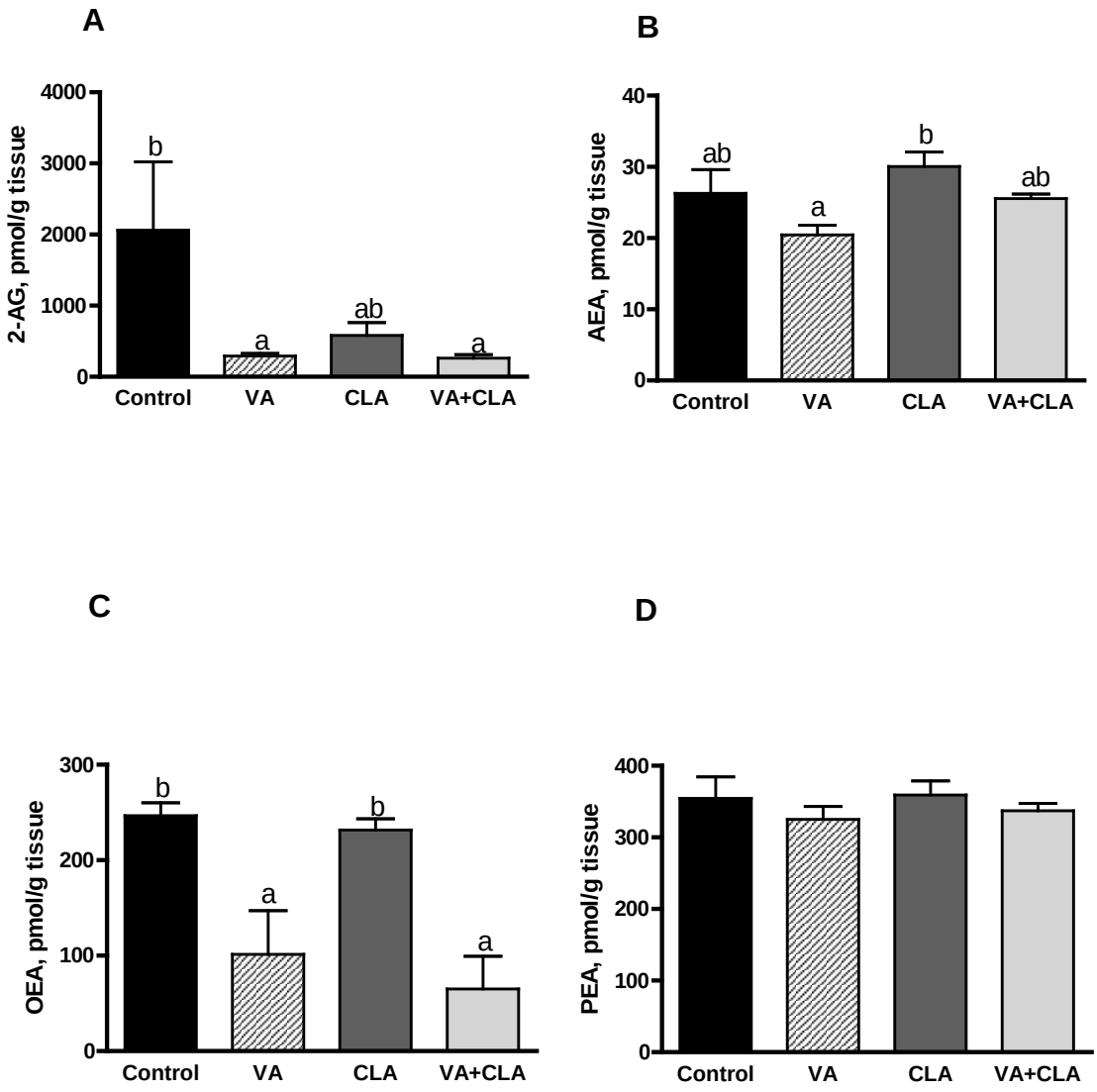

Figure 5-2 Endocannabinoid and *N*-acyl ethanolamide concentrations in VAT of JCR:LA-*cp* rats following dietary supplementation with VA, CLA or VA+CLA. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ ($p<0.05$).

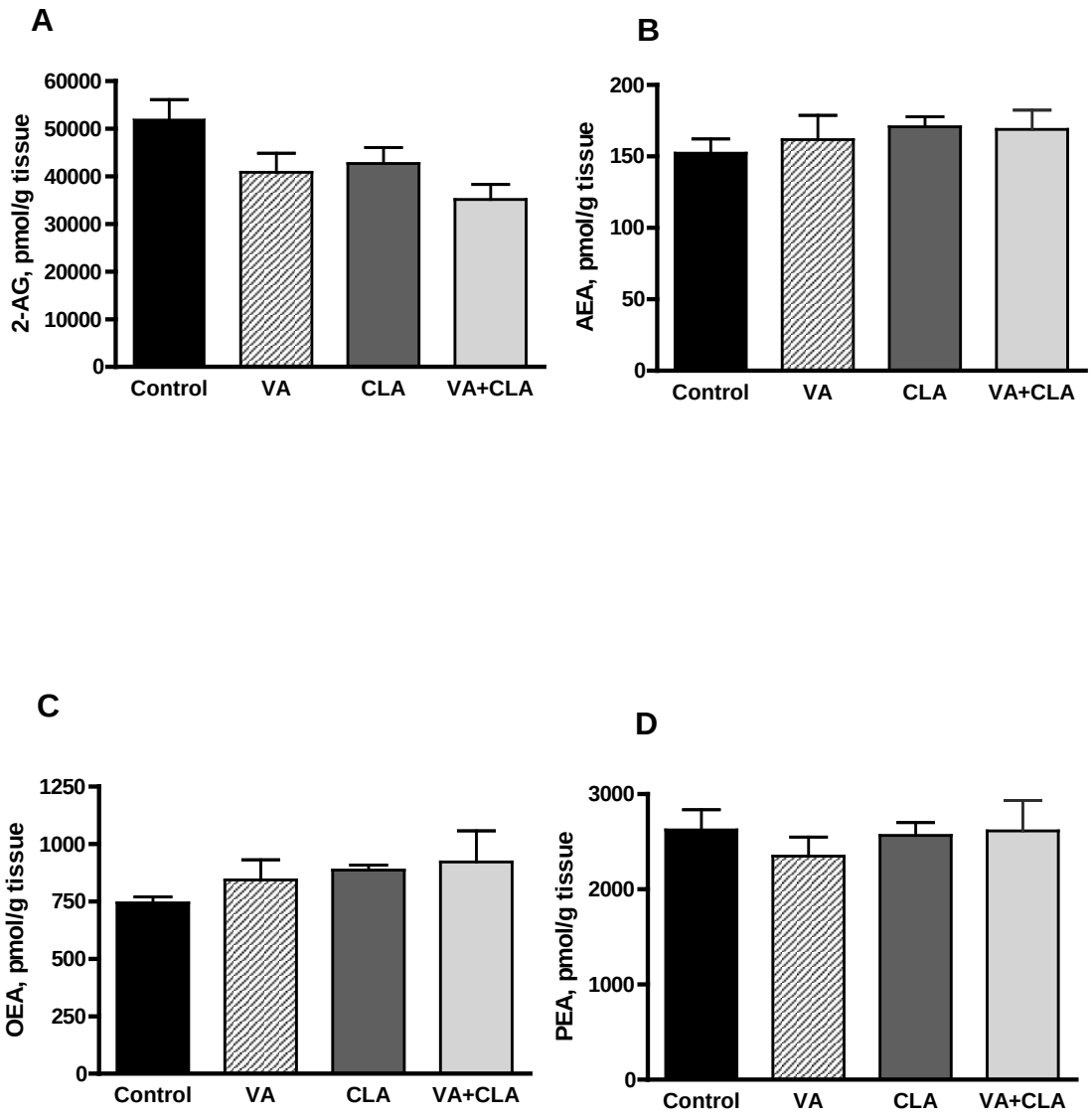

Figure 5-3 Endocannabinoid and *N*-acyl ethanolamide concentrations in the hypothalamus of JCR:LA-*cp* rats following dietary supplementation with VA, CLA or VA+CLA. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ ($p<0.05$).

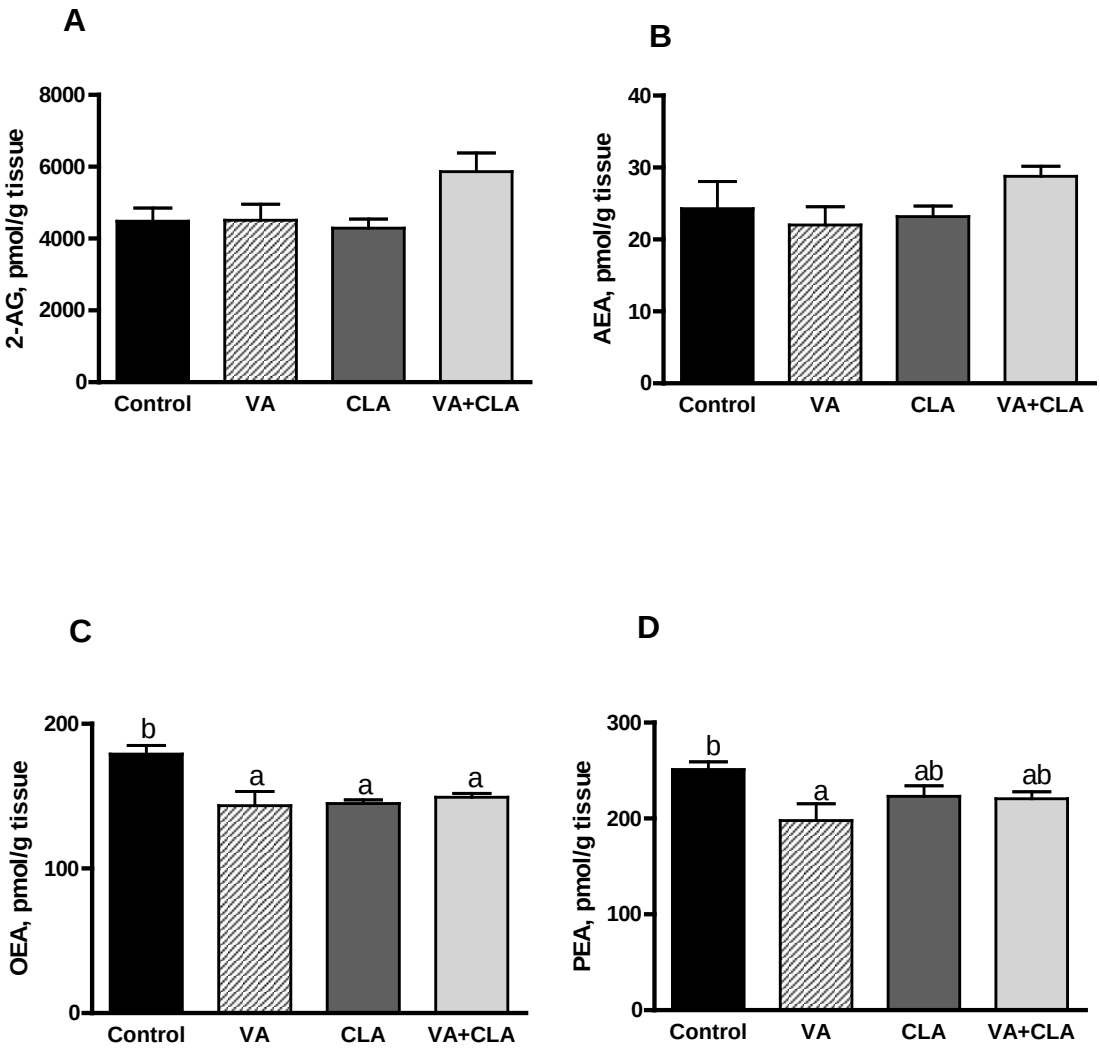

Figure 5-4 Endocannabinoid and *N*-acyl ethanolamide concentrations in the skeletal muscle of JCR:LA-*cp* rats following dietary supplementation with VA, CLA or VA+CLA. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ ($p<0.05$).

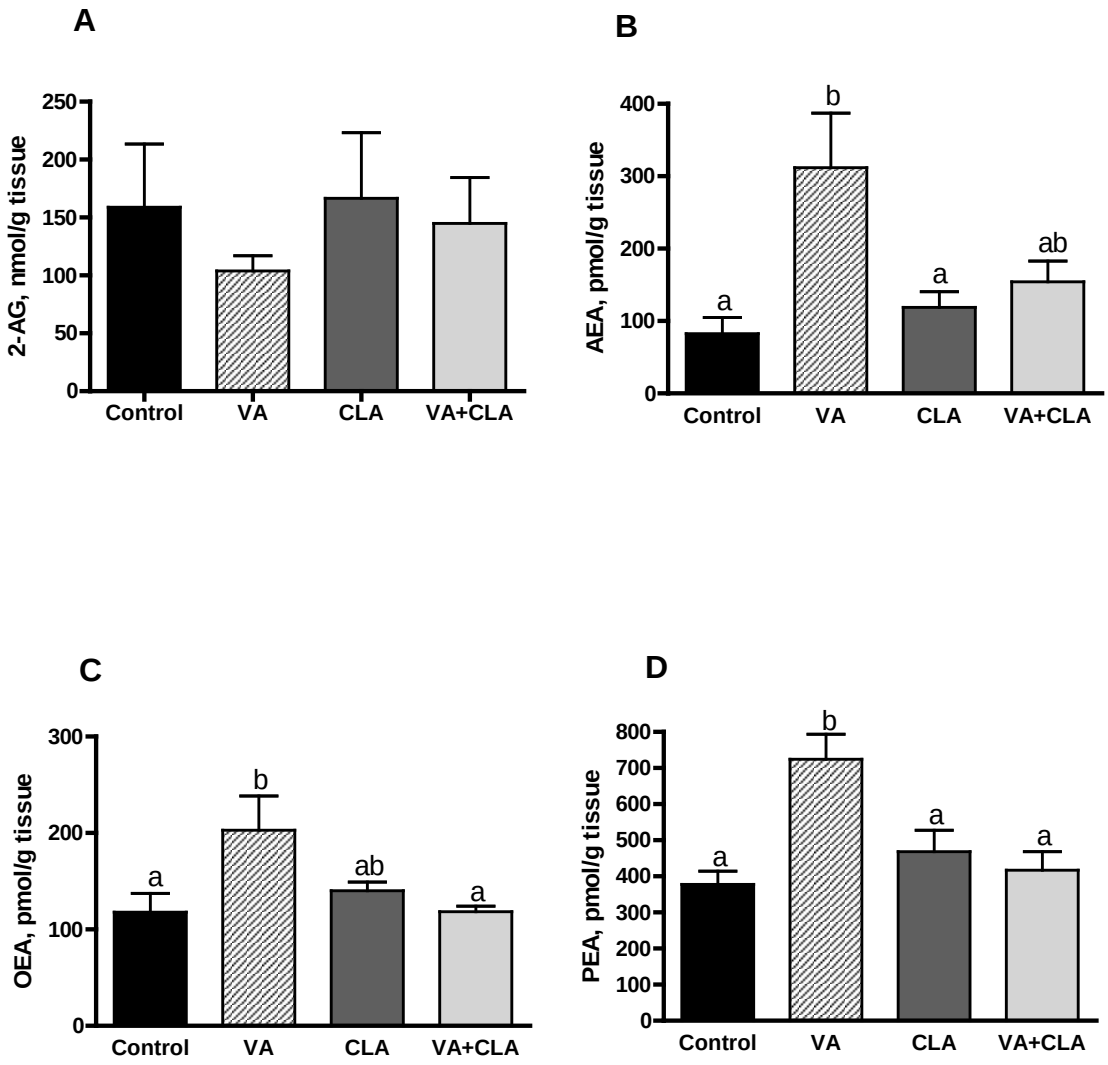

Figure 5-5 Endocannabinoid and *N*-acyl ethanolamide concentrations in the jejunum of JCR:LA-*cp* rats following dietary supplementation with VA, CLA or VA+CLA. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ ($p<0.05$).

5.3.3 Effects of dietary supplementation with VA, CLA or their combination on tissue PL fatty acid composition

We analyzed tissue PL fatty acid composition to determine whether changes in tissue EC (anandamide and 2-AG) and N-acyl ethanolamides (OEA and PEA) were due to alterations in the availability of their biosynthetic precursors in membrane PL (***Table 5-2***). Interestingly, we found that VA supplementation increased the EC precursor, AA in liver (30%, $P<0.001$) and skeletal muscle (11%, $P<0.05$) PL compared to control diet. VA+CLA fed-rats also had increased amounts of AA (20%) in liver PL relative to control rats ($P<0.01$). The incorporation of AA in VAT, jejunal and hypothalamus PL was not affected by any experimental diet (VA, CLA or VA+CLA) relative to control ($P>0.05$). VA and VA+CLA fed rats had lower concentrations of the OEA precursor, oleic acid (-35% and -40%, respectively) while VA-fed rats only had lower amounts of the PEA precursor, palmitic acid (-21%) in liver PL relative to control rats ($P<0.001$). CLA and VA+CLA fed rats had lower ($P<0.001$) amounts of oleic acid (-15% and -17%, respectively) in VAT PL, while only VA+CLA rats had lower concentrations of oleic acid (-33%, $P<0.01$) in jejunal PL relative to control rats. Collectively, findings from fatty acid analysis in tissue PL suggest that the regulatory effect of VA on tissue EC could not be explained by changes in their biosynthetic membrane PL precursor (AA).

