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The Hypolipidemic benefits of *trans*-11 vaccenic acid in a rat model of dyslipidemia and metabolic syndrome

by

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This Dissertation is dedicated to my parents

Weixiang and Haiying

and to all my family

Abstract

Trans-11 vaccenic acid (VA) is the predominant trans fatty acid in dairy fat and is the major precursor to endogenous synthesis of *cis*9,*trans*11-conjugated linoleic acid (CLA) in humans and animals. Epidemiological studies have shown the positive association between trans fat intake and incidence of coronary heart disease. Nevertheless, CLA, categorized as a group of trans fatty acids, has been shown to possess anti-carcinogenic, hypolipidemic and anti-diabetic benefits in several animal models as well as certain human populations, possibly via activating peroxisome proliferator-activated receptor (PPAR) related metabolic pathways. The subsequent effort in enriching CLA in dairy products (e.g. butter) has led to a concomitant increase in VA, whose bioactivity and health implications were not fully appreciated. Interestingly, VA is the major natural trans fat found in the diet. Therefore, the objectives of this thesis were to assess the effect of dietary supplementation of synthetic VA on lipid metabolism especially during conditions of dyslipidemia and metabolic syndrome, and to delineate the intestinal and hepatic metabolic pathways potentially modulated by VA. The JCR:LA-*cp* rat model, when homozygous for the cp trait (*cp/cp*), develop leptin receptor deficiency which leads to symptoms of metabolic syndrome and pre-diabetes including obesity, insulin resistance, hepatic steatosis, hypertriglyceridemia and exacerbated production of hepatic very low-density lipoproteins and intestinal chylomicrons (CM). Gas chromatography analysis on nascent lymph shows that VA was effectively absorbed into the intestine. In addition, VA from natural source (i.e. beef fat) showed higher intestinal bioavailability compared to synthetic VA. Dietary supplementation of 1.0% (w/w) synthetic VA to JCR:LA-*cp* rats (but not lean healthy controls) demonstrated a profound reduction in plasma triglyceride, total cholesterol, low-density

lipoprotein-cholesterol, non-esterified fatty acid and haptoglobin concentrations (51%, $p<0.001$; 40%, $p<0.001$; 50%, $p<0.05$; 20%, $p<0.05$ and 50%, $p<0.001$; respectively), as well as improvement in hepatic steatosis and postprandial lipaemia. Gastric infusion of VA also resulted in an acute reduction in CM secretion in response to a fat load ($p<0.05$). We also found that the overall hypolipidemic benefits of VA might be partially contributed by suppression of hepatic de novo lipogenesis, activation of PPAR-α activity as well as up-regulation of PPAR-α and PPAR-γ expression in the intestine. In conclusion, VA as a natural trans fat, possesses beneficial properties in a rat model of dyslipidemia and metabolic syndrome, suggesting potential for the prevention of cardiovascular disease risk.

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List of Abbreviations

AA	Arachidonic acid
ABC	ATP-binding cassette transporter
ACAT	Acyl-CoA cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
AGPAT	1-acyl-sn-glycerol-3-phosphate acyltransferase
Apo	Apolipoprotein
APOBEC1	ApoB-editing catalytic component 1
BBM	Brush border membrane
bHLH	Basic-helix-loop-helix-leucine zipper
cAMP	Byclic adenosine monophosphate
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CM	Chylomicrons
CM-r	Chylomicron remnants
COPII	Coat protein complex II
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DG	Diglycerides
DGAT	Diacylglycerol acyltransferase
DGATpa	DGAT associated with the phosphatidic acid pathway
EGIR	European Group for the Study of Insulin Resistance
EPA	Licosapentaenoic acid

ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
FABPpm	FATP bound to plasma membrane
FACS	Fatty acyl-CoA synthase
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster determinant 36
FATP	Fatty acid transport proteins
FFA	Free fatty acids
G-3-P	Glycerol-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPAT	Glycerophosphate acyltransferase
GLUT	Glucose transporter
HDL	High density lipoproteins
HL	Hepatic lipase
HSL	Hormone sensitive lipase
I-FABP	Intestine-type fatty acid binding
IC_{50}	Half maximal inhibitory concentration
IDL	Intermediate density lipoprotein
IL	Interleukin
IRS	Insulin receptor substrate
iTFA	Industrial *trans* fatty acids
LBD	Ligand binding domain
LCFA	Long-chain fatty acids
L-FABP	Liver-type fatty acid binding
LCAT	Lecithin cholesterol acyl transferase

LDL-r	LDL receptor
LDL	Low density lipoproteins
LRP	LDL receptor-related protein
LXR	Liver-X receptor
MetS	Metabolic syndrome
MG	Monoglyceride
MGAT	Monoacylglycerol acyltransferase
MI	Myocardial infarction
MMP	Monocyte chemoactive protein
MTP	Microsomal triglyceride transfer protein
NCEP ATP Panel	National Cholesterol Education Programme Adult Treatment
NPC1L1	Nieman Pick C-1 like1
nSREBP	N-terminus of SREBP
OA	Oleic acid
PAI-1	Plasminogen activator inhibitor-1
PCTV	Pre-chylomicron transport vesicles
PHVO	Partially hydrogenated vegetable oil
PKB	Protein kinase B
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response elements
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RXR	Retinoid X receptor
PAPH	Phosphatidic acid phosphohydrolase

rTFA	Ruminant *trans* fatty acids
S1P	Site 1 protease
S2P	Site 2 protease
SCAP	SREBP cleavage-activating protein
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acids
SR-BI	Scavenger receptor class B type I
SREBP	Sterol regulatory element binding proteins
SSD	Sterol sensing domain
TC	total cholesterol
TFA	*Trans* fatty acids
TG	Triglycerides
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TR-FRET	Time-resolved fluorescence resonance energy transfer
TRL	TG-rich lipoproteins
VA	Vaccenic acid
VAMP	Vesicle-associated membrane protein
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoproteins
WHO	World Health Organization

Chapter 1 Literature Review

1.1 *Trans* fatty acids

1.1.1 Introduction

Trans fatty acids (TFA) refer to a class of unsaturated fatty acids with at least one double bond in the *trans* configuration. It was estimated in 2002 that approximately 80-90% of all dietary TFA was derived from industrially-produced TFA (iTFA) present in partially hydrogenated vegetable oil (PHVO), while the remaining 10-20% was derived from ruminant sources such as beef and milk, thus termed as ruminant TFA (rTFA) (Elias *et al.* 2002, Wolff *et al.* 2002). The predominant rTFA in ruminant fat are *trans*11-18:1 (vaccenic acid or VA) and *cis*9, *trans*11-18:2 (conjugated linoleic acid or CLA), but the composition of *trans* fat in PHVO can vary substantially depending on the types of vegetable oils and processing conditions (e.g. temperature, catalysts) (Ratnayake *et al.* 2009)

The manufacture of PHVO can be traced back to about a century ago when it was discovered that soybean oil could be better preserved in a hydrogenated solid form. Although animal fats such as butter, lard and beef tallow have historically been the major source of dietary fat, the increasing use of hydrogenated PHVO has reduced their contributions to total fat intake. Compared to animal fats, PHVO have a lower production cost, a longer shelf life, superior spreadability and better baking performance. During the 1980's the public health campaign advocating that a diet high in cholesterol and saturated fatty acids (SFA) would increase the risk of heart disease, rapidly lead to the replacement of animal fat with PHVO (e.g. lard) (Richard *et al.* 1985). In developed countries, the major sources of PHVO include bakery products (e.g. cakes, cookies and pies), packaged snacks (e.g. popcorn), margarines and crackers. In developing countries, PHVO are mainly incorporated into cooking oils that are widely used and commonly subsidized by the government (Teegala *et al.* 2009, Butt *et al.* 2009). During the hydrogenation reaction, 30-50% of unsaturated double bonds are converted from the natural *cis* configuration to the *trans* configuration. However, it was not until the mid 1980's that dietary *trans* fat was first reported to be positively associated with incidence of cardiovascular disease (CVD) such as ischemic heart disease (Kritchevsky *et al.* 1982).