We also looked at the PL incorporation of VA and CLA in all tissues analyzed. VA and VA+CLA fed-rats had higher ($P<0.001$) concentrations of VA in skeletal muscle (12 fold), liver (3.2 fold), VAT (4.8 fold) and jejunal (3.8 fold) PL compared with rats fed the control diet. However, VA was not detected in the hypothalamus irrespective of dietary treatment. Consistent with previous reports (Santora *et al*., 2000), CLA was not detected in tissues of VA-fed rats, suggesting limited incorporation of CLA (produced from VA desaturation) into tissue PL. Furthermore, CLA was

only incorporated in the liver, VAT and jejunal PL of CLA fed-rats and to a lesser extent in the liver and VAT PL of rats fed the VA+CLA diet ($P<0.05$).

5.3.4 The magnitude of incorporation of VA in membrane PL is tissue-dependent

To associate the tissue-specific effects of VA with its magnitude of incorporation, we conducted a comparison of VA in tissue membrane PL (***Figure 5-6***). VA was efficiently incorporated in VAT (1.8 g/100 g fatty acids), followed by the liver (1.6 g/100 g fatty acids), jejunum (1.5 g/100 g fatty acids) and skeletal muscle (1.5 g/100 g fatty acids). VA was not detected in hypothalamic membrane PL.

Table 5-2 PL fatty acid composition in tissues of JCR:LA-cp rats fed control or experimental diets for 8 wk**.**

Fatty acids (g/100 g total fatty acids)	Control		VA		CLA		VA+CLA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver								
16:0	16.4[b]	±0.7	12.9[a]	±0.3	15.7[b]	±0.4	14.6[ab]	±0.5
*cis*9 18:1 (OA)	6.0[b]	±0.3	3.9[a]	±0.1	5.2[b]	±0.2	3.6[a]	±0.1
*trans*11-VA	0.2[a]	±0.1	1.6[c]	±0.0	0.7[b]	±0.0	1.6[c]	±0.1
*cis*9, *trans*11-CLA	ND	—	ND	—	0.6[b]	±0.0	0.3[a]	±0.0
20:4 *n*6 (AA)	19.7[a]	±0.7	25.2[b]	±0.5	18.5[a]	±0.7	24.2[b]	±0.6
VAT								
16:0	10.8	±1.2	9.5	±0.1	11.3	±1.3	10.3	±0.1
*cis*9 18:1 (OA)	11.8[b]	±0.3	11.6[b]	±0.2	10.0[a]	±0.2	9.8[a]	±0.2
*trans*11-VA	0.4[a]	±0.1	1.8[c]	±0.0	0.7[b]	±0.1	1.9[c]	±0.0
*cis*9, *trans*11-CLA	ND	—	ND	—	0.8[b]	±0.1	0.6[a]	±0.0
20:4 *n*6 (AA)	15.0	±1.3	13.9	±0.2	14.5	±0.9	14.1	±0.3
Hypothalamus								
16:0	17.6	±0.3	17.1	±0.3	17.2	±0.4	18.2	±0.4
*cis*9 18:1 (OA)	21.4	±0.3	21.9	±0.2	21.0	±0.7	20.7	±0.6
*trans*11-VA)	ND	—	ND	—	ND	—	ND	—
*cis*9, *trans*11-CLA	ND	—	ND	—	ND	—	ND	—
20:4 *n*6 (AA)	9.9	±0.2	9.6	±0.2	10.2	±0.5	10.3	±0.6
Muscle								
16:0	19.8	±1.9	21.3	±0.7	21.0	±0.6	17.1	±0.8
*cis*9 18:1 (OA)	5.3	±0.4	5.3	±0.4	5.7	±0.4	4.6	±0.4
*trans*11-VA	0.1[a]	±0.1	1.0[b]	±0.0	0.1[a]	±0.1	1.2[b]	±0.1
*cis*9, *trans*11-CLA	ND	—	ND	—	ND	—	ND	—
20:4 *n*6 (AA)	11.7[a]	±0.3	13.0[b]	±0.3	11.8[ab]	±0.4	10.8[a]	±0.3
Jejunum								
16:0	13.2	±0.5	11.6	±0.3	13.0	±0.3	12.5	±0.8
*cis*9 18:1 (OA)	8.3[b]	±0.1	7.2[ab]	±0.4	7.7[b]	±0.6	5.6[a]	±0.4
*trans*11-VA	0.4[a]	±0.0	1.5[c]	±0.1	0.6[a]	±0.1	1.4[c]	±0.1
*cis*9, *trans*11-CLA	ND	—	ND	—	0.5	±0.0	ND	—
20:4 *n*6 (AA)	14.5	±0.6	18.3	±1.5	14.7	±1.5	20.0	±1.6

OA, oleic acid; AA, arachidonic acid; ND, not detectable. Values are means, with ± standard errors (SEM), n=5. Values are means +/- SEM, n=5. Means in a raw with superscripts without a common letter differ, $P<0.05$.

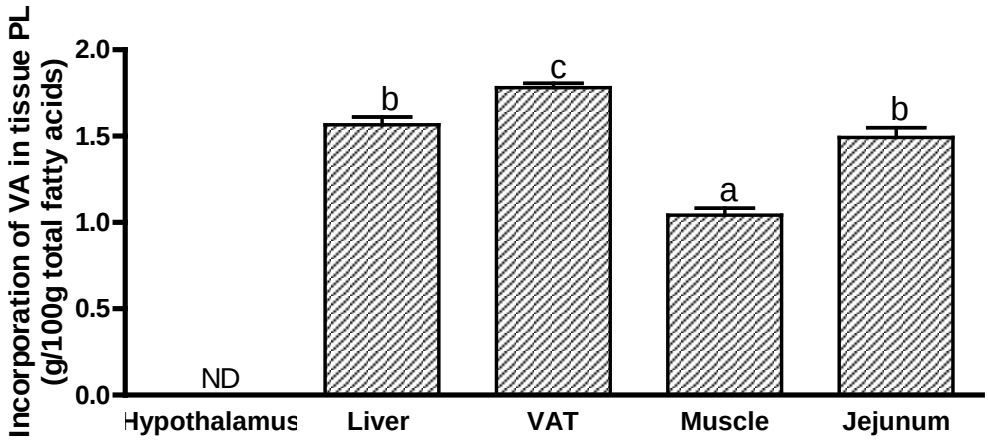

Figure 5-6 Incorporation of VA in tissue PL in JCR:LA-*cp* rats supplemented with VA for 8 wk. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ, P<0.05.

5.3.5 Dietary supplementation with VA alters the mRNA and protein expression of FAAH but does not affect the mRNA expression of CB_1 receptor in the jejunum

To determine whether selective increase of jejunal anandamide, OEA and PEA by VA could be associated with degradative pathways common to these three *N*-acyl ethanolamides, we measured the expression of FAAH, the primary enzyme hydrolyzing anandamide, OEA and PEA. Interestingly, VA and VA+CLA fed rats had higher jejunal mRNA expression of FAAH compared to the control group (1.4 fold, P<0.05 and 1.6 fold, P<0.01, respectively). In contrast, the protein abundance of this enzyme was reduced in VA fed-rats only (-34%, P<0.05) (***Figure 5-7A, 5-7B and 5-7C***). Collectively, these results suggest that VA may act in the intestine by reducing the degradation of anandamide, OEA and PEA via selective inhibition of FAAH activity in this tissue. We also found that VA

tended to lower jejunal mRNA expression of CB_1 relative to the other diets; however, this did not reach statistical significance (P>0.05) (***Figure 5-7*** **D**).

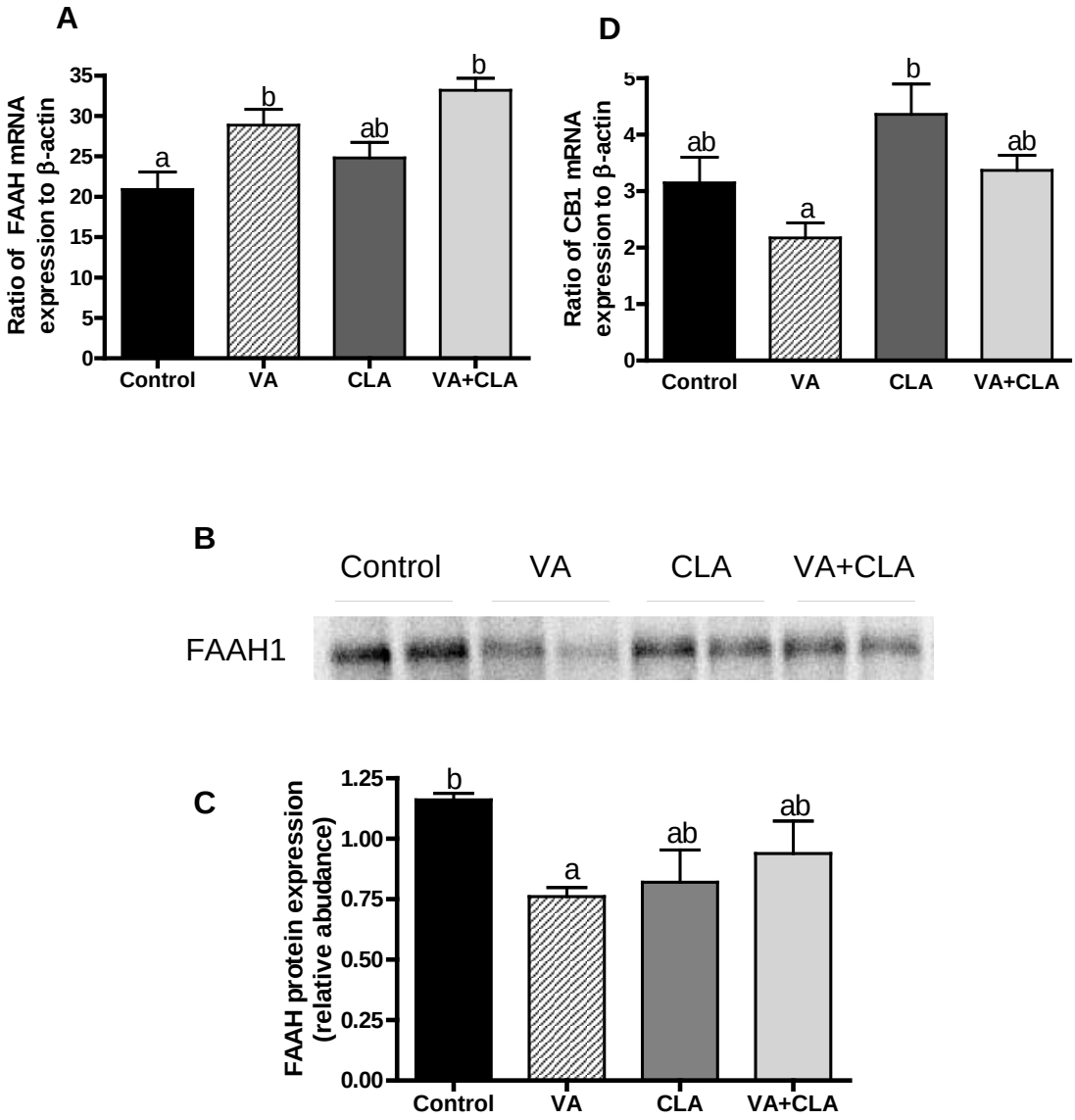

Figure 5-7 Jejunal mRNA expression of FAAH and CB_1 and protein abundance of FAAH in JCR:LA-cp rats fed control or experimental diets for 8 wk. The mRNA expression for FAAH (A) and CB_1 (D) is relative to the housekeeping gene β-actin. A representative blot (B) and graph (C) for FAAH relative protein abundance are shown. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ, P<0.05.

5.3.6 Dietary supplementation with VA reduced the mRNA expression of pro-inflammatory cytokines in the jejunum

The endocannabinoid system (ECS) is upregulated in human inflammatory bowel diseases and experimental models of colitis and colorectal cancer growth (Guagnini *et al*., 2006; D'Argenio *et al*., 2007; Massa *et al*., 2004; Ligresti *et al*., 2003). During these conditions, an overactive ECS is proposed to be an adaptive response to counteract the consequences of inflammation, such as T cell-mediated aberrant immune response (D'Argenio *et al*., 2007). Therefore, we wanted to explore whether the increase in jejunal anandamide we observed was associated with intestinal inflammation. Consistent with previous results from dietary VA supplementation in JCR:LA-*cp* rats (Blewett *et al*., 2009), the expression of pro-inflammatory cytokines (TNFα (***Figure 5-8A***) and IL-1β (***Figure 5-8B***)) was significantly lower in rats fed the VA supplemented diet compared with the control rats (-80% and -64%, respectively; $P<0.05$).

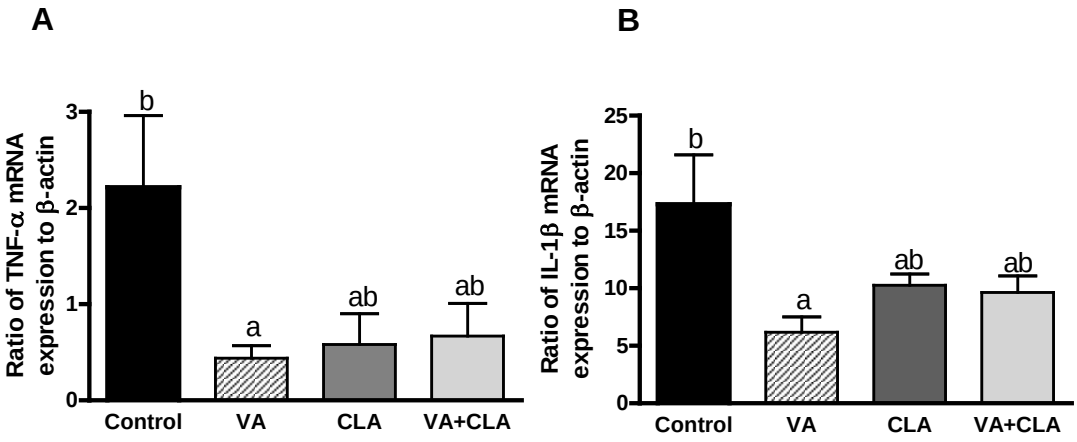

Figure 5-8 Jejunal mRNA expression of pro-inflammatory cytokines in JCR:LA-*cp* rats fed control or experimental diets for 8 wk. The expression of TNF-α (A) and IL-1β (B) is relative to the housekeeping gene β-actin. Values are means +/- SEM, represented by vertical bars (n=5). Means without a common letter differ, P<0.05.

5.4 Discussion

5.4.1 Dietary supplementation with VA reduces liver and VAT 2-AG without altering the availability of PL biosynthetic precursor

Several studies have linked the ECS to obesity and associated metabolic abnormalities in animal models as well as in humans. Upregulation of the ECS either by increased receptor expression and/or increased EC levels has been shown to lead to insulin resistance, fatty liver disease and dyslipidemia in animal models of obesity (Starowicz *et al*., 2008; Osei-Hyiaman *et al*., 2005; Alvheim *et al.,* 2012). Increased plasma EC concentrations have been found to be positively correlated with visceral fat mass and waist circumference in humans (Engeli *et al*., 2005; Bluher *et al*., 2006; Cote *et al*., 2007). Therefore, the ECS has been proposed as a critical target for the treatment of abdominal obesity and associated metabolic abnormalities in the MetS. While pharmacological blockade of

the CB1 receptor with Rimonabant has shown clinical success, the adverse psychiatric side effects associated with this drug have led to its withdrawal as a treatment option (Christensen *et al*., 2007). Recently, a causal role of dietary-induced EC dysregulation associated with an increased availability of AA in membrane PL in the development of obesity has also been reported (Alvheim *et al.,* 2012). Therefore, nutritional interventions that target the EC biosynthetic precursor AA, have been proposed as alternative therapeutic strategies. In this study, we targeted lipidomic analyses of the brain and peripheral tissues to explore putative differences in the EC, anandamide and 2-arachidonoylglycerol (2-AG) following dietary VA treatment. We also analyzed two non-CB receptor ligands, the *N*-acyl ethanolamides OEA and PEA which only differ from anandamide by their acyl chain and have shown to induce satiety (Fu *et al*., 2003; Schwartz *et al*., 2008) and exert anti-inflammatory effects through activation of PPARα receptor (Lo Verme *et al*., 2005).

To our knowledge this is the first report to demonstrate that dietary supplementation with VA can effectively decrease liver and VAT 2-AG concentrations in a rat model of MetS (***Figure 5-1 and 5-2***). Our findings resemble those reported for n3-long chain (LC)-PUFA (in the form of krill oil) in the Zucker *fa/fat* rat (Batteta *et al*., 2009) and may also suggest a putative mechanism of action for the ability of VA to decrease hepatic lipid accumulation and TG secretion previously shown in JCR:LA-*cp* rats (Chapter 4). The rationale for this hypothesis is that CB receptor activation in the liver and VAT leads to increased *de novo* lipogenesis and visceral adiposity (Osei-Hyiaman *et al*., 2005; Osei-Hyiaman et al., 2008; Pagano *et al*., 2007), both of which are attenuated by VA (Wang *et al*., 2009; Chapter 4). However, effects of VA on tissue EC concentrations cannot be explained by changes in AA levels in membrane PL but rather due to incorporation of VA (***Table 5-2***). In addition, findings from the present study suggest a potential regulatory effect of VA on the biosynthetic or

degradative enzymatic pathways controlling EC concentrations in the liver and VAT of JCR:LA-*cp* rats.

5.4.2 Effects of VA on tissue EC concentrations are associated with its incorporation into tissue PL

Previous studies in the JCR:LA-*cp* rat have shown that the lipid-lowering effects of CLA are enhanced by the addition of VA when compared to dietary supplementation with CLA alone (Chapter 3). In this study we provide evidence that VA *per se* can independently reduce tissue 2-AG concentrations corresponding with its magnitude of incorporation into tissue PL when compared to CLA (***Table 5-2***). Our findings have also revealed that effects of VA on EC concentrations depends on the tissue and parallel the extent of VA incorporation into respective tissue membrane PL (***Figure 5-6***). However further studies are needed to determine the mechanism of how incorporation of VA into tissue PL mediates a lowering of EC concentrations in liver and VAT.

5.4.3 VA increases jejunal anandamide and anandamide analogues associated with a down-regulation of FAAH protein expression

We have observed that VA re-equilibrates intestinal and hepatic lipid homeostasis while exerting different transcriptional regulation in both organs, as reflected in mRNA levels of SREBP1 and FAS (Chapter 4). In this study we found a consistent effect of VA on decreasing EC concentrations in the liver and VAT. In contrast, jejunal concentrations of anandamide and its analogues (OEA and PEA) were selectively increased following VA treatment alone (***Figure 5-5***). This selective increase of jejunal *N*-acyl ethanolamides by VA could not be explained by changes in their biosynthetic PL precursor AA but was associated with a reduction in protein expression of their hydrolyzing enzyme, FAAH (***Figure 5-7A, 5-7B and 5-7C***).

To the best of our knowledge this is the first study to show that VA can exert a selective effect on EC concentrations in the intestine. Interestingly, feeding n-3 LCPUFA is associated with an increase in the FAAH inhibitor, arachidonoyl-serotonin (AA-5-HT) and other jejunal LCPUFA-serotonins (also capable of inhibiting FAAH activity *in vitro*) in mice (Verhoeckx *et al.,* 2011). Therefore, it is plausible that VA supplementation may stimulate the formation of intestinal-specific lipid mediators that regulate FAAH activity and further studies are needed to elucidate the exact mechanisms.

Anandamide is found to be increased in human intestinal inflammatory diseases and is proposed to be an adaptive response to counteract the inflammatory milieu during these conditions (Ligresti *et al*., 2003; Guagnini *et al*., 2006; D'Argenio *et al*., 2007). In contrast to the pathological role of the ECS in the liver and VAT (Starowicz *et al*., 2008; Osei-Hyiaman *et al*., 2005; Alvheim *et al.,* 2012), CB1 or CB2 receptor activation by anandamide has been shown to reduce macroscopic damage scores of colonic inflammation in animal models of colitis (Massa *et al*., 2004; Storr *et al*., 2008; Storr *et al*., 2009). Additionally, the N-acyl ethanolamide PEA has also been shown to exert anti-inflammatory effects (reduction or pro-inflammatory cytokine production) through activation of PPARα (Di Paola *et al*., 2012). We therefore examined the mRNA expression of two key inflammatory markers, TNF-α and IL-1β in the intestine to explore a potential association with the increased anandamide and PEA concentrations observed in the jejunum. Indeed, dietary VA supplementation significantly reduced the mRNA expression of the pro-inflammatory cytokines, TNF-α and IL-1β (***Figure 5-8***). These findings are consistent with previous effects of dietary VA in the JCR:LA-*cp* rodent model of MetS (Blewett *et al*., 2009) and provide further evidence for the independent anti-inflammatory effects of VA. It is likely that these effects may be mediated by VA activation of PPARγ-regulated anti-inflammatory pathways (Jaudszus *et al*., 2012; Wang *et al*., 2012). Alternatively, the potential ability of VA to decrease EC degradation (via inhibition of FAAH)

in the intestine may offer an additional mechanistic explanation for the anti-inflammatory effects of VA and this warrants further investigation. The rationale for this hypothesis is based on reports that targeting the ECS (via inhibition of FAAH) has therapeutic potential in the treatment of inflammatory diseases (Storr et al., 2008; Storr *et al*., 2009).

In conclusion, dietary supplementation with VA effectively reduces liver and VAT 2-AG concentrations corresponding with its previously observed properties to beneficially modulate lipid storage compartments. We have also provided further evidence that VA can act independently of CLA, which seems to be associated with its incorporation into tissue PL. Additionally, the present findings delineate a unique regulation of VA on jejunal anandamide and its *N*-acyl ethanolamide analogues that cannot be explained by changes in their biosynthetic PL precursors. Rather, our results suggest a selective inhibitory effect of VA on the activity of FAAH in the intestine. Collectively, findings from this study have provided a potential novel mechanism of action for the health benefits of VA and highlight the need for further investigations to explore the efficacy of VA on intestinal inflammatory diseases.

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Chapter 6 Final Discussion

6.1 Summary of Findings

6.1.1 VA enhances the lipid-lowering effects of CLA during conditions of abnormal lipid metabolism.

Experiments in Chapter 3 (published in Nutrition and Metabolism, 2010) were essential to validate that VA has lipid-lowering effects independent from the bioactivity of CLA *per se*. Indeed, during the course of this thesis, complementary mechanistic studies have been published by our group, as well as studies by other groups affirming these conclusions (Wang *et al*., 2008; Wang *et al*., 2008; Wang *et al*., 2012; Bassett *et al*., 2010; Tyburczy *et al*., 2009; Herrera-Meza *et al*., 2013). Results from my studies in this chapter provide evidence that both 'ruminant' *trans* fatty acids (rTFA, VA+CLA) can act synergistically to improve abnormal lipid metabolism. The rationale for the synergistic effects of VA and CLA is based on the fact that VA is the only source for endogenous synthesis of CLA in the human body and can additionally facilitate mechanisms of action of CLA.

One of the more interesting findings from this Chapter also included the observation that the combination of both VA and CLA stimulated food intake in our animal model, yet beneficially lowered body weight and decreased hepatic lipid accumulation, as compared to control diets. These observations were key to the need for studies to assess the indirect calorimetric and body composition experiments in Chapter 4, leading to the hypotheses that VA may favourably promote whole body energy utilization and storage.

One potential clinical translational consideration that emerged from Chapter 3 findings was enriching dairy products with both rTFA (VA and CLA) may provide an additional health benefit during abnormal metabolic conditions, such as the metabolic syndrome. However, this finding went

against current dogma regarding the adverse benefits of trans fats and was different to effects observed with other dairy-derived fatty acids, such as SFA. SFA's have historically been associated with negative health effects, although this remains contentious (Mente *et al*., 2009; Siri-Tarino *et al*., 2010; Howard *et al*., 2010; Micha and Mozaffarian, 2010). Of course, rTFAs are an integral part of dairy fat, and one of the caveats of studies performed in Chapter 3, is that vegetable oils (not animal or other dairy fats) were used to carefully balance the fatty acid profile of experimental diets. While this experimental approach was needed to understand the select bioactive properties of VA and CLA *per se*, it did not represent the conditions in which these rTFAs are found naturally in foods.

In order to consider further the potential implication of findings from Chapter 3 and how this data might relate to rTFA found in the food matrix (i.e. dairy products), it was important to validate the bioactive properties of VA using a control diet which was more reflective of the regular consumption of rTFA in foods in the diet. Consequently, one of the main objectives of Chapter 4 was to generate custom butter oil isolates in order to carefully balance diets (with and without VA) by using a control diet that contained these animal derived fats.

The overall findings from Chapter 4 confirmed that enriching dairy products with VA (either naturally or by fortification) was a sound approach to maximize the health value of dairy-derived fats and this has significant relevance to the agriculture food and livestock sector.

6.1.2 Adding VA to a background diet containing dairy-derived SFA alleviates abnormal lipid metabolism

Findings from experiments in Chapter 4 demonstrated that enriching dairy-derived fat with VA (akin to product fortification) resulted in additional improvement in lipid abnormalities compared to low-VA containing dairy derived fat. Moreover, the findings also revealed *novel* properties of VA to

favorably modulate adipose storage compartments while simultaneously reducing ectopic lipid accumulation in the liver and the intestine. Indeed, we were also surprised when the indirect calorimetric studies showed VA enriched diets increased metabolic rate concomitant with an increased preference of whole body glucose utilization as a substrate for oxidation.

Another key finding from Chapter 4 was the extent to which both the liver and intestine became involved in re-equilibrating lipid homeostasis in the presence of VA. Our data suggest an intriguing opposing transcriptional regulation between both the intestine and the liver. We have defined this observation as the gut-liver-adipose axis, where the interplay of these organs in lipid metabolism requires further investigation (such as experiments conducted under 'fasted' versus 'fed' conditions described below in Sections 6.1.2.1 and 6.3.2).

In contrast to our original hypothesis, results from Chapter 4 suggested that reduced lipid accumulation in the liver following dietary VA enrichment was likely to be independent of PPARα-mediated lipid oxidation. These observations emphasized that VA may modulate energy use and storage by alternative pathways and led us to propose a mechanism of action convergent with these metabolic effects. Considering the documented ability of long chain fatty acids to regulate lipid-derived messengers, such as endocannabinoids, involved in energy metabolism, we proposed that VA's activity may also resemble these regulatory effects. Therefore, in Chapter 5, we targeted lipidomic analyses of the brain and peripheral tissues to explore putative differences in endocannabinoids following dietary VA treatment (see Chapter 5, summary section 6.1.3).

6.1.2.1 Effect of fasted *versus* fed states on intestinal lipid secretion

In chapter 4, *in vivo* 'secretion experiments' were designed to determine the lipid-lowering effects of VA on hepatic *versus* intestinal TG secretion during *fasting* and *fed* conditions, respectively. However, gene expression

and tissue lipid mass measurements were conducted in tissues (both liver and intestine) collected after an overnight fast. Therefore, these quantitative analyses are only reflective of the physiological status during the fasted state.

Future studies are therefore needed to elucidate the tissue-specific effects of VA during both fasted and fed state. For example, measuring intestinal lipid storage and lipid synthesis during the fed state may yield an explanation for the effects of VA on decreased CM-apoB48 and CM-TG secretion. For instance, it has been proposed that attenuation of postprandial lipemia by fish oil feeding, specifically LCPUFA 22:5 and 22:6 n-3, can be explained by decreased CM assembly (due to limited lipid synthesis/availability) *in the fasted state* (Levy *et al*., 2006), or by a citoplasmic accumulation of lipids in the enterocytes *during fed conditions* (Larsen *et al*., 2003). It is therefore plausible to suggest that reduced intestinal lipid accumulation by VA *in the fasted state* (as observed in the studies described in Chapter 4), may induce a transient citoplasmic lipid accumulation in enterocytes *in the fed state*; that ultimately might explain delayed CM/TG secretion by the intestine into the circulation. This might be the connecting link between gut-liver-adipose axis since it is known that delayed CM secretion results in partitioning of dietary fat towards oxidation rather than towards storage (while protecting against fatty liver) (Yen *et al*., 2009).

6.1.2.2 Effect of fasted *versus* fed states on fuel oxidation

I also wish to point out that the indirect calorimetric experiments (Chapter 4) were performed in rats fed *ad libitum;* therefore, the hyperphagic condition of our rat model suggests that animals had increased fuel availability (mainly carbohydrate) during these measurements. We found that VA feeding increased the preference of glucose utilization for oxidation (increased RER), and since glucose oxidation is positively

associated with the rate of glucose disposal (Galgani *et al*., 2008), we proposed that the increased RER in VA-fed rats could be associated with improved glucose uptake capacity (insulin sensitivity). Indeed, VA-fed rats presented with reduced fasting insulin and post-prandial glucose concentrations after a meal tolerance test, suggestive of improved insulin sensitivity compared to controls. Based on these findings, it is conceivable that VA may act by improving the normal physiological response to match fuel oxidation to fuel availability during fasted and fed conditions. Whether VA can directly increase the ability of skeletal muscle to switch between glucose and fat oxidation in response to nutrient availability and other hormonal signals (also called metabolic flexibility (Kelley and Mandarino, 2000) is unknown. Measuring energy expenditure and fuel oxidation in the fasted *versus* fed state may offer a line of investigation to determine the insulin-sensitizing effects of VA and associated reduced ectopic lipid accumulation.

For example, if VA can improve metabolic flexibility we should be able to observe an increase in whole body energy expenditure and fatty acid oxidation (over glucose) following an overnight fast. Perhaps, this may help explain the reduced hepatic lipid accumulation we observed in overnight-fasted rats (Chapter 4). In contrast, in the fed state, we might anticipate VA-fed rats (presumably more insulin sensitive) to display increased energy expenditure with a preference towards increased glucose utilization (over lipid) as a fuel. To complement this approach, it might be interesting to use non-invasive magnetic resonance techniques (e.g. magnetic resonance imaging) to monitor intrahepatic or intramuscular lipid content while conducting these experiments in the fed and fasted state. These additional experiments may provide evidence that increasing glucose uptake and oxidation in skeletal muscle of VA-fed rats (in the fed state, for instance), may explain the decreased availability of glucose as a substrate for hepatic *de novo* TG synthesis and subsequent hepatic lipid storage. Measuring the insulin-activated signaling pathways

in response to a carbohydrate meal would further provide evidence for the insulin sensitizing effects of VA in skeletal muscle.

6.1.3 VA exerts a selective regulation of tissue endocannabinoids that is associated with its metabolic effects

Experiments in Chapter 5 have revealed interesting and novel properties of VA to potentially regulate lipid signaling pathways involved in the endocannabinoid system that is convergent with our metabolic observations. Results presented herein have shown that VA can substantially reduce endocannabinoid (EC) concentrations in the liver and visceral adipose tissue (VAT), which may help explain the benefits of VA on adipose storage compartments and hepatic lipid accumulation (described in Chapter 4). However, these regulatory effects of VA on tissue EC (2-AG and anandamide) did not correspond with changes in the phospholipid (PL) precursors (arachidonic acid-containing PL). This suggests VA mediated regulation of EC may be at the level of mRNA and protein expression, and activity of the biosynthetic or degradative enzyme pathways. Surprisingly, VA was also shown to differentially regulate EC and anandamide analogues in the intestine *compared to* VAT and liver. In contrast to the liver and VAT, VA was shown to increase the concentrations of anandamide and its analogues, PEA and OEA, concomitant with decreased abundance of the enzyme responsible for their degradation (fatty acid amide hydrolase, FAAH). Based on the literature, we have proposed these findings may explain the anti-inflammatory properties of VA in this organ.

Collectively, findings presented in Chapter 5 have shown for the first time that VA can effectively regulate tissue endocannabinoid concentrations and this may have potential therapeutic applications. Results support a selective activation of protective pathways of the endocannabinoid system (ECS) in the intestine, and highlight the need for further investigation to

explore the efficacy of VA on intestinal inflammatory diseases. For example, results have led to discussions on the plausibility of VA-derived lipid mediators with capacity to inhibit the activity of intestinal FAAH, which would help ameliorate inestinal inflammation. The future directions of this work are discussed in more detail in Section 6.2.3.

6.1.4 Summary of the pleiotropic effects and proposed mode of action of VA

I have introduced data that spans a number of metabolic pathways encompassing regulation of lipid metabolism (e.g. absorption, partitioning, and storage), energy expenditure and fatty acid bioactivity in multiple organs. These observations are highly reflective of a complex interaction between physiological pathways and the following diagram (Figure 6-1) was generated to provide an overview of the effects of VA in different pathways that were studied in this thesis. In particular, it demonstrates the pathophysiology of the metabolic syndrome and the modes of action of VA that were discovered in my thesis research.