1.1.2 Health implications of *trans* fat

Since the 1980's, epidemiological data from retrospective case-control, prospective cohort and nested case-control studies consistently support the strong associations between TFA consumption and coronary heart disease (CHD) (Micha *et al.* 2009, Uauy *et al.* 2009, Willett *et al.* 1993, Oomen *et al.* 2001). In particular, the increased CHD risk has been related to the intake of both total TFA and foods known as major sources of TFA such as popcorn and baking products (Oomen *et al.* 2001). In a meta-analysis of four prospective cohort studies, every 2% increase of TFA intake was associated with 23% higher risk of myocardial infarction and CHD (Mozaffarian *et al.* 2006). When PHVO were replaced with other fat such as vegetable oils, there was an estimated 50% reduction in overall CHD risks (Mozaffarian *et al.* 2009). Further evaluation of CHD risk indices has also revealed that, TFA intake from PHVO was positively associated with critical risk factors of CVD development (Innis *et al.* 2006, Mozaffarian *et al.* 2009). The mechanism by which TFA exacerbates CVD risk factors has been extensively studied. TFA has been shown to negatively affect atherogenic lipoprotein profiles in numerous observational studies and clinical trials by increasing serum low density lipoprotein-cholesterol (LDL) as well as LDL to high density lipoprotein (HDL) ratio. TFA have also been indicated to influence other non-lipid parameters including systemic inflammation, endothelial dysfunction, adiposity and insulin resistance, all of which are independent CVD risk factors (Mozaffarian *et al.* 2009).

1.1.3 *Trans* fat regulations

In the last decade, based on cumulative evidence demonstrating the detrimental effect of *trans* fat consumption, government policies have been increasingly focused on reducing *trans* fat intake to as low as possible to minimize potential health risks (Eckel *et al.* 2007). In 2002 the panel on Macronutrients of the U.S. National Academies of Science, Institute of Medicine, recommended that *trans* fat consumption be as low as possible in a nutritionally adequate diet. Subsequently, in 2003 the World Health Organization recommended that *trans* fat intake be limited to less than 1% of overall energy consumption. As of December 2005 in Canada and January 1st 2006 in the United States,

manufacturers of conventional foods and supplements were required to list the content of TFA on the nutrition labels. Foods with less than 0.5 g of TFA per serving in the United States and 0.2 g in Canada can be listed as 0 g and promoted as “trans fat free” foods. Nevertheless, regulations for food prepared and served at restaurants, grocery stores, bakeries, cafeterias and other fast food outlets are still working on compliances with these new policies because of the difficulty in executing quality control and reproducibility with alternative fat sources. As of June 2006, increasing effort has been made to develop restrictions for the use of TFA in cooking oil (less than 2% of total fat from TFA) as well as TFA in total fat content (less than 5% of total fat from TFA) for those food prepared on site (Ratnayake *et al.* 2009).

1.1.4 Estimation of *trans* fat consumption

The estimated average intake of TFA in Western countries prior to 2000 was 2.5-13 g/day per person, and was generally higher in US and Canada than European countries (Craig-Schmidt *et al.* 2006). In the Nurse Health Study (initiated in the 1980s), the average TFA intake of over 100,000 female participants was reported to be 2.7 g/day with the highest quintile reaching 3.9 g/day (Mozaffarian *et al.* 2004). In countries where PHVO was used extensively for cooking (e.g. Iran), the average daily intake was accounted for 4.2% of all calories consumed (or approx. 12.3 g/day)(Mozaffarian *et al.* 2007). Ever since legislation has been implemented with regard to food labeling regulations and the amount of TFA allowed in foods, there has been a global decline of total TFA consumption, from an average of 10 g/day worldwide a decade ago to 3-4 g/day in North America; 2-4 g/day in northern European countries such as Denmark and the Netherlands; 1-3 g/day in Mediterranean countries and as low as 1 g/day in east Asian countries, as measured during the past few years (Hulshof *et al.* 1999, Craig-Schmidt *et al.* 2006).

1.1.5 Limitations in *trans* fat regulation

The public health campaign advocating for a reduction in *trans* fat consumption has resulted in a steady decline in total TFA consumption around the world (Friesen *et al.* 2006, Craig-Schmidt *et al.* 2006, Ratnayake *et al.* 2009). However, to date there has been little or no discrimination between natural and industrial

trans fat in legislatorial documents (i.e. during the promotion of *trans*-fat free diet by health professionals, or in population-based studies which built up the scientific basis for official *trans* fat regulations). Given the emerging evidence that suggests a neutral or even beneficial effect of rTFA-enriched dairy fat on blood lipid profile, it seems premature to eliminate all TFA isomers without further consideration of their respective bioactivity. Indeed, the Codex Alimentarius standard and the official dietary recommendations of several countries including Canada, Denmark and US have acknowledged the neutral-to-beneficial effect of ruminant CLA and have excluded it from the *trans* fat definition. However, ruminant CLA only makes up a small proportion of rTFA compared to the more predominant VA, whose bioactivity was largely unappreciated in the development of these recommendations.

1.2 Natural *trans* fatty acids

Although not as abundant as PHVO-derived iTFA in the diet, natural TFA that are derived from ruminant fat have been estimated to contribute to up to 20% of total TFA intake in the Western diet (Wolff *et al.* 2002). The two predominant 'natural' isomers in ruminant-derived foods include *trans*11-VA and *cis* 9,*trans*11-CLA (Palmquist *et al.* 2005). Both CLA and VA are produced during the biohydrogenation of PUFA, primarily linoleic acid and α-linolenic acid, by rumen bacteria (Lock *et al.* 2004). Indeed, VA is the major biohydrogenation intermediate that is produced in the rumen and later absorbed and accumulated in tissues. On the other hand, *cis*9,*trans*11-CLA is considered as an intermediate during luminal hydrogenation. Over 60% of CLA's adipose storage pool is endogenously synthesized by ∆9-desaturase [primarily stearoyl-CoA desaturase (SCD)], using VA as the predominant substrate (Griinari *et al.* 2000). SCD is the primary enzyme involved in the biosynthesis of palmitoleic acid and oleic acid by introducing a *cis* double bond at the 9^{th} carbon. Although the highest activity is found in the liver and adipose tissue, SCD is also present in the intestinal epithelium catalyzing the conversion of VA to *cis*9,*trans*11-CLA (Reynolds *et al.* 2008). The overall conversion rate of VA to *cis*9,*trans*11-CLA has been estimated to range from 5-12% in rodents and 11-30% in humans (Turpeinen *et al.* 2002). The luminal and in-vivo synthesis of rTFA is summarized in Figure 1-1.

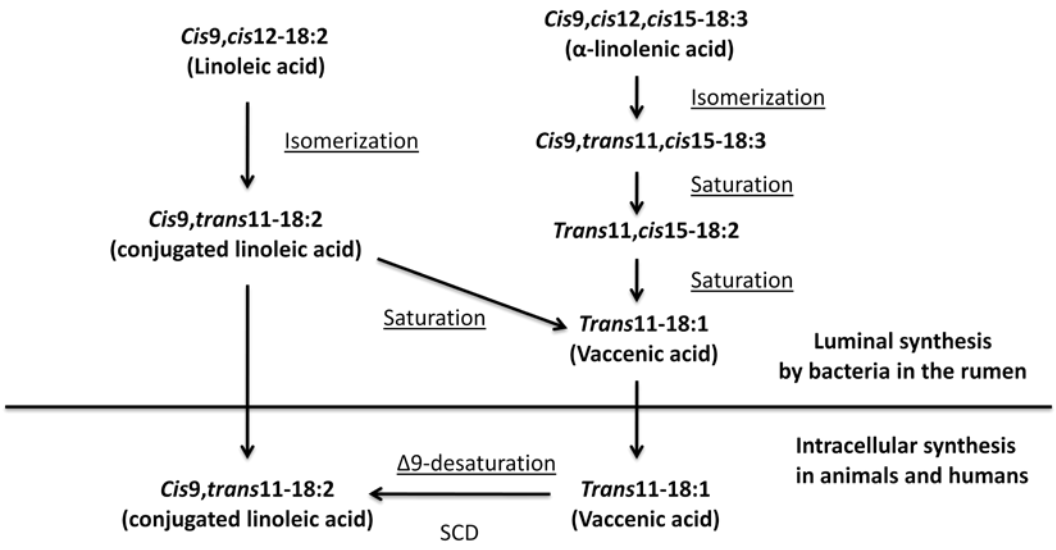

Figure 1-1. Biosynthesis of VA and *cis*9,*trans*11-CLA by bacteria in the lumen of ruminant animals and *in-vivo* conversion of VA to *cis*9,*trans*11-CLA by SCD in animals and humans. Adapted from Enjalbert *et al.* 2009.

Traditionally, rTFA has been considered to constitute a rather small part of the fatty acid composition of dairy fat (2% to 5% of total fatty acids) or beef and lamb (3%-9%) (Precht *et al.* 1996). However, it is now appreciated that the fatty acid composition is largely dependent on the bovine feeding practice and can vary substantially due to geographical and/or seasonal difference. For example, alpine pastures in northern European countries yield greater concentration of rTFA than lowland pastures (Precht *et al.* 1995). Milk produced during the long daylight of summer often contains more total rTFA than milk produced during the winter months (Precht *et al.* 1995). Moreover, modification to agricultural practices have been developed to enhance the content of rTFA, such as supplementing feed with oil seeds (Rego *et al.* 2009), changing the microflora of the rumen (Boeckaert *et al.* 2008) and pre-selecting cows that are genetically high 'rTFA producers' (Taniguchi *et al.* 2004). As a consequence, TFA content as high as 12% w/w of total fat is now a regular occurrence in dairy products (with additional reductions in saturated fat) (Palmquist *et al.* 2005, Mendis *et al.* 2008). The increase in the proportion of rTFA in dairy-derived products has confounded the premise for minimizing total dietary TFA and sparked a critical need to better understanding the bioactivity of specific rTFA isomers (e.g. VA).

While considerable evidence seems to support a positive association between TFA intake and CHD risk, very few studies have attempted to differentiate between iTFA and rTFA. There is however a limited number of epidemiological studies that imply a positive association with CHD risk only exists between TFA isomers generated by industrial means (i.e. *trans* isomers of C18:1 and C18:2) and not those TFA isomers formed through bio-hydrogenation reactions such as *trans*-C16:1 (Mozaffarian *et al.* 2004 and 2005, Lopez-Garcia *et al.* 2005). Interestingly, there has been some acknowledgment that rTFA isomers (e.g. *cis*9,*trans*11-CLA) have different health effects than most of the PHVO-derived iTFA with regard to CVD development. Supplementation of CLA alone or in combination with VA as in the enriched dairy fat has been shown to reduce plasma triglyceride (TG) and cholesterol concentrations, improve insulin sensitivity, reducing visceral fat deposition and aortic lesion area, as well as immune-regulatory effects (Bhattacharya *et al.* 2006). One proposed mechanism of the pleiotropic effect of CLA is by agonizing peroxisome proliferator-activated receptor (PPAR) pathways which regulate the transcription of key factors in insulin and lipid metabolism (Toomey *et al.* 2006, Biscetti *et al.* 2009). Being the major precursor to *cis*9,*trans*11-CLA, VA has been shown to be metabolized in a similar manner to that found for CLA (Banni *et al.* 2002). Therefore, findings regarding CLA's benefits could lend indirect support to the possibility that VA, the most abundant rTFA, may have protective effects on CVD risk factors, which was one of the major objectives of my doctoral thesis.

1.3 Cardiovascular disease

1.3.1 Introduction

CVD remains the leading cause of death and is responsible for an estimated 28% of total mortalities globally each year (Lloyd-Jones *et al.* 2010). It has a complex etiology with contributions from insulin resistance, hypertriglyceridemia, hypercholesterolemia, inflammatory dysregulation, hypertension and infectious diseases that cumulatively affect vascular and heart function (Yusuf *et al.* 2001). Further damage to the microvascular system could lead to complications including retinal damage, glomerular sclerosis, polycystic ovary disease and pathological changes in other organ (Lanza *et al.* 2010).

Atherosclerosis is regarded as the initial stage in CVD development, characterized by fatty plaques in the intima of arteries. The accumulation of plaques narrows vessel lumen and gradually interferes with blood circulation. More severe events can occur if the advanced unstable plaques rupture, releasing thrombulus particles which may block blood supply to vital organs, causing a series of ischemic complications including cardiac arrhythmia, myocardial infarct and cerebrovascular incidents (both thrombotic and hemorrhagic) (Libby *et al.*2006).

The infiltration of cholesterol-dense lipoproteins into the subendothelial space of the vessels initiates cholesterol accumulation and atheromatous plaque formation (Figure 1-2, Warnakula *et al.* 2010). Cholesterol-dense lipoproteins are primarily composed of the end-products of chylomicrons (CM) and very low density lipoproteins (VLDL) hydrolysis in the blood circulation, namely chylomicron remnants (CM-r) and LDL respectively. The delivery of cholesterol-rich lipoproteins across the arterial wall occurs via transcytosis and is dependent on particle size. Transcytosis refers to a process in which lipoprotein-containing vesicles are formed on the intimal surface of endothelial cells and translocate to the subluminal surface and release the contents by exocytosis (Nordestgaard *et al.* 1992, Simionescu *et al.* 1991). Lipoproteins with diameters less than 70-80 nm can be accommodated in the transport vesicles, and these residual cholesterol-rich lipoprotein particles are small enough to travel across arterial wall (Proctor *et al.* 1996, Mamo *et al.* 1994, Simionescu *et al.* 1991). In addition to transcytosis, proteoglycans present on the surface of endothelial cells may also assist the translocation of cholesterol-rich lipoprotein particles through the arterial wall (Ji *et al.* 1995).

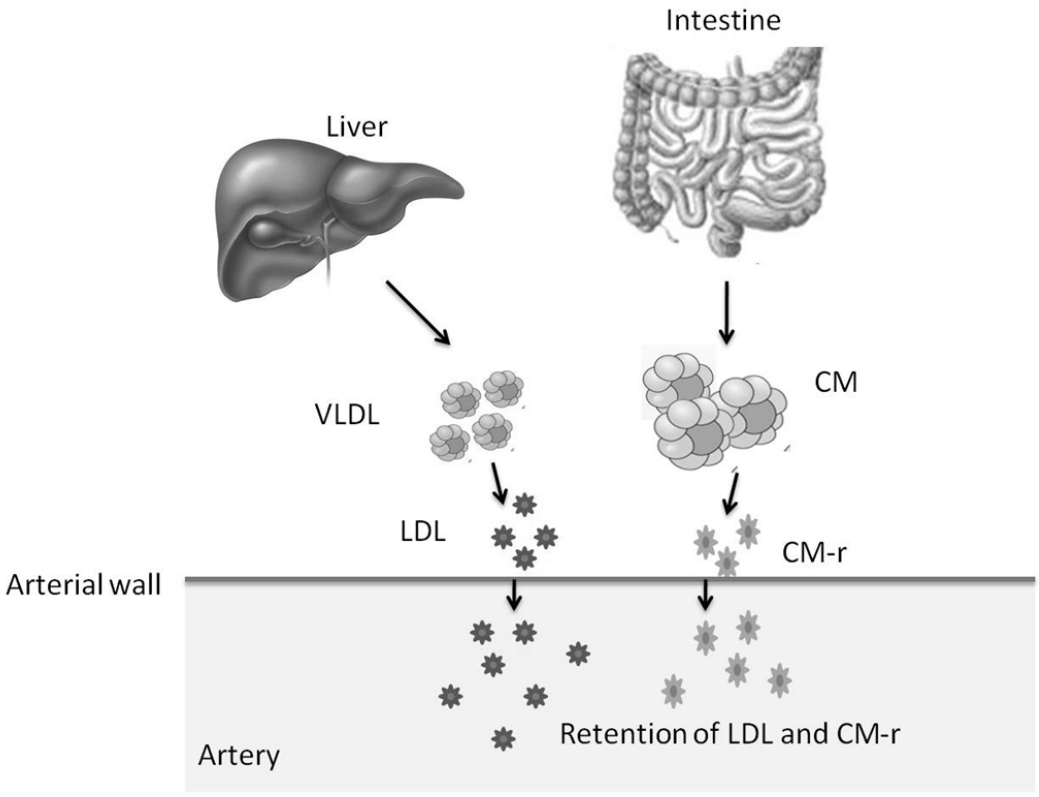

Figure 1-2. The infiltration of cholesterol-dense lipoproteins into the sub-endothelial space of the blood vessel (adapted from Warnakula *et al*. 2010). PG: proteoglycans, GAG: glycosaminoglycan.