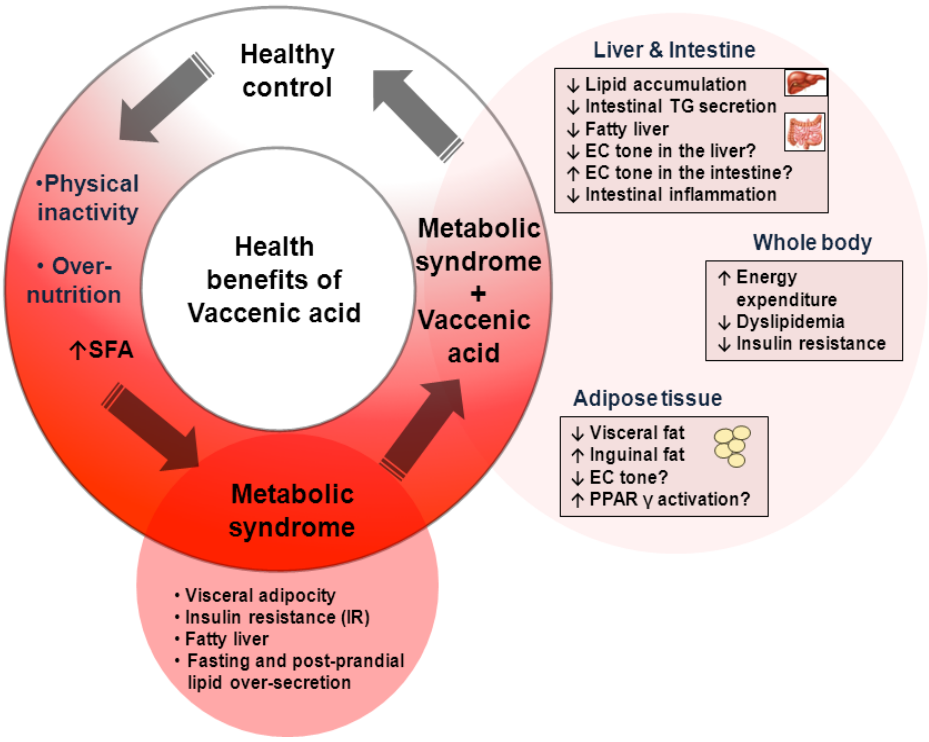

Figure 6-1 The pleiotropic metabolic effects of VA during MetS. Obesity, insulin resistance and physical inactivity are underlying risk factors for the MetS**.** The continuous provision of energy via select macronutrients (e.g. TFA and SFA) that characterize western food intake patterns further exacerbate the risk for CVD in individuals with MetS. Enriching dairy fat (containing SFA) with VA may improve the ability of adipose tissue (subcutaneous) to appropriately store excess energy away from peripheral tissues such as the liver. VA supplementation may also act to reduce the absorption of TG from the intestine and reduce classic metabolic risk factors such as visceral fat deposition, insulin resistance and dyslipidemia. The efficient incorporation of VA into membrane PL and its processing into bioactive lipid signaling molecules (e.g. PPAR activators, regulators of EC metabolism) may provide a potential mechanistic explanation for VA's physiological effects.

6.2 Future Directions

6.2.1 Effects of VA on energy metabolism in diet-induced obesity

Studies conducted in this thesis have uncovered novel properties of a common ruminant trans fatty acid (*trans-11* vaccenic acid [VA]) to modulate whole body energy metabolism in the JCR:LA-cp rodent model which has a leptin receptor defect and renders it prone to the metabolic syndrome. It is also important to acknowledge that many of the same bioactive properties of VA reported by this thesis have also been reported by other groups using different animal models, including LDL knockout mice, spontaneously hypertensive rats and the Syrian golden hamster (Bassett *et al*., 2010; Herrera-Meza *et al*., 2013; Tyburczy *et al*., 2009). It would also be pertinent to explore this regulatory effect of VA in an animal model of diet induced obesity (DIO). For example, feeding a high fat-palatable diet in a DIO animal model would lead to increased food intake and body weight which may be attenuated by increased energy expenditure in VA-supplemented animals. It is also possible that VA-supplemented animals may not be fully protected against obesity but rather might exhibit a more favorable body composition. Furthermore, given that leptin suppresses insulin secretion (Cases *et al*., 2001) and lipogenic enzymes such as SCD-1 (Biddinger *et al*., 2006), the leptin receptor defect in the JCR:LA-*cp* rat may prevent complete normalization (act as conounding factor) of hyperinsulinemia and lipogenesis following VA treatment. Therefore, using a DIO animal model (with functional leptin receptor) would further clarify effects of VA on insulin resistance and/or lipidogenesis in different dietary conditions.

6.2.2 Effects of VA on adipose tissue in *in vitro* studies and an adipose tissue-specific PPARy knock out mice model

VA has been shown to activate PPARy-regulated pathways *in vitro* (Wang *et al*., 2012; Jaudszus *et al*., 2012). The results shown in Chapter 4 of this thesis have suggested *in vivo* activation of PPARy in adipose tissue (adipose re-distribution, and decreased adipocyte size) in the JCR:LA-*cp* rat. Therefore, it would be worthwhile to investigate the effects of VA on adipose tissue remodeling. *In vitro* studies using primary and/or secondary cultures of adipocytes may be useful to study the direct effects of VA on macrophage infiltration and the associated inflammatory response as well as markers of adipose tissue differentiation and browning. This could provide direct support to the proposed PPARy-induced healthy remodeling of adipose tissue by VA.

In chapter 4, I discussed that VA may improve the ability of adipose tissue to appropriately store lipids away from insulin sensitive tissues (such as the liver), possibly by activating PPARy-downstream pathways in adipose tissue. To confirm that beneficial effects of VA on NAFLD progression are due to PPARy activation in adipose tissue *per se*, an adipose tissue-specific PPARy knock out mice model could be particularly useful. If this hypothesis is correct, knock out mice would exhibit increased lipid deposition in both the liver and skeletal muscle, resulting in increased insulin resistance following VA treatment compared to VA-fed wild type mice.

Based on reports in the literature that CM production can be stimulated by plasma FFA (Duez *et al*., 2008; Nogueira *et al*., 2012), this experimental design could also contribute to our understanding of the involvement of adipose tissue-induced intestinal CM secretion and the role of VA on modulating interactive crosstalk between these organs.

6.2.3 Potential role of VA in alleviating inflammatory bowel diseases

One of the most novel findings from this thesis is that VA has differential regulation in the intestine *versus* VAT and liver on EC and this is convergent with its beneficial metabolic and physiological effects. However, this regulatory effect of VA could not be explained by changes in biosynthetic membrane PL precursors of EC. Rather, findings have suggested a selective inhibitory effect of VA on the activity of the fatty acid ethanolamide hydrolyzing enzyme (fatty acid amide hydrolase, FAAH) in the intestine. Given the protective role of the anandamide against intestinal inflammation (D'Argenio *et al*., 2007; Storr *et al*., 2008) and colorectal cancer growth (Ligresti *et al*., 2003), an approach to decrease EC degradation (via inhibition of FAAH activity) in the intestine (by VA) has potential therapeutic implications in intestinal inflammatory diseases.

It is plausible that the conjugation of VA into bioactive lipid signaling molecules (e.g vaccenyl-ethanolamide or vaccenyl-serotonin (VA-5-HT)) could provide a potential mechanistic explanation for such effects of VA in the intestine. The rationale for this hypothesis is that the FAAH inhibitor arachidonoyl-serotonin (AA-5-HT) and its fatty acid analogues with similar bioactivity can be endogenously synthesized in tissues with high contents of serotonin (such as the intestine) (Verhoeckx *et al.,* 2011). For instance, a novel class of LCPUFA-serotonins with capacity to inhibit FAAH activity *in vitro,* were found to be induced by fish oil feeding in mice (Verhoeckx *et al.,* 2011). It is conceivable that VA feeding could stimulate the formation of intestinal-specific lipid mediators with capacity to regulate FAAH activity.

To confirm the efficacy of VA on intestinal inflammatory diseases, investigating the role of VA in cell proliferation and inflammation using the Caco-2 cell line and IBD mice models may be particularly useful. Using CB receptor agonist/antagonists (local infusion for the *in vivo* studies)

and/or FAAH inhibitors in these experimental models would elucidate whether anti-inflammatory effects of VA in the intestine are related to activation of protective pathways of the endocannabinoid system. Furthermore, including PPARα and PPARγ antagonists in these experiments would provide evidence for alternative anti-inflammatory properties of VA via PPAR activation.

Quantifying putative VA-derived mediators (e.g. vaccenyl-ethanolomide or vaccenyl-serotonin (VA-5-HT)) and investigating their biological activities would require the development of custom made compounds.

6.3 Significance and Impact of Thesis Findings

6.3.1 VA and lipid lowering effects in humans

Currently, there is sufficient evidence to suggest that rTFA intake of up to 4 g/day is safe and has neutral effects in normolipidemic conditions (Tholstrup *et al*., 2006; Chardigny *et al*., 2008; Motard-Belanger *et al*., 2008; Lacroix *et al*., 2012). The putative beneficial effects of rTFA in dyslipidemic individuals warrant further investigation via randomized clinical controlled trials. However, clinical dose-response studies are first needed in order to determine beneficial effects on blood lipids. Indeed, a recent study in hypercholesterolemic subjects has shown that consumption of cheese (90g/d) naturally enriched in VA and CLA significantly reduced plasma endocannabinoid and LDL-C concentrations after three weeks of intervention (Pintus *et al*., 2012). These effects were only observed with a 90 g/d (~2.8 g/d rTFA) but not with a 45 g/d (~1.4 g/d rTFA) intake of cheese, suggesting a dose-dependent effect. Furthermore, dose-dependent studies with synthetic forms of VA should also be conducted in order to determine hypolidemic effects and potential side effects. To this end, using large animal models may provide better suitability for translation of findings to human consumption of VA enriched foods and synthetic VA.

6.3.2 Potential therapeutic use of VA in NAFLD

It is clear from the studies presented in this thesis, at least in a rodent model of obesity and insulin resistance, that VA has potent beneficial and therapeutic effects in NAFLD. Currently, dietary modifications and exercise remain the best therapeutic options for preventing the progression of NAFLD (Naniwadekar, 2010). Therefore, bioactive properties of VA to improve hepatic and visceral adiposity as well as insulin resistance are very promising and warrant randomized controlled clinical trials to determine the effectiveness of VA in this metabolic disease. Outcomes that could be measured in NAFLD patients to determine the effectiveness of VA treatment could include hepatic enzymes (such as AST and ALT) or hepatic TG content by imaging technologies such as proton magnetic resonance spectroscopy (Browning *et al*., 2004).

It is also important to note that the underlying mechanisms of action of VA in NAFLD *per se* remain unclear. VA was shown in Chapter 4 to reduce hepatic lipid accumulation but this observation was not explained at the transcriptional level and was independent of PPARα-mediated lipid oxidation. For instance, VA feeding did not affect the expression of genes involved in lipid synthesis or oxidation such as SREBP1, FAS and CPT1α (to mention a few) in the liver. However, previous findings (Wang *et al*., 2009) have demonstrated lower protein expression of hepatic lipogenic enzymes (FAS and ACC-1) in our animal model. Therefore, these observations have led to propose that VA may regulate *de novo* lipid synthesis at a functional level but this may result in a compensatory transcriptional response by the liver.

Considering the pleiotropic effects of VA, physiological studies using tracer and multi-compartmental modeling technologies would also provide a better understanding of the beneficial regulatory effects of VA in NAFLD.

For instance, measuring hepatic *de novo* lipogenesis and fatty acid oxidation while tracing the flow of lipids from the intestine and adipose tissue *in vivo* (such as those studies performed by Donnelly *et al*., 2005), would delineate the physiological mechanisms that potentially contribute to alleviate NAFLD progression following VA treatment.

6.3.3 Potential implications of thesis findings in the development of novel foods

One of the major findings from studies presented in Chapter 5 is VA can significantly modulate tissue EC concentrations and these effects are associated with the level of incorporation of VA into tissue PL. Similar intake of CLA did not produce the same extent of incorporation of CLA into PL, nor did CLA have the same bioactivity on modulating EC concentrations. There have been studies to suggest that the bioactivity of fatty acids may depend on the chemical form in which they are administered (e.g. PL *versus* TG) (Rossmeisl *et al.,* 2012; Batteta *et al*., 2009). This property may determine the extent of incorporation into cell membrane PL and the subsequent processing of fatty acids into bioactive lipid signaling molecules leading to differential physiological effects (Rossmeisl *et al.,* 2012). For instance, krill oil (n3-long chain PUFA in the PL form) has been shown to be more effective than fish oil (n3-long chain PUFA in the TG form) in modulating tissue EC and improving parameters of MetS in the Zucker rat (Batteta *et al*., 2009) and DIO mice (Rossmeisl *et al.,* 2012). Therefore, it is possible that administration of VA in the FFA form may enhance its bioactivity. *Whether VA should be supplemented in the FFA or TG form is an important consideration for potential applications in the development of novel foods (discussed in the following Section).*

6.3.3.1 Nutraceuticals and fortified foods

The chemical form in which VA is administered may have a direct impact on its bioactivity, and therefore this needs to be considered for potential industrial applications. For example, in the experiments in Chapter 5, rTFA were administered as FFA or TG, and exhibited differential incorporation into PL. Therefore, it could be speculated that VA as FFA may be more bioactive that VA in the TG form. However, the bitter taste typically associated with FFA may render food fortification with VA in the FFA form undesirable. Perhaps, encapsulation, nanoparticle technology or emulsification methods of VA in the FFA form may be a suitable approach to prevent the off-taste at high concentrations (Onwulata, 2012). Alternatively, esterification of VA in the PL form may be preferable for food fortification, as this may prevent undesirable sensory characteristics while maintaining increased bioavailability and bioactivity. However, development of novel foods that include purified forms of VA may be unfeasible to date, as this requires specialized technologies for mass production of VA. Perhaps, the putative synergistic actions of nutrient components in foods as they are found naturally (as opposed to isolated components) (Jacobs *et al*. 2012), may be more important to consider when developing novel foods. A more practical and perhaps nutritionally-preferable option may be to increase the concentrations of VA and CLA in ruminant-derived products by natural processes such as modified feeding practices (Mele *et al*., 2011) or technological approaches such as dry fractionation (O'shea *et al*., 2000) . The development of naturally enriched VA and CLA products may also avoid food and drug regulatory hurdles compared to the use of purified VA fortification in food products.

6.3.3.2 Naturally enriched dairy products

The fatty acid composition of meat and dairy products can be modified by feeding practices allowing new opportunities for the health value of

animal-derived fats and food products (Dewhurst *et al*., 2006; Mele, 2009; Mele *et al*., 2011; Aldai *et al*., 2010). Ruminants that are exclusively pasture fed (and/or supplemented with seeds) produce 'healthier' milk and dairy products with less cholesterol-elevating SFA (such as myristic and palmitic acids), and greater levels of omega-3 fatty acids, VA and CLA compared to those grain fed (White *et al*., 2001; Kraft *et al*., 2003; Mele *et al*., 2011). The significance of such enrichment has been confirmed by a recent study in Italy, showing that a naturally enriched cheese can improve plasma lipid profile (reducing LDL-C) in hypercholesterolemic subjects (Pintus *et al*., 2012). To our knowledge this has been the first randomized controlled trial to show acute lipid benefits of naturally enriched dairy products. Additional well controlled randomized clinical trials have shown neutral effects of rTFA intake (< 4 g/day); however, these studies were conducted in healthy subjects (Tholstrup *et al*., 2006; Chardigny *et al*., 2008; Motard-Belanger *et al*., 2008; Lacroix *et al*., 2012). These observations support the hypothesis that the hypolipidemic activity of rTFA may be limited to but also beneficial to study participants with existing hyperlipidemia. Another important finding from the referred study (Pintus *et al*., 2012) is that supplementation with synthetic CLA at a dose comparable to that attainable from its daily intake with the enriched cheese, did not provide the same benefits. Therefore, the changes detected with the enriched cheese could not be attributed to CLA *per se*. It is important to note that the fatty acid composition of the enriched and control cheese diets was not matched with respect to SFA. Therefore the results from the study by Pintus *et al*. (2012) provide evidence for a potential synergistic action of VA and CLA along with the reduced SFA content in the enriched cheese. Whether increasing the ratio of VA to SFA in animal-derived fat can provide an additional value in dyslipidemic populations warrants further investigation and randomized controlled trials.

Results from this thesis support the concept that the ratio of VA to SFA in dairy foods may be important for health and hypolipidemic properties *in*

vivo. However, important considerations are needed before such enriched animal-derived products are produced in large-scale and are available in the food supply (as discussed below).

6.3.4 Implications on current labeling regulations and policies

From an evolutionary perspective, rTFA from meat and dairy produce have been in the human food supply for as long as animals have been consumed and domesticated. Population-based studies to date have shown no harm associated with the consumption of rTFA (Willett *et al*., 1993; Ascherio *et al*., 1994; Pietinen *et al*., 1997; Oomen *et al*., 2001; Jakobsen *et al*., 2008). Based on the discussion above, a naturally enriched dairy product may be considered as a 'healthier' alternative choice to attain the 2 servings/day recommended in a nutritionally-adequate diet (Health Canada Dietary Recommendation Guidelines). However, the enrichment process cannot be placed into practice until nutrition guidelines and policies support rTFA enrichment (Mapike *et al*., 2012).

It is important to note that the current international definition of TFA does currently acknowledge the distinctive health effects of TFA derived from ruminant and industrial food sources. This definition includes all fatty acids containing *trans* double bond (s) except CLA and was established by the Codex Alimentarius to guide nutritional and legislatorial regulations to reduce TFA consumption. The successful implementation of food-labeling regulations in North America (FDA, 2006; Health Canada, 2005) has resulted in a significant decline in TFA intake (Vesper *et al*., 2012). However, because rTFA (other than CLA) remain included on food labels in North America, a decrease in TFA intake may not be limited to industrial forms but may also impact the intake of TFA from ruminant sources.

6.3.4.1 Considerations for labeling TFA based on the source as opposed to all TFA *per se*

The enrichment of dairy products has further complicated the recommendations to minimize total TFA intake. Enriching dairy products would result in a relatively high cumulative daily intake of rTFA which may result in exceeding the recommended limit for total TFA by Health Canada (2 g/day). For example, the test cheese used in the study by Pintus *et al*., (2012) provided a daily intake of 2.8 g rTFA which would exceed the current recommendations in Canada. A serving (50 g) of this cheese would contain approximately 1.6 g of rTFA. *Therefore, a dairy product derived from grass-fed/supplemented ruminant animals could not be labeled as trans fat free and this may be perceived as detrimental in health value.* In this context, there have been some recent discussions as to whether unprocessed whole-fat dairy products should be excluded from the current Codex TFA definition. However, potential detrimental effects associated with supplemental CLA (synthetic forms) have resulted in concerns regarding the correctness of excluding CLA from the TFA definition, which has further complicated these discussions.

Regarding CLA, it has been consistently documented that supplemental and ruminant sources of CLA have different bioactivity (Wanders *et al*., 2010; Moloney *et al*., 2004; Raff *et al*., 2009; Risérus *et al*., 2004; Steck *et al*., 2007; Arbonés-Mainar *et al*., 2006; Roche *et al*., 2002; Valeille *et al*., 2005; Pintus *et al*., 2012). Therefore, it could also be argued that different sources of CLA should be considered separately with respect to health regulations and/or nutritional guidelines. Furthermore, the profile of fatty acids from industrial and ruminant food sources is inherently different in terms of isomer distribution and absolute abundance. Collectively, labeling TFA based on the source as opposed to all TFA *per se* would potentially minimize confusion for public health messaging and food labeling, particularly in Canada. The results from this thesis are consistent with

previous pre-clinical studies suggesting that VA, the most abundant rTFA, possesses potent therapeutic properties that are important to consider under metabolically abnormal conditions. Indeed, these findings are consistent with those reported by Pintus *et al*., (2012) that suggest VA may be beneficial to those with hypercholesterolemia. Therefore, findings from this thesis have contributed to the growing literature that supports the need for nutrition guidelines/policies focused on eliminating industrial forms of *trans* fat from processed foods, as opposed to all TFA *per se*, in particular natural ruminant TFA.

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Increased hypolipidemic benefits of *cis*-9, *trans*-11 conjugated linoleic acid in combination with *trans*-11 vaccenic acid in a rodent model of the metabolic syndrome, the JCR:LA-*cp* rat

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Abstract

Background: Conjugated linoleic acid (*cis*-9, *trans*-11 CLA) and *trans*-11 vaccenic acid (VA) are found naturally in ruminant-derived foods. CLA has been shown to have numerous potential health related effects and has been extensively investigated. More recently, we have shown that VA has lipid-lowering properties associated with reduced hepatic lipidogenesis and chylomicron secretion in the JCR:LA-*cp* rat. The aim of this study was to evaluate potential additional hypolipidemic effects of purified forms of CLA and VA in an animal model of the metabolic syndrome (the JCR:LA-*cp* rat).

Methods: Twenty four obese JCR:LA-*cp* rats were randomized and assigned to one of three nutritionally adequate iso-caloric diets containing 1% w/w cholesterol and 15% w/w fat for 16 wk: 1) control diet (CD), 2) 1.0% w/w *cis*-9, *trans*-11 CLA (CLA), 3) 1.0% w/w VA and 1% w/w *cis*-9, *trans*-11 CLA (VA+CLA). Lean rats were fed the CD to represent normolipidemic conditions.

Results: Fasting plasma triglyceride (TG), total cholesterol and LDL-cholesterol concentrations were reduced in obese rats fed either the CLA diet or the VA+CLA diet as compared to the obese control group ($p < 0.05$, $p < 0.001$; $p < 0.001$, $p < 0.01$; $p < 0.01$, $p < 0.001$, respectively). The VA+CLA diet reduced plasma TG and LDL-cholesterol to the level of the normolipidemic lean rats and further decreased nonesterified fatty acids compared to the CLA diet alone. Interestingly, rats fed the VA+CLA diet had a higher food intake but lower body weight than the CLA fed group ($P < 0.05$). Liver weight and TG content were lower in rats fed either CLA ($p < 0.05$) or VA +CLA diets ($p < 0.001$) compared to obese control, consistent with a decreased relative protein abundance of hepatic acetyl-CoA carboxylase in both treatment groups ($P < 0.01$). The activity of citrate synthase was increased in liver and adipose tissue of rats fed, CLA and VA+CLA diets ($p < 0.001$) compared to obese control, suggesting increased mitochondrial fatty acid oxidative capacity.

Conclusion: We demonstrate that the hypolipidemic effects of chronic *cis*-9, *trans*-11 CLA supplementation on circulating dyslipidemia and hepatic steatosis are enhanced by the addition of VA in the JCR:LA-*cp* rat.

Introduction

Conjugated linoleic acid (CLA) is a term that refers to diverse positional and geometrical isomers of linoleic acid and its numerous health related effects have been extensively investigated. CLA was first described as a potent anti-carcinogenic component and more recently has been associated with improving dyslipidemia, insulin sensitivity and the pro-inflammatory state related to obesity and the metabolic syndrome [1,2]. However, some animal studies (specifically those using mouse models), in addition to a handful of clinical trials, have indicated that the major isomers found in CLA mixtures (*cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) are responsible for different physiological effects [1-10].

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CLA is found naturally in ruminant-derived lipids and *cis*-9, *trans*-11 CLA is the major natural isoform, accounting for about 80-90% of the total CLA isomers [11]. *Trans*-11 vaccenic acid (VA) is the precursor to endogenous synthesis of the *cis*-9, *trans*-11 CLA isomer in rats [12,13] and humans [14], and is the predominant isomer of the total *trans* fatty acids found in ruminant-derived fats such as dairy and meat products. We have shown previously that unlike industrially produced *trans* fatty acids, VA has lipid-lowering properties associated with reduced hepatic lipogenesis and chylomicron secretion in the obese and insulin resistant JCR:LA-*cp* rat [15]. Interestingly, our observations indicate that VA has neutral effects under normolipidemic conditions and induces hypotriglyceridemic effects under conditions of dyslipidemia [16]. We also observed that VA supplementation for 16 weeks had a greater potential to influence lipoprotein metabolism [15] compared to a shorter term feeding [16]. These findings are supported by several clinical [17-19] and animal studies [20-24] showing that dietary *trans* fats derived from ruminants have either neutral or beneficial effects on cardiovascular disease risk factors compared to industrially produced *trans* fats.

As a result of the increasing evidence associating CLA and more recently VA with health benefits, there has been a growing interest to increase the concentrations of these natural *trans* fats in meat and dairy products [25-28]. Interestingly, both VA and CLA (*cis*-9, *trans*-11) can account for more than 15% of the total fat in naturally enhanced dairy products [26] which could provide an additional health value to animal-derived fats. Consequently, in this study we hypothesized that chronic supplementation with both CLA and VA would enhance the lipid lowering effects to improve whole body lipid metabolism. Therefore, the aim of this study was to evaluate the effect of dietary supplementation with purified forms of both *cis*-9, *trans*-11 CLA and VA on impaired lipid metabolism in an established animal model of the metabolic syndrome, the JCR:LA-*cp* rat.

Materials and methods

Animals and diets

All experimental procedures were approved by the University of Alberta Animal Ethics Committee and conducted in accordance with the Canadian Council on Animal Care. Twenty four male obese JCR:LA-*cp* rats (*cp/cp*) were raised in our established breeding colony at the University of Alberta as previously described [29]. At 3 wk of age, rats were transferred from the isolated breeding colony areas to an individually ventilated caging environment (TecniplastTM, Exton PA, USA) and had access to a standard rat chow diet (5001, PMI Nutrition International) (see Additional file 1). At 8 wk of age, rats (n = 8) were randomized and assigned to one of three diets for 16 wk (control and experimental diets) and had free access to food and water. Age and weight matched lean littermates (n = 8) were fed the control diet to mimic cholesterol/high fat feeding under normolipidemic conditions. Food intake and body weight (BW) were monitored weekly throughout the study. At 23 wk of age, a meal tolerance test (MTT) as previously described [30], was performed in four randomly chosen rats from control and treatment groups in order to determine plasma glucose and insulin concentrations after a meal. We also performed an oral fat challenge test (OFC), as previously described [29] in four additional rats from each group. At the end of the treatment period (24 wk of age), rats were fasted overnight and anesthetized using isofluorane anesthesia. Plasma was sampled from the left ventricle and heart, liver and fat pads were excised, weighted and immediately frozen at -80°C until analysis. Adipose fatty acid composition was measured from total triglyceride on the epidydimal fat pad as previously described [16].

Three iso-caloric diets were prepared with a constant polyunsaturated to saturated fatty acid ratio (P:S) of 0.4. A control diet (CD) was supplemented (w/w) with 1% cholesterol and contained 42% of energy from carbohydrate, 23.7% from protein and 34.3% from fat. Experimental diets were prepared by adjusting the lipid composition of the CD to provide 1.0% w/w of *cis*-9, *trans*-11 CLA alone (CLA), both 1% of VA and 1% w/w of *cis*-9, *trans*-11 CLA (VA+CLA). Semi-purified *cis*-9, *trans*-11 CLA (G-c9t11 80:20) containing 59.8% of *cis*-9, *trans*-11 CLA and 14.4% of *trans*-10, *cis*-12 CLA was kindly provided by Lipid Nutrition. The amount of CLA and VA (1% w/w) was chosen based on previous studies allowing for metabolic sufficiency while maintaining a normal dietary fatty acid proportion [15-17,31]. Purified VA was synthesized by a chemical alkali isomerisation from linoleic acid-rich vegetable oil [32]. The diet mixture was extruded into pellets, dried at RT°C and stored at 4°C. Fatty acid composition of the three diets was confirmed by gas chromatograph analysis [28] of the fat blend samples (Table 1).

Plasma biochemical components

The concentration of biochemical parameters in fasting plasma from lean and obese groups were assessed using commercially available homogenous, enzymatic colorimetric assays. Triglyceride (TG) (Wako Pure Chemical Industries, catalog no. 998-40391, 0.01 mmol/L minimum), Total cholesterol (TC) (Wako Pure Chemical Industries, catalog no. 993-00404, 0.002 mmol/L minimum), LDL cholesterol (LDL-C) (Wako Pure Chemical Industries, catalog no. 993-00404, 0.03-10.4 mmol/L)

Table 1 Fatty acid composition (% of total fatty acids) of control and experimental diets

Fatty acid	Control diet (CD)	CLA diet	VA+CLA diet
C16:0	9.1	8.5	9.1
C18:0	47.3	46.9	44.3
18:1 *t*-11 (VA)	ND	ND	5.6
18:1 *c*-9 (OA)	17.3	11.4	10.5
18:1 *c*-11	ND	0.5	0.5
C18:2 n6 (LA)	23.3	23.4	20.4
C18:3 n3 (ALA)	1.6	1.7	1.6
CLA *c*-9, *t*-11	0	5.2	3.9
CLA *t*-10, *c*-12	ND	1.1	0.8
other CLA	ND	0.3	0.3
Summary			
∑ total SFA[2]	57.2	56.3	54.2
∑ C12:0, C14:0, C16:0[3]	9.1	8.5	9.1
∑ *cis* MUFA[4]	17.4	12.0	11.1
∑ PUFA[5]	25.0	25.1	22.0
∑ n-6 PUFA	23.4	23.4	22.0
∑ n-3 PUFA	1.6	1.7	1.6
P/S ratio[6]	0.4	0.4	0.4
∑ CLA	0.0	6.6	5.0

[1.] No detectable

[2] Sum of all saturated fatty acids

[3] Sum of lauric, myristic and palmitic acids

[4] *cis* MUFA, sum of all monounsaturated excepting *trans* fatty acids

[5] Sum of all polyunsaturated fatty acids excepting CLA

[6] Ratio of polyunsaturated to saturated fatty acids

and nonesterified fatty acids (NEFA) (HR Series NEFA-HR, catalog no. 999-34691, Wako Diagnostics) were measured using direct colorimetric chemical enzymatic reactions. Plasma glucose was measured as per the glucose oxidase method (Diagnostic Chemical, catalog no. 220-32, 0.03-33.3 mmol/L) and plasma insulin was determined using commercially available enzymatic immunoassays for rodents (Ultrasensitive rat insulin ELISA, Mercodia, catalog no. 80-INSRTU-E01, 0.03-1.0 pmol/L). Samples were analyzed in triplicate using assay kits from a single lot and performed in one single batch.

Tissue homogenization and hepatic TG

Liver and adipose tissue samples (0.5 g) were homogenized in 200 μL lysis buffer [PBS (pH 7.4) with 1.5% TritonX-100 and 1% protease inhibitor cocktail (Sigma)] and and hepatic TG levels were determined by a commercially available enzymatic colorimetric assay (Wako Pure Chemical Industries, catalog no. 998-40391, 0.01 mmol/L minimum), using an aliquot of the whole homogenate and adjusting by the protein concentration of the homogenate [15]. The remainder of the homogenate was centrifuged at 700 g for 15 min and the supernatant was collected and stored at -80C for western blot and citrate synthase activity analysis.

Hepatic and adipose tissue citrate synthase activity

Citrate synthase activity in liver and adipose tissue samples was determined using a commercially available kit from Sigma (catalog no. CS0720). The coefficient of variation of this assay in our laboratory is < 10%. The citrate synthase activity was expressed as μmol/min/g protein.

Relative protein abundance of lipogenic enzymes

Hepatic acetyl-CoA carboxylase-1 (ACC-1) and fatty acid synthase (FAS) were determined by western blot analysis as described elsewhere [33] with few modifications [15]. ACC-1 and FAS relative abundance were normalized based on the respective β-actin protein mass (internal control).

Statistical analysis

Statistical analysis was performed using the Graph pad Prism software, version 4.0. Data was tested for normal distribution and one-way ANOVA followed by Tukey post-hoc tests were used to identify differences among both lean and obese controls and treatment groups (CLA and VA+CLA). Post-prandial glucose and insulin metabolism as well as post-prandial TG response were assessed by area under the curve (AUC) analysis. Fasting concentrations of these parameters were further subtracted from the total AUC to yield the incremental area under the curve (iAUC). Results are expressed as means ± SEM and the level of significance was set at $p < 0.05$.

Results

Food intake, body weight and body composition

Obese rats fed the combination of VA+CLA showed increased food intake compared to those obese rats fed either the CD or CLA diet. Paradoxically, the VA+CLA fed rats showed reduced body weight ($p < 0.001$) compared to the CLA group (Table 2). Despite the higher body weight of rats fed the CLA diet as compared to obese control, no difference was observed in absolute and relative heart weights or fat pad deposition ($p > 0.05$), as measured by the amount of absolute and relative perirenal and inguinal fat pad weights compared with the obese rats fed the CD (Table 2). In contrast, feeding either the CLA or the VA+CLA diet resulted in a lower absolute liver weight by 15% and 26%, respectively, and both diets reduced the ratio of liver weight to total body weight by 22% as compared to obese rats fed the CD ($p < 0.001$).

Citrate synthase activity in liver and adipose tissue

The activity of citrate synthase in liver and adipose tissue is shown in Figure 1. There was a higher ($p < 0.001$) citrate synthase activity in liver and inguinal adipose

Table 2 Food intake, body weight and body composition of rats in dietary groups

	Dietary groups			
	Lean control	**Obese control**	**Obese CLA**	**Obese VA+CLA**
Food intake (g/day)	19.7 ± 0.3^{c}	32.4 ± 0.5^{b}	31.4 ± 0.6^{b}	36.1 ± 0.6***^{a}
BW/16 wk (g)	384 ± 8.2^{c}	646 ± 9.3^{b}	702 ± 17.9^{a}*	624 ± 9.5^{b}
Heart (g)	0.89 ± 0.0^{b}	1.19 ± 0.0^{a}	1.26 ± 0.0^{a}	1.23 ± 0.0^{a}
weight, %BW	0.23 ± 0.0^{a}	0.18 ± 0.0^{b}	0.18 ± 0.0^{b}	0.20 ± 0.0^{b}
Liver (g)	9.1 ± 0.4d	23.1 ± 0.7^{a}	19.7 ± 0.6***^{b}	17.2 ± 0.3***^{c}
weight, %BW	2.4 ± 0.1^{c}	3.6 ± 0.1^{a}	2.8 ± 0.1***^{b}	2.8 ± 0.0***^{b}
Perirenal FP[1] (g)	1.3 ± 0.1^{c}	7.4 ± 0.4^{ab}	8.6 ± 0.8^{a}	6.7 ± 0.4^{b}
weight, %BW	0.33 ± 0.0^{b}	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.1 ± 0.1^{a}
Inguinal FP (g)	1.4 ± 0.2^{b}	17.9 ± 1.1^{a}	18.5 ± 1.0^{a}	15.7 ± 1.0^{a}
weight, %BW	0.36 ± 0.0^{b}	2.8 ± 0.2^{a}	2.6 ± 0.1^{a}	2.5 ± 0.1^{a}

Values are means ± SEM, n = 8. Means in the same raw with different symbol are significantly different as compared to obese control; *P < 0.05, **P < 0.01, ***P < 0.001. Means in the same raw with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA).
FP[1]= Fat pad

tissue after feeding either CLA or VA+CLA diets as compared to lean and obese rats fed the CD. Interestingly, the citrate synthase activity in liver and adipose tissue did not differ between lean and obese rats fed the CD ($p > 0.05$).