When cholesterol-rich lipoproteins are retained inside the intima, the phospholipids within the particles are further subjected to tissue oxidative stress brought about by fatty acid oxidation, hyperglycemia or hypertension. High circulating blood glucose also increases the extracellular proteoglycan production, thus enhances the chances of cholesterol-rich lipoproteins being trapped within the subendothelial matrix (Little *et al.* 2008). Oxidized LDL, for example, signals endothelial cells to produce a cluster of inflammation mediators such as cell adhesion molecules, monocyte chemotactic proteins and monocyte colony-stimulating factors. Consequently, leukocytes (e.g. monocytes) are recruited to the intima of the arterial wall and transformed into macrophages (Watanabe *et al.* 1985). Macrophages internalize and over-accumulate lipids and sterols at the focal site, eventually transforming into lipid-laden foam cells (Ishigaki *et al.* 2009). Notably, small dense LDL particles tend to be considered more atherogenic due

to their higher total surface area and thus increased susceptibility to oxidation (Krauss *et al.* 1995).

Inflammation is one of the important causes of CVD and a key participant during the development of atherosclerosis. Chronic low-grade inflammation, typically seen in individuals with excess visceral fat, is regarded as a strong predictor of CVD development (Mathieu *et al.* 2010). Circulating pro-inflammatory cytokines [e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-6] and acute phase protein such as C-reactive protein (CRP) indirectly promote atherogenesis by increasing the production of cell adhesion molecules, IL-6, plasminogen activator inhibitor-1 (PAI-1) and a prothrombotic protein, all of which promote atherogenesis and thrombosis (Lippy *et al.* 2006). CRP may also be involved in the activation of complement cascade and form cell transformation (Hage *et al.* 2010). Recent evidence has linked dysregulated inflammation with postprandial lipaemia. *In vitro* studies further demonstrated enhanced expression of adhesion molecules and PAI-1 in endothelial cells treated with post-prandial plasma from hypertriglyceridemic patients (Inoue *et al.* 1998, Doi *et al.* 2000, Shin *et al.* 2004, Park *et al.* 2005).

Accumulating evidence suggest that subjects diagnosed with hypertriglyceridemia and metabolic syndrome (MetS) are at high risk of coronary atherosclerosis (Hayden *et al.* 2002, Jacobson *et al.* 2007, Bayturan *et al.* 2010). Therefore, it is essential to thoroughly understand the interaction between elevated circulating TG and the development of cardiovascular complications.

1.3.2 Hypertriglyceridemia predicts cardiovascular disease risk

Due of the essential involvement in the initiation and progression of atherosclerotic plaque formation, LDL is traditionally regarded as an important indicator of CVD risk. However, several major statin (cholesterol synthesis inhibitors) trials have also consistently reported CVD occurrence among patients who already managed to drastically lower their blood LDL levels (Neeli *et al.* 2009, Alagona *et al.* 2010). Indeed, up to 40% of the subjects diagnosed with CHD have normal plasma LDL concentration (Mediene-Benchekor *et al.* 2001, Colhoun *et al.* 2004). These findings not only suggest the possibility of other sources of cholesterol contributing to atheromatous cholesterol, but also weaken

the applicability of using plasma LDL concentration as a CVD risk biomarker, particularly during MetS. The pursuit of more dependable and sensitive CVD predictors has led to significant findings that both fasting and non-fasting hypertriglyceridemia are independently associated with the pathogenesis of CVD.

Hypertriglyceridemia, characterized by elevated plasma TG, is a distinctive feature of dyslipidemia. A recent meta-analysis involving over 10,000 CVD incident cases from 29 western prospective studies suggests that the association between fasting plasma TG and CHD risk is moderate but consistently significant (Sarwar *et al.* 2007). In a prospective cohort of school children, after up to 30 years of follow-up, fasting plasma TG concentrations appeared to be independently associated with CVD events in later life stages (Morrison *et al.* 2009). On the other hand, non-fasting (i.e. post-prandial) hypertriglyceridemia has emerged during the past decade with comparable power in predicting CVD development (Bansal *et al.* 2007, Langsted *et al.* 2008). The clinical significance of postprandial hypertriglyceridemia was recently vitalized by several large-scale cohorts confirming the strong association with end-stage myocardial infarction, ischemic heart disease and related death (Nordestgaard *et al.* 2007, Freiberg *et al.* 2008).

The mechanism by which hypertriglyceridemia accelerates CVD development has been extensively studied. Hypertriglyceridemia is primarily caused by high circulating TG-rich lipoproteins (TRL) composed of hepatically derived VLDL and intestinally derived CM (Jacobson *et al.* 2007). Overproduction and/or delayed clearance of TRL not only raise plasma TG, but also lead to increased circulating remnant lipoproteins (i.e. CM-r and LDL) that are smaller in diameters and tend to be more atherogenic (Tanaka *et al.* 2004). Another lipid abnormality often accompanying hypertriglyceridemia is low circulating HDL concentration (Schaefer *et al.* 1978, Avogaro *et al.* 1991). Increased circulating TRL particles stimulate the activity of cholesteryl ester transfer protein (CETP) and accelerate the transfer of TG from TRL to HDL. Overloaded HDL particles are rapidly cleared from the circulation by the liver (Tato *et al.* 1997). Reduced plasma HDL concentrations would interfere with reverse cholesterol transport pathways, leading to the accumulation of cholesterol in peripheral endothelial cells and macrophages, which may in turn exacerbates atherosclerotic plaque formation.

Although the positive association between hypertriglyceridemia and CVD risk may be weakened in some studies after adjusted for HDL, a number of prospective studies with an average of 10-year follow-up still indicated a significant and independent association between fasting plasma TG and cardiovascular events such as CHD and myocardial infarction (MI), even after adjusted for other coronary risk factors including HDL (Hokanson *et al.* 1996, Ginsberg *et al.* 2002, Fujioka *et al.* 2009). A clinical condition combining high plasma TG and low HDL profile may strongly predicts future CVD development, which is typically referred to as the metabolic syndrome.

1.3.3 Metabolic Syndrome—a cluster of metabolic disorders featuring hypertriglyceridemia and insulin resistance

Metabolic syndrome (MetS) represents an earlier stage in the development of CVD, with a cluster of metabolic disorders. Diagnosis of MetS is regarded as a strong predictor of future CVD development. It is estimated that one fourth of the world's adult population have MetS and are 2-3 times as likely to die from heart attack or stroke compared to people without MetS (Nesto *et al.* 2003, *et al.* 1994). Although gender difference did not seem to be apparent in the overall prevalence (Ford *et al.* 2002), the occurrence of obesity, MetS and type-2 diabetes appear to have shifted to earlier stages of life over the past few decades (Ode *et al.* 2009).

World Health Organization (WHO), the European Group for the Study of Insulin Resistance (EGIR) and the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII) have published separate definitions of MetS that are commonly used in clinical and research background. The WHO and EGIR definitions require the evaluation of insulin resistance via complicated and labor-intensive measurements (e.g. hyperinsulinaemic-euglycaemic clamp), which are therefore more often used in research. On the other hand, NECP ATPIII definition is more applicable in a clinical application and forms the basis of the recently updated diagnostic criteria for MetS by International Diabetes Federation (Table 1-1).

Table 1-1. Diagnostic criteria of the MetS based on the latest definition by International Diabetes Federation[1]

Metabolic disorders		Criteria		
Central obesity			Male	Female
		Europids	≥ 94 cm	≥ 80 cm
		Asians	≥ 90 cm	≥ 80 cm
Any two of the four factors	Hypertriglyceridemia	≥ 150 mg/dL (1.7 mmol/L) or specific treatment		
	Hyperglycemia	≥ 100 mg/dL (5.6 mmol/L) or type-2 diabetes		
	Reduced HDL cholesterol	< 40 mg/dL (1.03 mmol/L) in males <50mg/dL (1.29 mmol/L) in females or specific treatment		
	Hypertension	Systolic BP ≥ 130 mm Hg, or diastolic BP ≥ 85 mm Hg, or treatment of hypertension		

[1]Adapted from Zimmet *et al.* 2005.

Despite of the discrepancies between each definition, there are several shared characteristic properties: abdominal obesity, hypertriglyceridemia, low plasma HDL, insulin resistance and hypertension. It is critical to appreciate the fact that elevated plasma TG rather than LDL is used as one of the diagnostic criteria, which again implies the importance of hypertriglyceridemia in predicting the early stages of CVD progression.

1.3.4 Therapeutic strategies for cardiovascular disease treatment

Based on the discussion above, a clear strategy in reducing CVD risk is to improve symptoms of dyslipidemia and MetS. Non-pharmaceutical management of CVD is recommended by the WHO as the primary intervention. It focuses on lifestyle changes including: smoking cessation, weight control, regular physical activity, moderate alcohol intake and special attention to dietary habits and macronutrient intake. Dietary management proves to be effective in reducing hypertriglyceridemia which is fairly sensitive to energy balance. The American Heart Association (AHA) currently recommends choices of foods richer in poly-unsaturated fatty acids (PUFA), such as fatty fish, whole grains and nuts, and mono-unsaturated fatty acids (MUFA) such as olive oil, whilst restricting the intake of whole fat dairy and red meat products (Lichtenstein *et al.* 2006). For people at high risk for CVD and when lifestyle and dietary pattern changes are

insufficient to improve risk factors, drug therapy would be recommended as secondary intervention. The NCEP-ATPIII guidelines recommended nicotinic acid (vitamin B_3) as the drug of choice for hypertriglyceridemic patients which have been demonstrated to effectively reduce TG by 30-45%, reduce LDL by 15-20% and increase HDL by 15-25% (Viljoen 2009). However, nicotinic acid is poorly tolerated and may exacerbate insulin resistance. Fibrate derivatives (PPAR-α agonists), on the other hand, reduce hepatic VLDL secretion and improves hypertriglyceridemia with limited side-effects and has been approved by US Food and Drug Administration as another lipid-lowering drug (Lalloyer *et al.* 2010). Other drugs have been used targeting at insulin signaling pathways. Metformin therapy in patients with insulin resistance led to delayed development of diabetes most likely by enhancing the function of insulin receptors (Hasnain *et al.* 2010). Thiazolidinedione studies have provided convincing efficacy in insulin-sensitizing effect as well, possibly via activation of PPAR-γ signaling pathways (Lalloyer *et al.* 2010).

It is clear that both pharmacological and nutraceutical approaches that have proven to effectively reduce CVD risk are modulating either individual metabolic pathways or the major regulatory factors of lipid metabolism. Therefore, in order to assess the potential cardio-protective effect of dietary VA supplementation, it is essential to understand the digestion and absorption of dietary lipids, basic physiological pathways in lipid metabolism as well as key regulators in modulating these pathways.

1.4 Lipid metabolism

1.4.1 Introduction

Lipids are a diverse collection of biologically active molecules actively involved in various physiological functions such as energy supply (e.g. TG), cellular structure maintenance [e.g. phospholipids (PL)], signaling transduction (e.g. sphingosines) and metabolic regulations (e.g. eicosanoids, estrogen). The major classes of lipids present in the plasma are TG, free fatty acids (FFA), phospholipids, free cholesterol (FC) and cholesteryl esters (CE). Most lipids are insoluble in aqueous conditions that predominate in biological environments; therefore they need to be transported from one tissue to another by attaching to hydrophilic proteins.

Lipoproteins are such lipid-protein complexes produced primarily in the small intestine and the liver where hydrophobic lipids are combined with specific carrier proteins, called apolipoproteins (apo), to form a spherical structure. PL forms the hydrophilic surface of the lipoprotein particle that faces the aqueous environment and a tremendous amount of TG, FC and CE are embedded in the core. Based on particle density, lipoproteins are subcategorized into CM, VLDL, intermediate density lipoprotein (IDL), LDL and HDL (Table 1-2). As the particle density increases, the particle diameter and the proportion of TG decrease and the proportions of FC, CE and PL increase per particle (Kritchevsky *et al.* 1986). Each class of lipoproteins contains characteristic apolipoproteins that not only function as structural components but also facilitate respective physiological functions (Table 1-2).

Table 1-2. Major classes of lipoproteins and their physical properties

	Nascent CM	CM-r	VLDL	LDL	HDL
Density (g/mL)	<0.95	<1.006	0.095-1.006	1.019-1.063	1.063-1.21
Size (nm)	100-400	45-60	40-70	5-15	7.5-10
Major apolipoproteins	B48, C-I, C-II, C-III, E	B48, E	B100, C-I, C-II, C-III, E	B100	A-I, A-II, C-II, C-III
Triglyceride (% lipid)	80-95	70	55-80	5-15	5-10
Cholesterol (% lipid)	2-7	13	5-15	40-50	15-25
Phospholipids (% lipid)	3-9	11	10-20	15-25	20-30

Modified from Kritchevsky *et al.* 1986

The maintenance of lipid pool homeostasis is coordinated by lipid metabolism including absorption of dietary lipids, lipid synthesis and degradation, as well as lipid storage, mobilization and utilization. Genetic abnormalities and environmental stimulation (e.g. chronic energy imbalance) lead to disrupted balances and more severely, metabolic disorders such as obesity, CVD, cancer and type-2 diabetes. Several key regulators including insulin, leptin, sterol regulatory element binding proteins (SREBP) and PPAR are commonly implicated in maintaining this balance, and they will be discussed in details later in this chapter.

1.4.2 Digestion and absorption of exogenous lipids

Various types of lipids are consumed in the diet, among which the greatest quantity, regardless of its origin (animal or plant sources), is TG. Further, most of TG contains predominantly long-chain fatty acids (chain lengths of 14-20 carbons), with different degrees of saturation, esterified to the glycerol backbone. The intake of dietary fat is approximately 100 grams per day which accounts for an average of one third of daily total energy requirement (Tso *et al.* 2006). Phospholipids, the second most abundant dietary lipid class, contribute a significant portion of essential fatty acids and choline to support daily requirement. In addition to about 2 g of PL from typical Western diet, another 10-20 g of almost exclusively phosphatidylcholine come from bilary lipids produced by the liver (Tso *et al.* 2006). It is noteworthy that the absorption of PL directly affects the bioavailability of other dietary fat, since several stages in lipid absorption and transportation (e.g. micelle formation, CM biosynthesis) is dependent on the abundance of PL (Tso *et al.* 2006). Other dietary lipids such as CE also play vital roles in modulating metabolic activities although their daily intake is relatively low compared to TG.