Fatty acid profile in epididymal adipose tissue triglyceride

The fatty acid composition in adipose tissue triglyceride was shown to directly reflect the dietary fatty acid composition as shown in Table 3. Obese rats fed with the CLA and VA+CLA diets had a markedly increased proportion of the *cis*-9, *trans*-11 CLA isomer relative to the obese control group. The content of this isomer, expressed as a percentage of total fatty acids was different between the treated groups. The CLA diet showed the greatest incorporation of the cis-9, trans-11 CLA isomer (70 fold greater than control group) compared to the VA+CLA diet, which was only 46 fold higher than the obese control group. Rats fed VA+CLA diet showed a greater incorporation of *trans*-11 18:1 (VA) compared to CLA and obese control rats ($p < 0.001$). Interestingly, rats fed the VA+CLA diet had lower proportions of linoleic acid (18:2 n6), α-linolenic acid (18:3 n3) and arachidonic acid (20:4 n6).

Fasting plasma lipid, glucose and insulin concentrations

As shown in Table 4, fasting plasma TG, TC and LDL-C were significantly lower in obese rats fed either the CLA or the VA+CLA diet, as compared to the CD. However, feeding the VA+CLA diet further reduced plasma NEFA concentration relative to the CLA fed rats and lowered TG and LDL-C concentrations not different from lean rats fed the CD ($p > 0.05$). The CLA or VA+CLA diet lowered fasting insulin to that comparable of lean rats ($p > 0.05$) and reduced total insulin concentration (AUC) after the MTT ($p < 0.05$). There was no

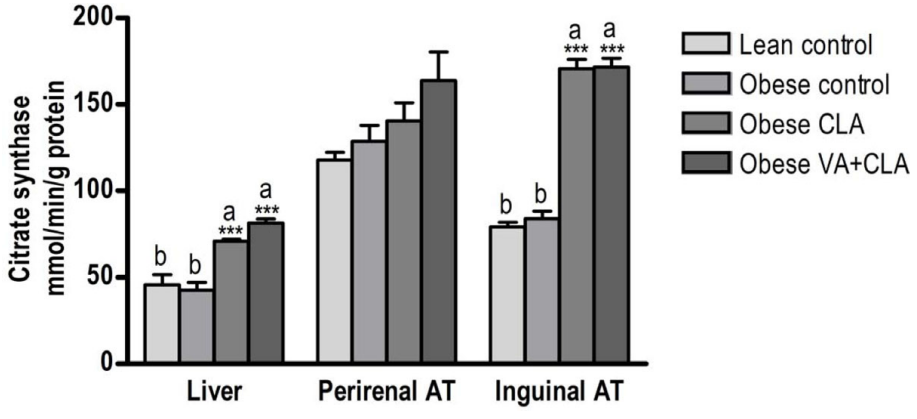

Figure 1 Citrate synthase activity in liver, perirenal and inguinal adipose tissue. Values are mean ± SEM, n = 8. Means with different symbol are significantly different as compared to obese control; *P < 0.05, **P < 0.01, ***P < 0.001. Means with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA). AT, adipose tissue.

Table 3 Fatty acid composition (% of total fatty acids) of triglyceride in epididymal adipose tissue

	Dietary groups			
	Lean control	**Obese control**	**Obese CLA**	**Obese VA+CLA**
Fatty acid				
C18:0	13.91 ± 0.71^{a}	6.36 ± 0.14^{b}	5.47 ± 0.07^{b}	6.31 ± 0.17^{b}
C18:1 *t*-11 (VA)	0.05 ± 0.01^{b}	0.02 ± 0.00^{b}	0.08 ± 0.00^{b}	1.72 ± 0.06***^{a}
C18:1 *c*-9	28.37 ± 0.14^{d}	37.9 ± 0.56^{a}	32.72 ± 0.32***^{c}	35.21 ± 0.49**^{b}
C18:2 n6	35.76 ± 0.22^{a}	20.51 ± 0.24^{b}	20.15 ± 0.25^{b}	15.06 ± 0.52***^{c}
C18:3 n3	1.2 ± 0.03^{a}	0.91 ± 0.02^{b}	0.96 ± 0.02^{b}	0.47 ± 0.06***^{c}
CLA *c*-9, *t*-11	0.05 ± 0.01^{c}	0.04 ± 0.01^{c}	2.85 ± 0.04***^{a}	1.91 ± 0.09***^{b}
C20:4 n6	0.45 ± 0.01^{a}	0.33 ± 0.25^{b}	0.32 ± 0.01^{b}	0.22 ± 0.0**^{c}

Values are mean ± SEM, n = 8. Means in the same raw with different symbol are significantly different as compared to obese control; *P < 0.05, **P < 0.01, ***P < 0.001. Means in the same raw with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA). Long chain n3 PUFA were not detectable.

statistical difference between groups for either glucose metabolism (fasting or iAUC) or the relative change (iAUC) in insulin.

Post-prandial plasma TG response

Obese rats had a higher post-prandial plasma TG response (iAUC) compared to lean rats following an OFC (Figure 2). AUC analysis showed an improved total TG concentration ($p < 0.05$) over the 10-h post-prandial period in rats fed either the CLA or VA+CLA diet (35 ± 5 and 38 ± 8 mmol/L.h, respectively), compared to obese rats fed the CD (62 ± 5 mmol/L.h). However, the post-prandial iAUC for TG was not different between obese control and obese treatment groups (CLA and VA +CLA).

Liver TG concentration and relative abundance of hepatic lipogenic enzymes

Liver TG concentration was higher in obese control rats compared to lean control rats. However, feeding the CLA diet resulted in a liver triglyceride concentration that was 22% lower than the obese rats fed the CD (Table 4). Interestingly, the VA+CLA diet further lowered liver TG concentration by 43% and 27% as compared to obese control and CLA groups, respectively. In addition, CLA and VA+CLA diets resulted in significantly lower hepatic ACC-1 protein abundance relative to obese control (34% and 38%, respectively) and was normalized to concentrations similar to lean rats fed the CD ($p > 0.05$) (Figure 3A, B). The relative abundance of hepatic FAS protein did not differ between the obese groups ($p > 0.05$) (Figure 3A, C).

Discussion

Combination of VA+CLA increases food intake and decreases liver weight

Dyslipidemia and insulin resistance are common features associated with cardiovascular disease and the metabolic syndrome. The homozygous obese (*cp/cp*), JCR:LA-*cp* rat, has a complete absence of the leptin receptor. As a consequence, the JCR:LA-*cp* rat spontaneously develops hyperphagia and obesity, associated dyslipidemia, insulin resistance, and macro-and microvascular dysfunction [34-36]. In the present study, we

Table 4 Fasting plasma lipid concentrations, glucose and insulin AUC after MTT and hepatic TG

	Dietary groups			
	Lean control	**Obese control**	**Obese CLA**	**Obese VA+CLA**
TG (mmol/L)	0.47 ± 0.0^{c}	3.28 ± 0.4^{a}	2.05 ± 0.3*^{b}	1.4 ± 0.1***^{bc}
NEFA (mmol/L)	0.20 ± 0.02^{c}	0.48 ± 0.02^{ab}	0.57 ± 0.03^{a}	0.42 ± 0.05^{b}
TC (mmol/L)	2.26 ± 0.0^{c}	6.18 ± 0.4^{a}	4.22 ± 0.2***^{b}	4.92 ± 0.2**^{b}
LDL-C (mmol/L)	0.97 ± 0.1^{c}	2.28 ± 0.2^{a}	1.56 ± 0.1**^{b}	1.2 ± 0.1***^{bc}
Fasting glucose (mmol/L)	5.94 ± 0.08	6.81 ± 0.4	5.94 ± 0.36	6.75 ± 0.72
Glucose iAUC (mmol/L.h)	45.19 ± 4.5	52.22 ± 7.8	70.25 ± 14.7	42.7 ± 33.05
Fasting insulin (μIU/L)	73.41 ± 27.4^{b}	629.2 ± 165.3^{a}	216 ± 13.6*^{b}	372.8 ± 98.24^{ab}
Insulin AUC (μIU/L.h)	8739 ± 1566 ^{c}	40437 ± 4384 ^{a}	24548 ± 3465*^{b}	23001 ± 2744*^{b}
Insulin iAUC (μIU/L.h)	4452 ± 1464	11805 ± 7603	11585 ± 3885	4451 ± 3015
Liver TG (mmol/g protein)	2.2 ± 0.3^{d}	21.2 ± 1.0^{a}	16.6 ± 1.1*^{b}	12.1 ± 1.3***^{c}

Plasma lipid and liver TG values are mean ± SEM, n = 8. Insulin AUC is mean ± SEM, n = 4. Means in the same raw with different symbol are significantly different as compared to obese control; *P < 0.05, **P < 0.01, ***P < 0.001. Means in the same raw with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA).

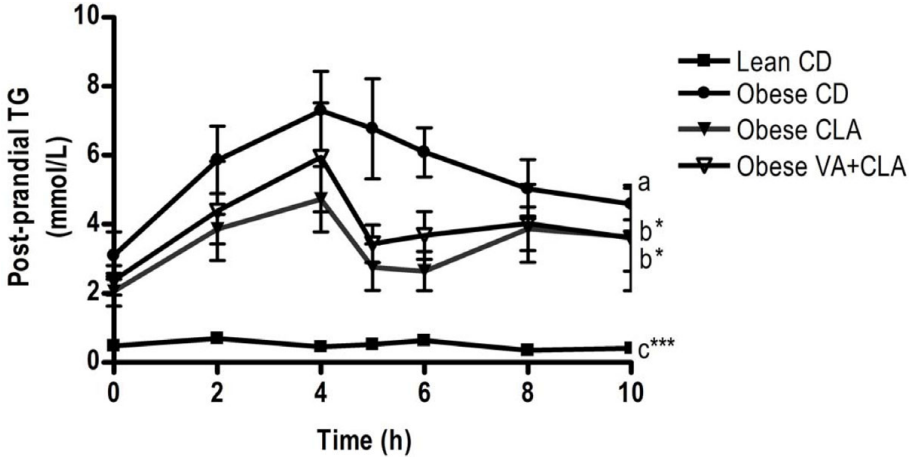

Figure 2 Post-prandial triglyceride response following an oral fat challenge. Values are mean ± SEM, n = 4. AUC differ relative to obese rats fed the CD; $^{b}P < 0.05$, $^{c}P < 0.001$. iAUC differ relative to obese rats fed the CD; ***P < 0.001.

used this unique animal model to evaluate hypolipidemic effects of *cis*-9, *trans*-11 CLA in combination with VA. Obese rats fed the CLA diet had a higher final body weight relative to obese control rats. However, this was not associated with an increase in fat deposition. *Cis*-9, *trans*-11 CLA has been observed to regulate metabolic pathways involved in fatty acid oxidation as well as energy production and thermogenesis [37-40]; therefore we investigated whether combination VA +CLA might promote fatty acid oxidation.

CLA and VA+CLA promote mitochondrial fatty acid oxidation in liver and adipose tissue

Peroxisome proliferator-activated receptor (PPAR)-agonists, such as thiazolidenediones (TZDs), are effective drugs for the treatment of type 2 diabetes by inducing adipogenesis as well as increasing the uptake and metabolism of free fatty acids in adipose tissue. Increased mitochondrial oxidative capacity of white adipose tissue has been observed after treatment with TZD [41,42]. CLA is a natural PPAR ligand [43-45] and has been observed to promote fatty acid oxidation [40] or TG synthesis in adipose tissue contributing to lower circulating NEFA and TG concentrations [7]. We observed a significant increase in citrate synthase activity in liver and inguinal adipose tissue following supplementation with either CLA or VA+CLA diet but not in perirenal adipose tissue. Interestingly, citrate synthase activity was not different between lean and obese control rats. It has been reported that insulin resistance is associated with an increase in muscle mitochondrial content and oxidative capacity [46-48]. Similarly, it has been demonstrated that mitochondrial biogenesis increases during adipose tissue differentiation [49]. Therefore, it can be proposed that JCR:LA-*cp* rats maintain similar mitochondrial fatty acid oxidation relative to lean rats but during increased dietary lipid consumption, it is insufficient to prevent TG deposition. Treatment with CLA or VA+CLA may stimulate mitochondrial fatty acid oxidation and in turn this may contribute to improvements in liver and adipose tissue metabolism in the JCR:LA-*cp* rat.

Incorporation of CLA and VA in adipose tissue triglyceride

It is well established that the fatty acid composition of adipose tissue is dependent on dietary intake. However, endogenous synthesis of fatty acids, fatty acid transport and inter-conversion processes (elongation and desaturation) are also significant contributing factors to the composition of adipose tissue [50]. As expected, supplementation with *cis*-9, *trans*-11 CLA (CLA diet) and VA (VA+CLA diet) increased the proportion of these fatty acids in adipose tissue from obese rats. We also wish to note that the endogenous synthesis of *cis*-9, *trans*-11 CLA from VA may have also occurred in this study. In both humans and animals, the conversion of dietary VA to CLA has been reported to be at a rate of approximately 12-19% [12 and 14]. We also note that the dietary ratio of VA:CLA in this study was ~1.5:1, respectively (Table 1) and that the resultant incorporation of these fatty acids into adipose tissue was found to be 1:1 following supplementation (Table 3). While we cannot infer a rate of conversion from VA to CLA *per se* from this data, it would support that previously published.

Combined dietary VA+CLA has a greater effect to reduce dyslipidemia and hepatic steatosis

One of the most striking effects of the combined treatment (VA+CLA) in the present study was the reduction

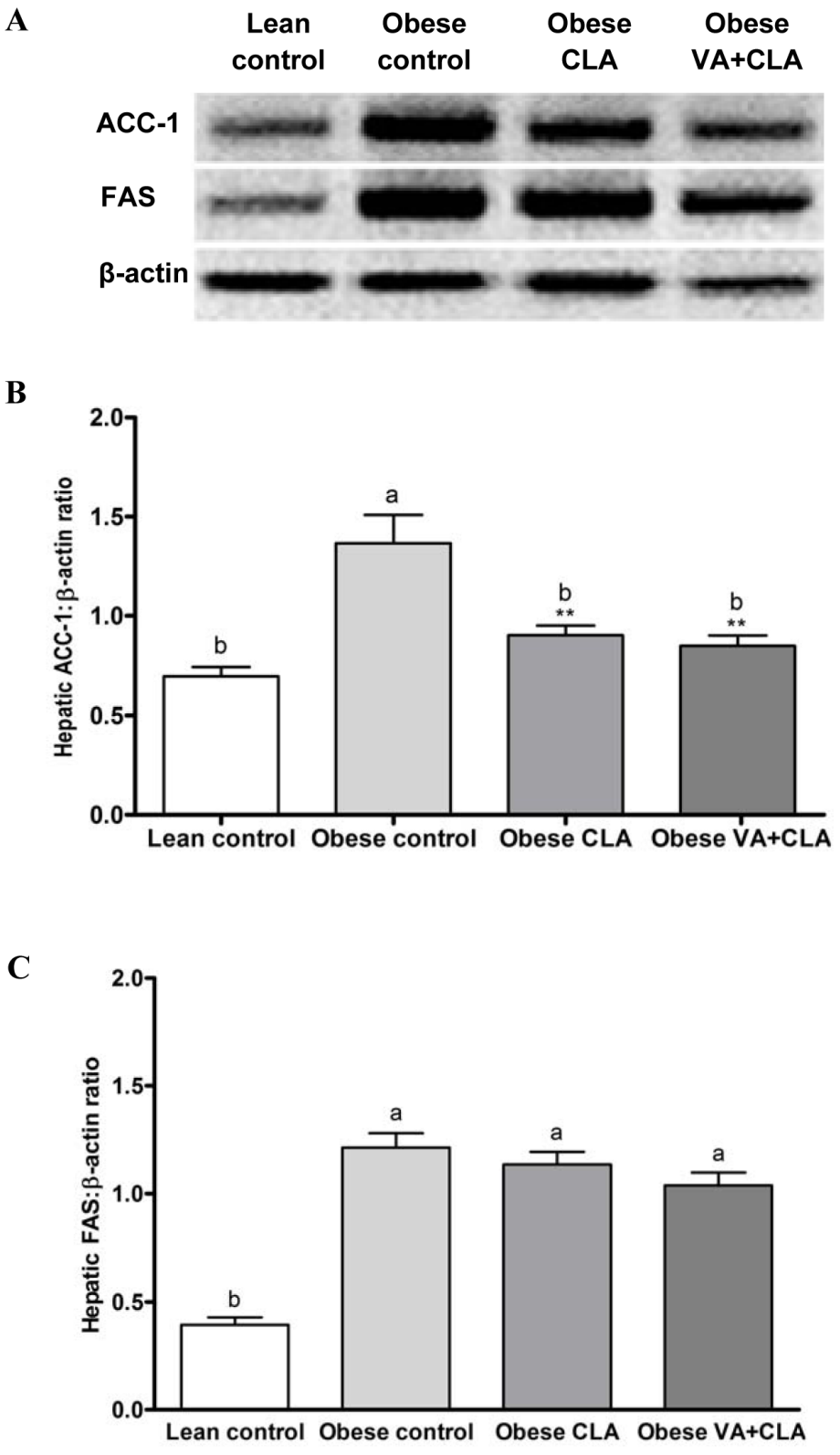

Figure 3 Effects of CLA and VA+CLA on hepatic protein abundance of lipogenic enzymes. Western blots of hepatic lipogenic enzymes (A), and relative abundance of ACC-1 (B) and FAS (C). Values are mean ± SEM, n = 8. Means with different symbol are significantly different as compared to obese rats fed the CD; *P < 0.05, **P < 0.01, ***P < 0.001. Means with different letter are significantly different among control (lean and obese) and obese treated groups (CLA and VA+CLA).

in hepatic TG concentration. The improvement in fasting lipid parameters by VA+CLA diet (i.e. TG and LDL-C) suggest an additional benefit with the combination diet and is consistent with previous observations that dietary VA has lipid lowering properties independent from CLA [15]. We also wish to note that both diets (CLA and VA+CLA) consistently contained 0.15 w/w of the *trans*-10, *cis*-12 CLA isomer, which is also known for its hypolipidemic properties.

ACC-1 and FAS are two key lipogenic enzymes involved in the synthesis of fatty acids and subsequent TG synthesis. TG is then either stored as lipid droplets within the hepatocyte, secreted into the blood compartment as VLDL or hydrolyzed via oxidation [51]. It is plausible that reduced hepatic lipogenesis may contribute (at least in part) to reduced hepatic TG in rats fed either the CLA or VA+CLA diet, which is supported by a lower hepatic ACC-1 protein abundance relative to obese rats fed the CD. We have reported previously that VA may act in part via ACC-1 and FAS pathways resulting in reduced VLDL secretion to decrease circulating concentrations of plasma TG and LDL-C [15]. However, in conditions of insulin resistance, hepatic steatosis is thought to be caused by an increased free fatty acid flux from adipose tissue into the liver [52]. As discussed above, rats fed the combined treatment (VA+CLA) showed lower circulating NEFA concentrations compared to rats fed the CLA diet alone. We propose that reduced NEFA may also contribute to a further decrease in hepatic TG. In addition, activation of ACC is regulated by phosphorylation/dephosphorylation [53] and thus, it may be possible that CLA and VA+CLA diets may differently regulate post-translational modifications of ACC-1.

Conclusion

In conclusion, results in this study confirm hypolipidemic effects of chronic supplementation with *cis*-9, *trans*-11 CLA alone or in combination with *trans*-11 VA in the dyslipidemic and insulin resistant JCR:LA-*cp* rat. Our data also support the hypothesis that a dietary formulation enriched with both CLA and VA may further enhance their hypolipidemic properties, particularly during conditions of hypertriglyceridemia, hypercholesterolemia and/or hepatic steatosis.

Additional material

Additional file 1: Changes in body weight throughout the study and schematic representation of the experimental design. Rats (n = 8) were fed a standard chow diet prior to the study (from 3-8 wk of age). Then, control and experimental diets were provided for 16 wk. An oral fat challenge test (OFC) and a meal tolerance test (MTT) were conducted on different rats (n = 4 in each test).

List of abbreviations

ACC-1: acetyl-CoA carboxylase-1; AUC: area under the curve; iAUC: incremental area under the curve; CD: control diet; CLA: *cis*-9, *trans*-11 CLA; FAS: fatty acid synthase; MTT: meal tolerance test; NEFA: nonesterified fatty acid; OFC: oral fat challenge; TC: total cholesterol; TG: triglyceride; VA: *trans*-11 vaccenic.

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Authors' contributions

MMJS, JL, CJF, DFV, and SDP contributed to research design; MJR and JS provided essential reagents; MMJS, JL, YW, MRR and DCW conducted research and analyzed data; MMJS, CJF, DFV, and SDP contributed to the writing of the manuscript; MMJS and SDP had primary responsibility for the final content. All authors read and approved the final manuscript.

Competing interests

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Appendix 'B'

Supp. Table 4-1 Fat composition of designed control and VA diets

Fat ingredient	Control diet	VA diet
	g/kg of diet	
Butter	48.75	57.3
Sunflower oil	14.25	21.6
Flaxseed	3.75	3.9
Fully hydrogenated canola oil	6.75	6.6
Olive oil	52.5	28.5
Lard	24	19.4
Vaccenic acid	0	12.66

Butter fat was processed from local milk provided by the Dairy Research and Technology Center, University of Alberta. VA was chemically synthesized by alkali isomerisation from linoleic acid-rich vegetable oil. Values are expressed as g per kg of diet.

Supp. Table 4-2 Fatty acid composition of control and VA diets[1]

Fatty acid	Control diet	VA diet
	g/100 FAME*	
C12:0	1.0	1.2
C14:0	3.6	4.2
C14:1	0.3	0.4
C16:0	19.0	18.1
C16:1	1.2	1.0
C18:0	11.0	10.9
C18:1 *trans*-9	0.5	0.5
C18:1 *trans*-11 (VA)	1.3	8.8
C18:1 *cis*-9 (oleic acid)	35.6	27.9
C18:1 *cis*-11	1.9	1.4
C18:2 n6	13.7	13.9
C18:3 n3	1.7	1.7
*cis*9, *trans*11 CLA	0.2	0.3
Summaries		
∑SFA	37.5	37.6
∑C12:0, C14:0, C16:0	23.6	23.5
PUFA	15.6	15.8
*cis*MUFA	40.0	31.6
*trans*MUFA	4.2	11.4
n6	13.8	13.8
n3	1.7	1.7
n6:n3 ratio	7.9	7.9
PUFA:SFA ratio	0.4	0.4

[1]Values are expressed as percentage of fatty acids

*FAME, fatty acid methyl esters

Supp. Table 4-3 Expression of genes known to affect intestinal lipid synthesis, oxidation and transport. Values are the fold change in mRNA expression relative to MetS control rats

	Lean	MetS	MetS+VA
Transcription factors			
PPARα	0.78 ± 0.07	1.05 ± 0.13	0.85 ± 0.10
PPARγ	1.28±0.27	1.16±0.26	1.24±0.14
LXRα	0.80 ± 0.05	1.02 ± 0.09	0.84 ± 0.07
LXRβ	1.01 ± 0.15	1.05 ± 0.13	0.99 ± 0.09
SREBP2	1.12 ± 0.11	1.02 ± 0.10	0.99 ± 0.07
FA synthesis and oxidation			
FAT/CD36	1.12 ± 0.19	1.03 ± 0.11	0.85 ± 0.05
FATP1	1.29 ± 0.30	1.03 ± 0.11	0.87 ± 0.08
FATP4	0.85 ± 0.11	1.02 ± 0.08	1.03 ± 0.05
L-FABP	0.69 0.04	1.02 ± 0.09	1.12 ± 0.05
I-FABP	1.00 ± 0.13	1.02 ± 0.09	0.99 ± 0.07
ACLY	1.08 ± 0.14	1.00 ± 0.03	0.88 ± 0.09
ME1	0.62 ± 0.08 [a]	1.01 ± 0.05 [b]	0.95 ± 0.09 [b]
ACC	1.15 ± 0.17	1.00 ± 0.03	0.93 ± 0.09
SCD1	0.08 ± 0.03 [a]	1.17 ± 0.28 [b]	0.87 ± 0.15 [b]
AMPKα	0.93 ± 0.10	1.02 ± 0.08	1.00 ± 0.08
CPT1α	0.87 ± 0.10	1.01 ± 0.08	0.97 ± 0.07
ACOX1	0.83 ± 0.11	1.02 ± 0.09	0.96 ± 0.08
MCAD	0.84 ± 0.05	1.01 ± 0.05	0.95 ± 0.05
LCAD	0.79 ± 0.05	1.02 ± 0.09	1.10 ± 0.08
VLCAD	0.83 ± 0.11	1.01 ± 0.07	1.13 ± 0.07
Lipid synthesis			
MGAT1	1.50 ± 0.30	1.34 ± 0.34	2.17 ± 0.19

MGAT2	0.96 ± 0.07	1.00 ± 0.04	0.90 ± 0.04
GPAT1	1.17 ± 0.18	1.02 ± 0.09	0.90 ± 0.10
GPAT3	0.78 ± 0.18	1.02 ± 0.10	0.91 ± 0.08
DGAT1	0.94 ± 0.03	1.01 ± 0.06	1.00 ± 0.03
DGAT2	0.89 ± 0.05	1.02 ± 0.07	0.97 ± 0.05
AGPAT2	0.87 ± 0.05	1.01 ± 0.07	1.03 ± 0.07
Cholesterol synthesis/transport			
HMGCoAr	1.18 ± 0.17	1.11 ± 0.18	0.84 ± 0.14
SCAP	1.09 ± 0.17	1.02 ± 0.10	0.88 ± 0.07
INSIG1	0.82 ± 0.10	1.04 ± 0.13	0.86 ± 0.07
NPC1L1	1.01 ± 0.04	1.01 ± 0.05	1.03 ± 0.10
ACAT	1.42 ± 0.12	1.06 ± 0.14	1.09 ± 0.06
ABCA1	1.20 ± 0.19	1.06 ± 0.16	1.07 ± 0.07
ABCG5	0.92 ± 0.03	1.03 ± 0.11	1.05 ± 0.03
ABCG8	0.89 ± 0.06	1.04 ± 0.13	0.90 ± 0.09
apoB	0.94 ± 0.09	1.02 ± 0.09	0.76 ± 0.05
MTP	1.00 ± 0.13	1.02 ± 0.09	1.05 ± 0.07
LDLR	0.77 ± 0.10	1.03 ± 0.10	0.83 ± 0.08

Values are means ± SEM, n=5. Means without a common letter differ ($P<0.05$) as assessed by one-way ANOVA followed by Tukey's post hoc test. Intestinal segments from lean, MetS and Met+VA were collected after an overnight fasting (16 hr). PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma; LXRα, liver X receptor alpha; LXRβ, liver X receptor beta; SREBP2, sterol response element binding protein 2; FAT/CD36, fatty acid translocase/cluster determinant 36; FATP1, fatty acid transporter 1; FATP4, fatty acid transporter 4; L-FABP, liver-type fatty acid binding protein; I-FABP, intestine-type fatty acid binding protein; ACLY, ATP citrate lyase; ME1, malic enzyme 1; ACC, acetyl-coenzyme A carboxylase alpha; SCD1, stearoyl-coenzyme A desaturase 1; AMPKα, AMP-activated

protein kinase alpha; CPT1α, carnitine palmitoyltransferase 1; ACOX1, acyl-coenzyme A oxidase 1, MCAD, medium chain acyl-coenzyme A dehydrogenase; LCAD, long chain acyl-coenzyme A dehydrogenase; VLCAD, very long chain acyl-coenzyme A dehydrogenase; MGAT1, monoacylglycerol O-acyltransferase 1; MGAT2, monoacylglycerol O-acyltransferase 2; GPAT1, glycerol-3-phosphate acyltransferase 1; GPAT3, glycerol-3-phosphate acyltransferase 3; DGAT1, diacylglycerol O-acyltransferase 1; DGAT2: diacylglycerol O-acyltransferase 2; AGPAT2, 1-acyl-sn-glycerol 3-phosphate O-acyltransferase 2; HMGCoAr 3-hydroxy-3-methyl-glutaryl-CoA reductase; SCAP, *SREBP* cleavage-activating protein; INSIG1, insulin induced gene1; NPC1L1, Nieman Pick C 1-like protein 1; ACAT, sterol O-acyltransferase 1; ABCA1, ATP-binding cassette A1; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; LDLR, low density lipoprotein receptor.

Supp.Table 4-4 Expression of genes known to affect hepatic lipid synthesis, oxidation and transport. Values are the fold change in mRNA expression relative to MetS control rats

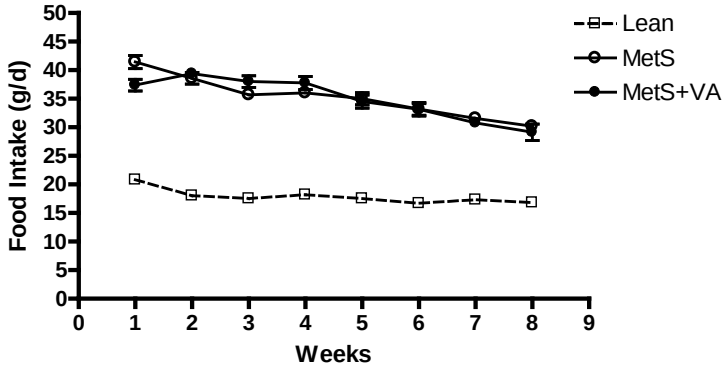
	Lean	MetS	MetS+VA
Transcription factors			
PPARα	1.32 ±0.18	1.04 ± 0.12	1.00 ± 0.09
PPARγ	0.53 ± 0.07	1.08 ± 0.32	1.34± 0.45
LXRα	0.82 ± 0.06[a]	1.01 ± 0.04[b]	1.07 ± 0.05[b]
LXRβ	1.04 ± 0.11	1.01 ± 0.06	1.02 ± 0.09
SREBP1	0.20 ± 0.05[a]	1.1 ± 0.25[b]	0.93 ± 0.03[b]
SREBP2	0.91 ± 0.15	1.02 ± 0.10	0.81 ± 0.05
FA synthesis and oxidation			
FAT/CD36	0.11 ± 0.01 [a]	1.05 ± 0.13 [b]	1.24 ± 0.13 [b]
FATP1	1.09 ± 0.11	1.01 ± 0.07	1.24 ± 0.07
FATP4	1.11 ± 0.10	1.02 ± 0.09	1.03 ± 0.09
L-FABP	1.63 ± 0.06[b]	1.02 ± 0.09[a]	1.14 ± 0.05[a]
ACLY	0.18 ± 0.03	0.78 ± 0.15	0.86 ± 0.11
ME1	0.23 ± 0.05 [a]	1.14 ± 0.30 [b]	1.63 ± 0.20 [b]
FAS	0.05 ± 0.01[a]	1.56 ± 0.82[b]	0.91 ± 0.13[b]
ACC	0.29 ± 0.07 [a]	1.06 ± 0.18 [b]	1.10 ± 0.12 [b]
SCD1	0.03 ± 0.02 [a]	1.11 ± 0.25 [b]	0.72 ± 0.08 [b]
AMPKα	0.86 ± 0.13	1.01 ± 0.04	1.05 ± 0.06
CPT1α	1.30 ± 0.22	1.02 ± 0.07	1.10 ± 0.10
ACOX1	0.92 ± 0.12	1.02 ± 0.10	1.05 ± 0.11
MCAD	1.06 ± 0.08	1.01 ± 0.07	0.97 ± 0.06
LCAD	0.94 ± 0.07	1.01 ± 0.06	1.08 ± 0.07
VLCAD	1.03 ± 0.10	1.01 ± 0.07	1.02 ± 0.09
UCP2	1.39 ± 0.49	1.07 ± 0.18	1.06 ± 0.19

Lipid synthesis			
MGAT1	0.69 ± 0.23	1.04 ± 0.15	0.57 ± 0.10
GPAT1	0.42 ± 0.09 [a]	1.11 ± 0.26 [b]	1.25 ± 0.26 [b]
GPAT3	0.99 ± 0.15	1.01 ± 0.07	1.40 ± 0.17
DGAT1	1.07 ± 0.10	1.02 ± 0.08	1.23 ± 0.09
AGPAT2	0.67 ± 0.05[a]	1.01 ± 0.06[ab]	1.32 ± 0.12[b]
Cholesterol synthesis/transport			
HMGCoAr	1.07 ± 0.17	1.03 ± 0.08	0.84 ± 0.08
SCAP	1.29 ± 0.07 [b]	1.02 ± 0.08 [a]	1.11 ± 0.03 [ab]
INSIG1	0.81 ± 0.13	0.83 ± 0.19	0.40 ± 0.04
ACAT	0.87 ± 0.12	1.02 ± 0.09	0.95 ± 0.03
ABCA1	0.88 ± 0.11	1.02 ± 0.07	0.93 ± 0.05
ABCG5	4.69 ± 0.45 [b]	1.08 ± 0.17 [a]	0.62 ± 0.10 [a]
ABCG8	6.36 ± 0.68 [b]	1.16 ± 0.23 [a]	0.82 ± 0.25 [a]
apoB	1.14 ± 0.10	1.01 ± 0.05	1.05 ± 0.06
MTP	0.75 ± 0.11[a]	1.00 ± 0.04[ab]	1.13 ± 0.08[b]
LDLR	0.75 ± 0.12	1.06 ± 0.16	0.90 ± 0.07

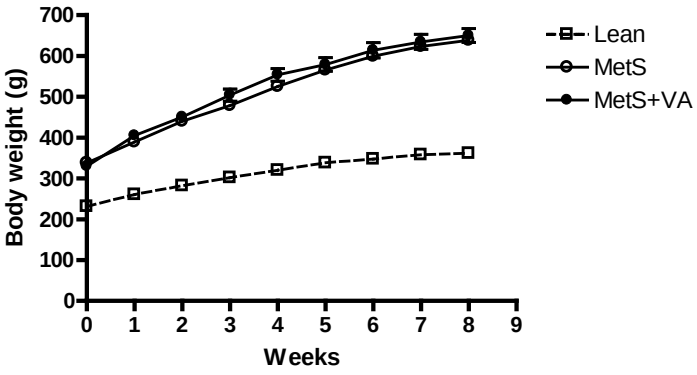
Values are means ± SEM, n=5. Means without a common letter differ ($P<0.05$) as assessed by one-way ANOVA followed by Tukey's post hoc test. Liver segments from lean, MetS and Met+VA were collected after an overnight fasting (16 hr). PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma; LXRα, liver X receptor alpha; LXRβ, liver X receptor beta; SREBP1, sterol response element binding protein 1; SREBP2, sterol response element binding protein 2; FAT/CD36, fatty acid translocase/cluster determinant 36; FATP1, fatty acid transporter 1; FATP4, fatty acid transporter 4; L-FABP, liver-type fatty acid binding protein; ACLY, ATP citrate lyase; ME1, malic enzyme 1; FAS, fatty acid synthase; ACC, acetyl-coenzyme A carboxylase alpha; SCD1, stearoyl-coenzyme A desaturase 1; AMPKα, AMP-activated protein kinase alpha;

CPT1α, carnitine palmitoyltransferase 1; ACOX1, acyl-coenzyme A oxidase 1, MCAD, medium chain acyl-coenzyme A dehydrogenase; LCAD, long chain acyl-coenzyme A dehydrogenase; VLCAD, very long chain acyl-coenzyme A dehydrogenase; UCP2, uncoupling protein 2; MGAT1, monoacylglycerol O-acyltransferase 1; GPAT1, glycerol-3-phosphate acyltransferase 1; GPAT3, glycerol-3-phosphate acyltransferase 3; DGAT1, diacylglycerol O-acyltransferase 1; AGPAT2, 1-acyl-sn-glycerol 3-phosphate O-acyltransferase 2; HMGCoAr 3-hydroxy-3-methyl-glutaryl-CoA reductase; SCAP, *SREBP* cleavage-activating protein; INSIG1, insulin induced gene1; ACAT, sterol O-acyltransferase 1; ABCA1, ATP-binding cassette A1; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; LDLR, low density lipoprotein receptor.