Taking rTFA-enriched butter fat as an example, chemical digestion of dietary lipids starts when the gastric lipase (lingual lipase for rodents) secreted into the stomach preferentially cleaves the fatty acid at the sn-3 position of the TG molecule (Tso *et al.* 2006). The grinding and mixing of chime promotes the dispersion of butter fat into smaller droplets. Stable emulsion droplets are formed at a ratio of TG:PL of 30:1 (Tso *et al.* 2006). The resulted increase in surface

area enhances direct interaction between hydrophilic enzymes and hydrophobic lipids on the oil-aqueous interface (Tso *et al.* 2006)

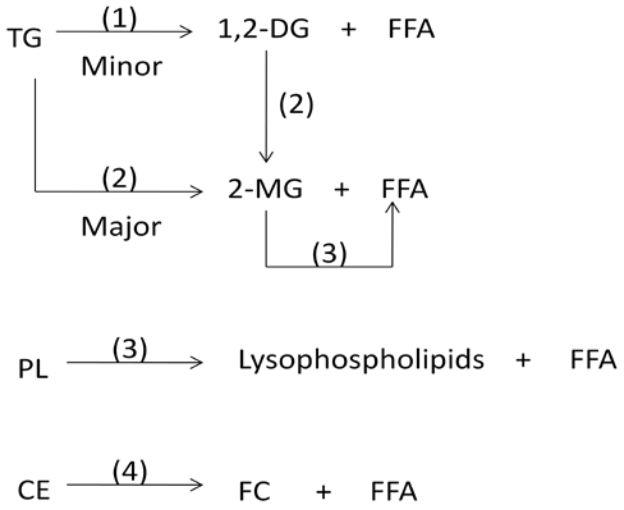

Figure 1-3. Enzymatic digestion of dietary lipids in the gastrointestinal tract. (1): gastric lipase, (2): pancreatic lipase+co-lipase, (3): phospholipase A_2 and (4) cholesterol esterase.

The major hydrolysis of TG, PL and CE from butter fat occurs in the duodenum, with the assistance of pancreatic lipase, colipase, phospholipase A_2 (PLA_2) and cholesterol esterase present in the pancreatic juice. The release of pancreatic juice is stimulated by cholecystokinin, a hormone produced by the stomach in response to food load. Pancreatic lipase acts primarily on the sn-1 and sn-3 positions of the TG [as well as 1,2-diglycerides (DG)] molecules to release one 2-monoglyceride (MG) and two free fatty acid (FFA) per TG molecule. However, the presence of colipase is a prerequisite for pancreatic lipase's function, since the bile salt-covered lipid droplets cannot get into contact with the lipase without first binding of colipase. PLA_2 preferentially releases fatty acids esterified to the sn-2 position of both dietary and bilary phospholipids to yield lysophospholipids. PLA_2 also acts on a small portion of 2-MG to liberate the sn-2 fatty acid from its glycerol backbone. Cholesteryl esters are hydrolyzed to FFA and FC by cholesterol esterase, which has low substrate specificity and acts on a variety of dietary lipids including phospholipids, esters of vitamins A and D, MG and TG without further differentiation of ester linkage positions. The predominant digestion products are FFA, 2-MG, FC and lysophospholipids, over 95% of which

are effectively and rapidly absorbed across the brush border membrane (BBM) into the enterocytes (Tso *et al.* 2006).

The absorption of digestion products of butter fat primarily takes place in the jejunum which has the characteristic villus structure at the epithelial layer lining the intestinal lumen. Intestinal villi, approximately 0.5-1 mm in length, are finger-shape projections facing towards the lumen. On the surface of each villus there are additional extensions called microvilli protruding from epithelial cells (Tso *et al.* 2006). The villus and microvillus structure drastically increase the surface area as well as absorptive area in order to allow considerably fast and efficient absorption. Capillaries and lacteals embedded within each villus collects nutrients from the basolateral side of epithelial cells, and transport them around the body. In the case of butter fat, absorbed glycerol, short-chain and medium-chain fatty acids directly diffuse out of the basolateral membrane of enterocytes into capillaries, whereas most of the long-chain fatty acids (LCFA) are re-esterified into PL, TG and CE, incorporated into CM particles, secreted into the lymphatic flow in the central lacteal which eventually drains into blood circulation at the thoracic duct. Rats are capable of absorbing at least 0.5 mM oleic acid per hour and convert 80% of absorbed FFA into TG within 30 seconds (Mansbach *et al.* 2000). Similarly, humans can absorb up to 600 g fat with 95% efficiency. Fat malabsorption would lead to steatorrhea, the excessive excretion of fat in the stool, and potentially fat-soluble vitamin deficiency.

Although the exact mechanism of how lipid digestion products move across BBM is not well understood, it has been proposed to involve two separate pathways—the protein-independent simple diffusion (the “flip-flop” model) and the protein-dependent transport (Mansbach *et al.* 2007). The formation of micelle structure in the presence of bile salts is the crucial step in fatty acid absorption regardless of the pattern of transmembrane activity. FFA embedded in the hydrophobic core dissociates as the micelles approach the unstirred water layer that covers BBM. FFA, PL and FC are protonized in the acid microclimate between unstirred water layer and microvillis. The addition of charge increases the water solubility and speeds up the transmembrane activity of fatty acids, either directly by passive diffusion or by binding to membrane transport proteins such as fatty acid binding protein (FABP) bound to plasma membrane (FABPpm) and fatty acid

translocase/cluster determinant 36 (FAT/CD36) (Tso *et al.* 2006). Nieman Pick C-1 like1 (NPC1L1) protein specifically functions as cholesterol transporter across BBM as well as intracellular trafficking to the endoplasmic reticulum (ER) and other organelles in the enterocytes (Sane *et al.* 2006). LCFA, lysophospholipids and MG bind to an intestinal-type and a liver-type fatty acid binding protein (I- and L-FABP) at the cytosolic side of BBM. L- and I-FABP are present in approximately equal amounts in rat enterocytes, whereas in humans L-FABP predominates (Pelsers *et al.* 2003). It has been suggested that I-FABP functions in removing FA from the BBM and delivering the ligands to ER membrane. L-FABP, on the other hand, acts as FA reservoir and is highly inducible with high fat feeding. Fatty acid transport proteins (FATP) are another family of protein carriers that have been implicated in fatty acid uptake and intracellular trafficking (Gimeno *et al.* 2007). FATP4 is the predominant form expressed in the enterocytes. Silencing of FATP4 mRNA has been shown to reduce FA uptake by 40-50% (Stahl A *et al.* 1999).

1.4.3 The essential role of the liver in lipid metabolism

The liver plays a central role in maintaining the homeostasis of fatty acid and cholesterol metabolism (Figure 1-4) (Canbay *et al.* 2007, Nguyen *et al. et al.* 2008). Liver is the major site of fatty acid and cholesterol *de novo* synthesis from non-lipid precursors such as glucose and amino acids. Bile acid is produced in the liver, secreted into the bile, stored in the gallbladder and drain into the small intestine to facilitate lipid emulsification and absorption. The luminal bile acids are either eliminated in the stool or recycled via the enterohepatic bile acid cycle. Bile acid synthesis is an important approach for the liver to excrete excess amount of cholesterol and keep blood cholesterol concentrations relatively stable. In the context of CVD development, the liver can be regarded as the central hub and an irreplaceable modulator in lipoprotein metabolism. Abnormal liver function such as overproduction of VLDL, delayed clearance of remnant lipoproteins from the circulation and the disrupted reverse cholesterol transport pathways lead to metabolic dyslipidemia and predict the commencement of atherosclerosis (Nguyen *et al.* 2008).

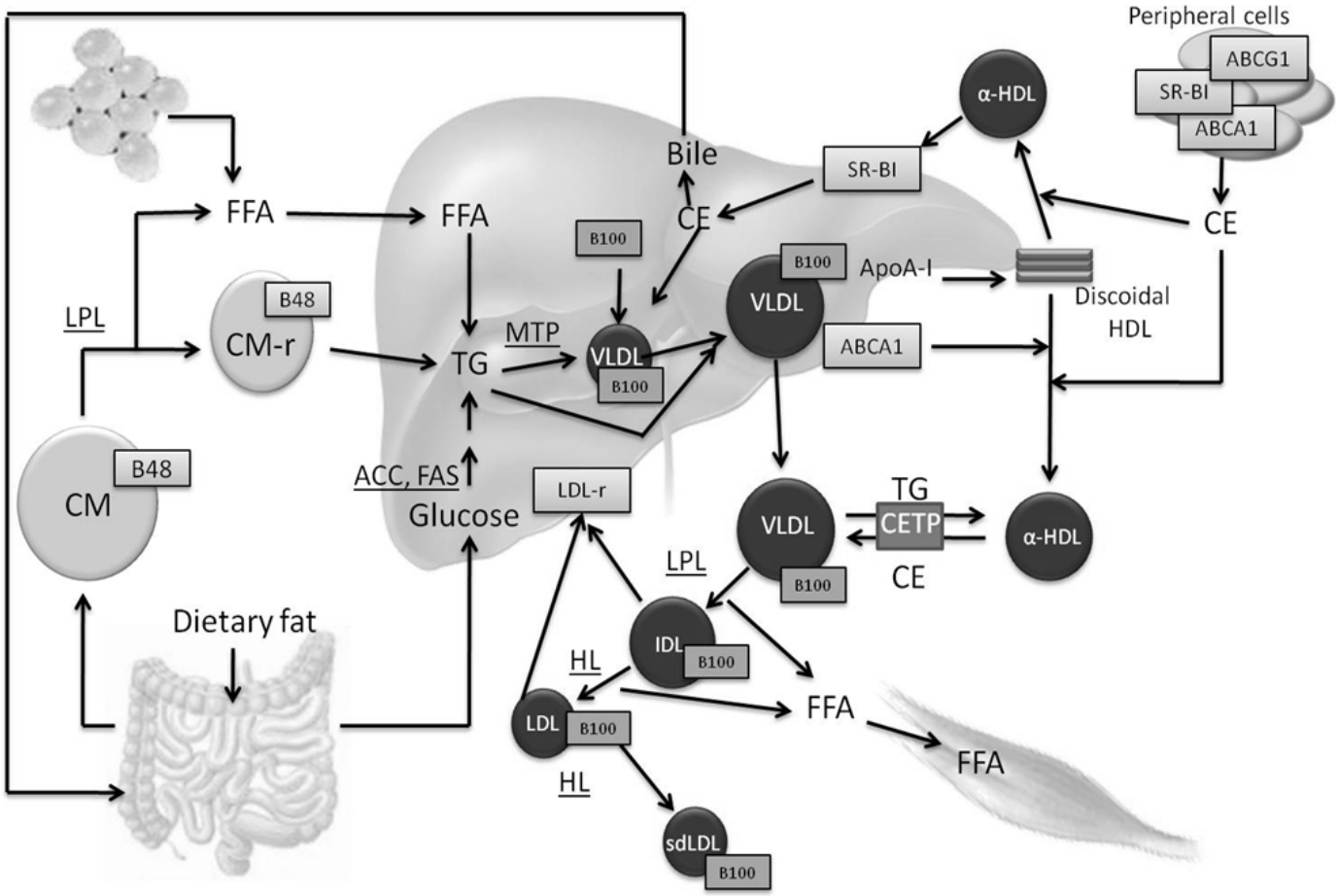

Figure 1-4. Schematic diagram of the central role of liver in lipid metabolism.

Liver is the major site of fatty acid and cholesterol *de novo* synthesis from non-lipid precursors such as glucose and amino acids. Bile acid is produced in the liver, secreted into the bile, stored in the gallbladder and drain into the small intestine to facilitate lipid emulsification and absorption. The luminal bile acids are either eliminated in the stool or recycled via the enterohepatic bile acid cycle. Bile acid synthesis is an important approach for the liver to excrete excess amount of cholesterol and keep blood cholesterol concentrations relatively stable. In the context of CVD development, the liver can be regarded as the central hub and an irreplaceable modulator in lipoprotein metabolism. Abnormal liver function such as overproduction of VLDL, delayed clearance of remnant lipoproteins from the circulation and the disrupted reverse cholesterol transport pathways lead to metabolic dyslipidemia and predict the commencement of atherosclerosis (Nguyen *et al.* 2008).

1.4.3.1 Hepatic *de novo* lipogenesis

Endogenous synthesis of fatty acids takes place in the liver, intestine and adipose tissue, but the liver has the highest capacity in producing LCFA (Sul *et al.* 2006, Hellerstein *et al.* 1999). The primary location for fatty acid synthesis is the cytosol and the production rate is subjected to feed-back, feed-forward and hormone controls (Figure 1-5). Acetyl-CoA carboxylase (ACC) catalyzes the very first and also the rate-limiting step, converting acetyl-CoA to malonyl-CoA. There are two isoforms of ACC: ACC-1 is involved in fatty acid synthesis and primarily expressed in the liver, intestine and adipose tissue. ACC-2 is present on the mitochondrial membrane of skeletal muscle, heart and the liver. It is involved in the synthesis of malonyl-CoA which controls the transfer of cytosolic LCFA to mitochondria for fatty acid oxidation. ACC is allosterically activated by citrate, the concentration of which increases during conditions favoring fatty acid synthesis. When production of acetyl-CoA from pyruvate is elevated in the mitochondria, the rate of citrate synthesized from acetyl-CoA in the citric acid cycle is also increased, resulting in the accumulation of intramitochondrial citrate. Excess citrate can be translocated to the cytosol and converted back to acetyl-CoA. This step has crucial physiological significance.

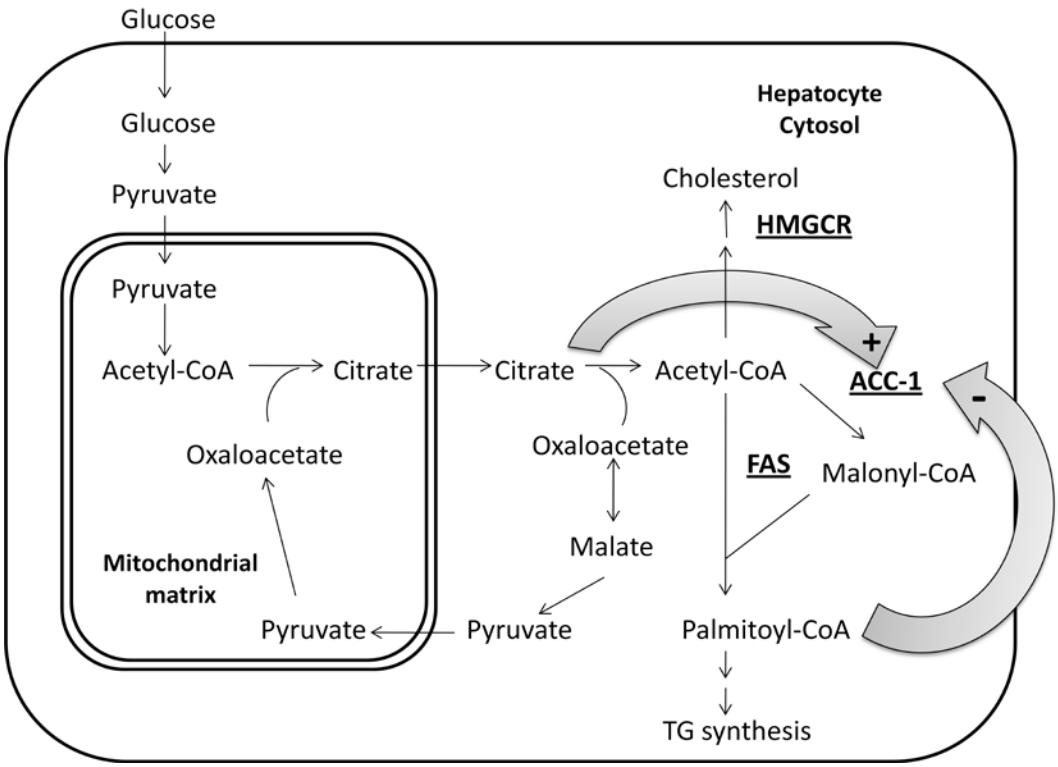

Figure 1-5. Schematic diagram of fatty acid and cholesterol *de novo* synthesis in the liver.

The inner mitochondrial membrane is impermeable to acetyl-CoA. Excess acetyl-CoA is physically trapped within the mitochondria and unavailable for the cytosolic fatty acid synthesis unless being converted to citrate. Therefore citrate serves not only as the ACC activator, but more importantly as acetyl-CoA "transporter" from mitochondria to cytosol. On the other hand, long-chain acyl-CoA allosterically inhibits ACC activity, which is higher during starvation and reduced in the fed state. The activity of ACC is also affected by covalent modification of several serine residues. When cellular energy state is low [indicated by high intracellular cyclic adenosine monophosphate (cAMP) concentration], AMP-activated protein kinase is allosterically activated by cAMP and in turn phosphorylates ACC, resulting in a decreased sensitivity to the stimulatory effect of citrate and an increased sensitivity to the inhibitory effect of long-chain acy-CoA. In addition to the regulation of ACC activity, change in the number of ACC molecules present in the cell also modulates fatty acid *de novo* synthesis (Sul *et al.* 2006, Hellerstein *et al.* 1999).