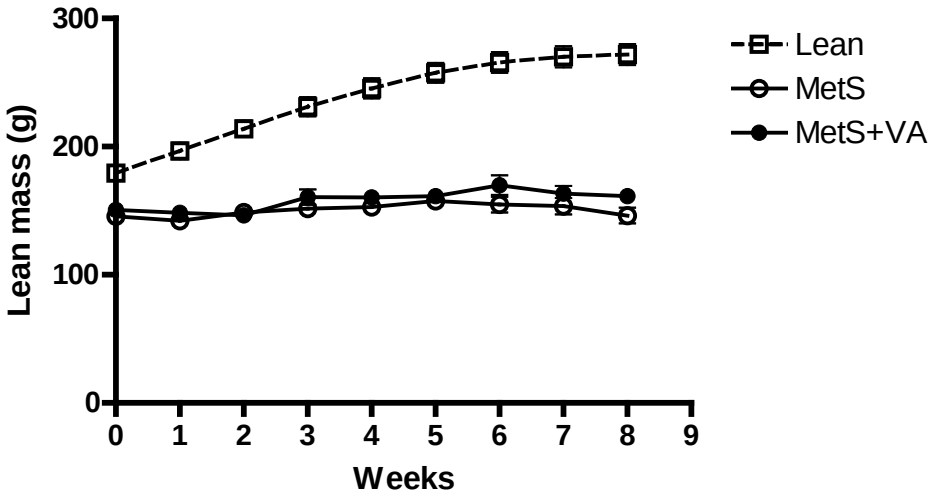
A)



B)



Suppl. Figure 4-1 Food intake (Panel A) and body weight (Panel B) of lean (open squares), MetS (open circles) and MetS+VA rats (black circles). Food intake and body weight did not differ between MetS and MetS+VA rats ($P>0.05$) as assessed by one-way ANOVA followed by Tukey's post hoc test.



Supp. Figure 4-2 Absolute lean mass of lean (open squares), MetS (open circles) and MetS+VA rats (black circles) determined by NMR. Values are means with standard error represented by vertical bars. Mean values did not differ between MetS and MetS+VA rats ($P>0.05$) as assessed by one-way ANOVA followed by Tukey's post hoc test.

Appendix 'C'

Protocol for Extraction and Analysis of Endocannabinoids and Fatty acid fractions (TG and PL) in Tissue

Folch Extraction

Materials and reagents:

- Chloroform
- Methanol
- Internal standard (IS) (preparation steps shown in the Table below)
- Double distilled water
- Glass screw top tubes
- Pasteur pipettes
- Graduated glass pipettes

a) Stock Solution Preparation

Initial concentration of commercial IS	Volume taken from Initial IS	Volume of solvent added to make the stock solution	Stock Solution, final concentration
2-AG-d5 (500ug/500uL)	50 uL*	500 uL (acetonitrile)	100ug/mL
AEA-d8 (100ug/100uL)	50 uL*	500 uL (methanol)	100ug/mL
PEA-d4 (100ug/100uL)	50 uL*	5000 uL (methanol)	10 ug/mL
OEA-d2 (100ug/100uL)	50 uL*	5000 uL (methanol)	10 ug/mL

*dry aliquots of IS under nitrogen before adding the corresponding solvent. Use micropipettes (glass capillaries) for measuring 50 and 100 uL of IS

b) IS 1st Working Solution

Initial concentration of commercial IS	Volume taken from Initial IS	Volume of solvent added to make the stock solution	Stock Solution, final concentration
2-AG-d5 (100ug/mL)	100 uL*	500 uL (acetonitrile)	20 ug/mL
AEA-d8 (100ug/mL)	50 uL*	2.5 mL (methanol)	2 ug/mL
PEA-d4 (10 ug/mL)	100 uL*	500 uL (methanol)	2 ug/mL
OEA-d2 (10 ug/mL)	100 uL*	500 uL (methanol)	2 ug/mL

*dry aliquots of IS under nitrogen before adding the corresponding solvent.

c) IS Working Solution (freshly prepared):
Take 100 uL of each of the above IS 1st working Solutions into a pre-labeled amber vial (5mL), dry under nitrogen and add 4 mL of methanol. So the concentration in each IS working solution is:
2-AG-d5: 500 ng/mL

AEA-d8, PEA-d4, OEA-d2: 50 ng/mL

d) Spike 96 uL of the above IS Working Solutions into each simple (as described in Step #3 below). So, the theoretical IS concentration in the final LC-MS samples is:

2-AG-d5: 200 ng/mL

AEA-d8, PEA-d4, OEA-d2: 20 ng/mL

1. Prepare Folch solution by adding chloroform and methanol (C/M) in a ratio of 2:1 (e.g. for 90 mL of Folch solution use 60 mL of chloroform + 30 mL of methanol).

2. Weight ~300 mg of tissue (record the weight) and place the sample into an appropriately labelled tube. 100-150 mg for brain and intestine.

3. Add in 96 uL of internal standard for endocannabinoids. The internal standard working solution is prepared following the steps in the Table above.

4. Add 9mL of Folch solution to each tube and vortex well and leave the tubes at room temperature (in the dark) for 1 hr.

5. Then add 3 mL of double distilled water to each tube. Mix by gentle inversion (this is crucial) and then let layers separate at room temperature (in the dark) for 1 hr.

6. Next, centrifuge the tubes at 3000 rpm at room temperature for 15-20 min.

7. Draw up 5 mL of the bottom layer with a graduated glass pipette and put into a clean, labelled tube. Alternatively, can aspirate the upper layer first (discard) by using a glass pipette connected to a vacuum flask system. Then can proceed to draw up 5 mL of the bottom layer with a graduated glass pipette and put into a clean, labelled tube.

8. Dry under nitrogen gas at ~30°C (heating block) in the fume hood (samples can be frozen at -20 ºC or stored in the fridge for short term at this point or before drying).

9. Reconstitute the sample with 500 uL of chloroform just before starting solid phase extractions.

Solid Phase Extraction (SPE)

Materials and reagents:

- Chloroform
- Methanol
- Chloroform: methanol (9:1) solution (e.g for 100 mL of solution use 90 mL of chloroform+10 ml of methanol)
- Solid Phase Extraction (SPE) columns (Phenomenex; Strata SI-1 Silica; 8B- S012-HCL; 55 um, 70A; 500 mg, 6 mL)
- Glass screw top tubes (collection tubes)
- Pasteur pipettes (glass transfer pipettes)
- Insulin syringes
- Syringe PTFE membrane filters (VWR International; Cat No. 28145-491; diameter 13 mm, pore size 0.2 um)

- Preparation of pre-measured solvents for each sample:

  To facilitate the process, pre-measurement of solvents saves time. For each sample, label three scintillation vials (20ml) as chloroform, methanol and chloroform:methanol 9:1 solution respectively. Measure 10ml of each of these solvents into the respective vials. Prepare such vials for each sample to be analyzed. *(This saves time as during the column washes one does not have to measure the solvents and simply dispense the premeasured amounts into the column using a glass transfer pipette).* *It is up to you whether to use disposable glass tubes or recyclable kimax glass tubes (need to wash them thoroughly-perhaps also leave them for a few hours in the AFNS furnace).*

- Preparation of pre-labeled tubes:
  It is preferable to label the collection tubes before starting the process to save time as well as to avoid mistakes. For each sample, label 3 glass tubes as TG (for the TG fraction), EC (for the endocannabinoid fraction) and PL (for the phospholipid fraction). Using sticky tape is helpful, so that the labels can be transferred from tube to tube to prevent any errors (mixing up the samples).
  Proceed with this protocol in batches of samples. So that at any stage all samples are undergoing the same treatment/step. For example, label all tubes first, prepare fresh solutions to prevent evaporation and dry the samples simultaneously. If handling too many samples then stagger them in batches so that during a batch all samples are treated simultaneously. Please see the picture below for set up.

10. Wash the SPE column with chloroform (approx. 500 uL by drawing some chloroform with a glass transfer pipette and dispensing it into the SPE column). Use a collection tube (or scintillation vials) underneath the column to avoid spills.

11. *Once the solvent has been dispensed add air pressure on the SPE column (For example by using a glass syringe that fits an adaptor on the neck of the SPE column snugly) to flush the solvent down the column.*

12. Transfer (load) the reconstituted sample (500 uL) (from step #9) onto the column by using a glass transfer pipette.

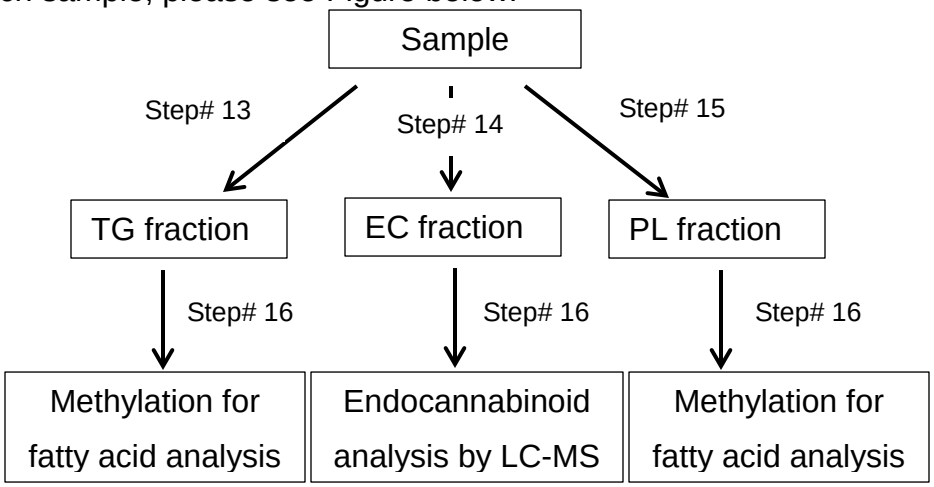
13. Then, wash out the ‘TG fraction’ from the column by transferring the pre-measured 10 mL **chloroform** with a glass transfer pipette. Make sure to fit the column on the top of the respective pre-labeled glass tube (to collect the solvent). Will need to load the solvent in two steps as the column won’t fit the 10 mL at once. Repeat step # 11 to flush the solvent down.

14. Next, wash out the ‘EC fraction’ from the column by transferring the pre-measured 10 mL **chloroform: methanol 9:1** solution with a glass transfer pipette and collect the wash out solvent in the respective pre-labeled glass tube (to collect the solvent). Again, will need to load the solvent in two steps while repeating step # 11 to flush the solvent down.

15. Finally, wash out the ‘PL fraction’ from the column by transferring the pre-measured 10 mL **methanol** with a glass transfer pipette and collect the wash out solvent in the respective pre-labeled glass tube (to collect the solvent). Again, will need to load the solvent in two steps while repeating step # 11 to flush the solvent down.

16. Dry each collected fraction under nitrogen gas at ~30°C (heating block) in the fume hood (samples can be frozen at -20 ºC or stored in the fridge for short term at this point or before drying). To facilitate the drying process it is recommended that all samples are at the same step of solvent addition and drying. For example If some samples have methanol only and others have chloroform only then their drying times will be different and this leads to increased nitrogen wastage.

 For further clarification of how to proceed further after drying each sample, please see Figure below.



17. After drying, the EC fraction is reconstituted in 200 uL of methanol, drawn from the glass tube with an 'insulin' syringe and passed through a syringe PTFE membrane filter while being transferred to a glass micro-insert in a GC vial. Samples are 'READY' for endocannabinoid analysis by LC-MS.

    The final concentration of endocannabinoids in tissues can be calculated shown below.

	MW	mg tissue	Calculated concentration (ug/mL)	ug/200 uL methanol*	ng/g tissue†	nmol/g tissue‡
2-AG	379	86.4	5.58	1.116	15500.0	40.9
AEA	348	86.4	0.0228	0.00456	63	0.2
OEA	326	86.4	0.1	0.02	277.8	0.9
PEA	300	86.4	0.386	0.0772	1072.2	3.6

*Concentration (ug) in 200 uL methanol= Calculated concentration x 200/1000

†Concentration (ng) per g tissue= (ug/200uL methanol/mg tissue) x (6/5) x10^6; *where 6 corresponds to the total volume of chloroform added to the sample (in the Folch solution) while 5 corresponds to the volume taken from the bottom layer after Folch extraction.*

‡ Concentration (pmol) per g tissue = ng/g tissue/MW of the respective molecule

Dried TG and PL fractions can undergo further methylation for fatty acid analysis, as described below. It is important to note that TG and PL fractions can also be separated by thin layer chromatography (TLC) (Procedure not included here).

Methylation of fatty acids in collected fractions

Materials and reagents:

- Methanolic Base 0.5 N (Sigma 33080) (for samples containing CLA)
- Hexane
- Sodium sulfate anhydrous
- Glass tubes
- Pasteur pipets
- Micro-inserts for GC

- Microvials for GC with caps

18. Add 1 mL hexane and 2 mL Methanolic Base to each tube. Vortex well (make sure to keep the same caps with the same tube).


work in fume hood

19. Put on heating block at 80°C for 15 min.


Make sure to cap each tube tightly and check for leaking; otherwise the solvent will evaporate. If the solvent evaporates, fatty acids will NOT be detected by GC (Perhaps due to evaporation of methyl esters?). If this is the case, a new sample would need to be extracted and fractionated again.

20. Let tubes cool.

21. Add 1 mL H_2O (double distilled) and 1 mL of hexane to each tube.

22. Vortex well and centrifuge at 2500 rpm for 5 min at room temperature.

23. Transfer the 'upper layer' containing the organic solvent using a glass pipette into a clean, labelled screw top glass tube containing 1 mL of double distilled water (this is an extra step just to make sure there is no traces of methanolic base in the final sample and therefore it is *optional* ).

24. Transfer the '**upper layer'** containing the organic solvent using a glass pipette ( be very careful not to draw up the bottom layer) into a clean, labelled screw top glass tube containing some sodium sulphate anhydrous (this is to dry the sample from any residual moisture that would damage the GC column).

25. Vortex well and centrifuge at 2500 rpm for 5 min at room temperature.

26. Take off hexane with glass pipette and put in clean tubes.

27. Dry under Nitrogen gas (in fume hood).

28. Add 100-200 uL hexane to each tube and vortex well.

29. Transfer to micro-insert tubes (inside microvials for GC) and cap the tubes.

if samples are too diluted then can dry them down and add 50 uL of hexane into each insert, if they are too concentrated, then dilute appropriately. Try to keep a low concentration of lipids (~0.5-1 mg/mL of hexane) in samples containing ruminant trans fats to enhance peak separation in the 18:1 region of the chromatogram.

30. Samples are READY for GC analysis

Instrument and GC column

Varian 3600, FID detector, Column: CP Sil 88 (CP7489, Varian) 100m X 0.25mm i.d. X 0.2 µm film thickness

Operating conditions (from Kramer's lab (Cruz Hernandez *et al*., J AOAC Int., 2004)

45 °C isothermal for 4 min, increase to 175 °C at 13 °C/min, isothermal for 27 min at this temperature then increase to 215 °C at 4 °C/min and hold isothermal for 35 min.

[45°C (4 min)--13°C/min--175°C (27 min)-- 4°C/min --215 °C (35 min), total time 86 min]

Injector and detector temperatures: 250 C

Injection volume: 1 uL