Using malonyl-CoA as the substrate, a multifunctional enzyme complex—fatty acid synthase (FAS), sequentially adds two-carbon fragments to a growing chain until a 16-carbon saturated fatty acid (i.e. palmitic acid) has been formed (Hellerstein *et al.* 1999). Similar to other enzymes involved in fatty acid synthesis such as ATP-citrate lyase, the activity and abundance of FAS is higher in the fed state (especially if the diet is high in carbohydrates) and lower in the fasting state, regulated at transcriptional and post-transcriptional levels (Hellerstein *et al.* 1999).

1.4.3.2 Hepatic triglyceride synthesis

There are two basic pathways involved in hepatic TG synthesis, the monoacylglycerol pathway and the phosphatidic acid pathway (Figure 1-6) (Lehner *et al.* 1996). The substrates used originate from catabolism of internalized remnant lipoproteins, hydrolysis of stored TG in the hepatocytes and adipose tissue, as well as those from cytosolic *de novo* lipogenesis (Lehner 1996). In addition to TG synthesis, the phosphatidic acid pathway is continuously committed to the glycerophospholipid biosynthesis (Lehner *et al.* 1996). Free fatty acids need to be activated to their CoA derivatives by ATP-dependent fatty acyl-CoA synthase (FACS) before undergoing further esterification or oxidation. FACS has been localized to microsomes, mitochondria and peroxisomes of both intestinal and hepatic cells (Sul 2006). Fatty acid transport protein (FATP) family also possesses acyl CoA synthase activity and thereby facilitates the activation of free fatty acid during cellular trafficking (Stahl A *et al.* 2001).

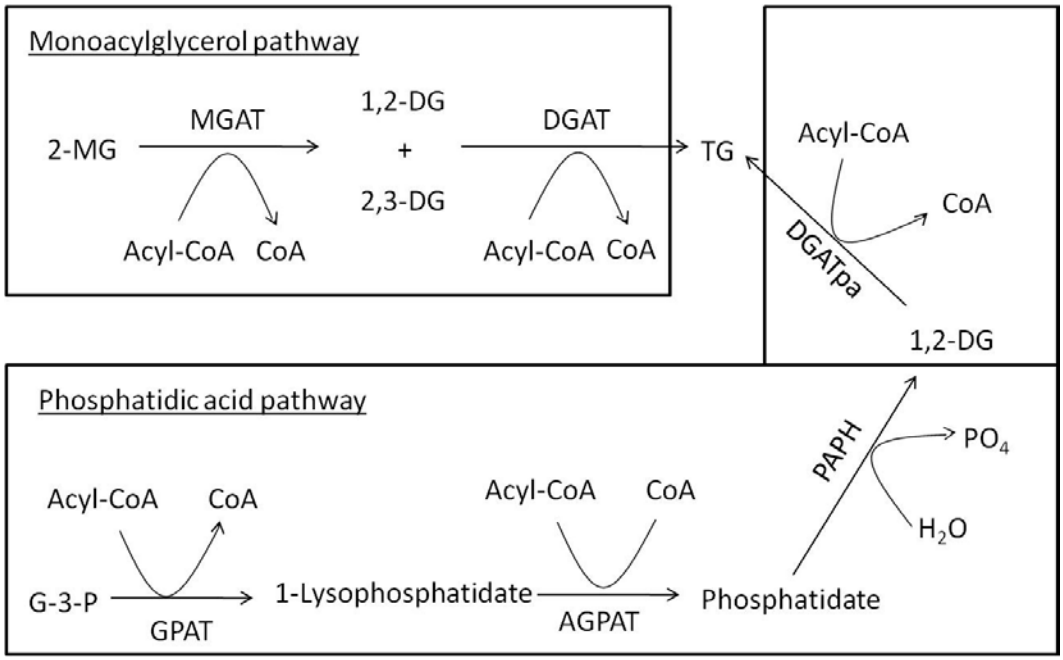

Figure 1-6. Schematic diagram of triglyceride biosynthesis.

The majority of TG is synthesized in ER. It has been suggested that the monoacylglycerol pathway is primarily associate with smooth ER whereas the phosphatidic acid pathway is localized to rough ER. Diacylglycerol acyltransferase (DGAT) catalyzes the last step in TG synthesis by adding a third fatty acyl-CoA to either sn-1 or sn-3 position of DG. The regulation of hepatic TG synthesis has been proposed to involve DGAT as it is the only unique enzyme involved in TG but not PL synthesis. The acylation stimulating protein directly interacts with DGAT and stimulates its activity by 90% (Yasruel *et al.* 1991). Moreover, the activity of DGAT may be regulated by phosphorylation and dephosphorylation mechanisms on its tyrosine residues (Rodriguez *et al.* 1992). Glucagon and cAMP inhibit hepatic microsomal DGAT activity in vitro by inducing phosphorylation of this enzyme (Haagsman *et al.* 1982). In addition to TG synthesis, monoacylglycerol acyltransferase (MGAT) and DGAT may also be involved in regulating dietary fat absorption, insulin sensitivity, satiety control and energy homeostasis (Shi *et al.* 2009).

1.4.3.3 Hepatic very low density lipoprotein production

VLDL is about one tenth the size of CM and is produced in the liver (Figure 1-7). Pre-VLDL particles are produced in two separate steps in ER lumen. Firstly an

initial PL shell is formed with density similar to HDL and consists of apo B100, apo A-IV, PL, FC and minor amounts or TG and CE. Apo B100 is constitutively synthesized in the ER, but is also co- or post-translationally degraded by ubiquitin-proteasome pathway if it is not lipidated on the luminal side of the ER membrane (Sakata *et al.* 1993, Liao *et al.* 1998). During translation, apo B100 slowly moves through the ER membrane and interacts with both the lipids and chaperones called microsomal triglyceride transfer protein (MTP). MTP is a lipid transfer protein containing two subunits and catalyzes the transport of TG, CE and PL between membranes (Wu *et al.* 1996). The small subunit of MTP is a multifunctional protein with both chaperone and disulfide isomerase activity. The small subunit helps retain MTP in the ER lumen, but its disulfide isomerase activity is not necessary for MTP's lipid transfer activity and is lost upon irreversible binding to the large subunit (Lamberg *et al.* 1996). The large subunit is responsible for associating with lipids (1 neutral lipid and 3 phospholipids per subunit), forming a transport and storage lipoprotein complex (Wetterau *et al.* 2000). During the process of lipid transfer, MTP first binds to a membrane from which it extracts individual lipid molecules. Then it dissociates from the membrane and diffuses to an acceptor membrane where it deposits lipid molecules to apo B100 during its translation (Atzel *et al.* 1993).

The pre-VLDL formed in the ER lumen travel to the *cis*-Golgi along the secretory pathway. The ER exit of the pre-VLDL particles is regarded as the rate-limiting step in the overall secretion from the liver (Mansbach *et al.* 2000). Due to the presence of lipolytic enzymes present in the cytosol (e.g. pancreatic lipase), it is essential that newly-synthesized pre-VLDL are protected from TG hydrolysis, which is achieved by the formation of VLDL transport vesicles that contains coat protein complex II and are formed via budding from ER membranes. Once fused with the Golgi membrane, pre-VLDL particle enters the Golgi lumen where it fuses with apo B-free lipid droplets pre-formed in smooth ER. Apo B100 is glycosulated and apo A-I is attached to the particle to form a mature VLDL that contains apo A-I, apo E and apo B100. Nascent VLDL receives additional apolipoproteins (e.g. apo C-II and apo C-III) from HDL particles in the plasma and at the same time apo A-I dissociates from VLDL into the plasma to participate in the reverse cholesterol transport (Siddiqi *et al.* 2000).

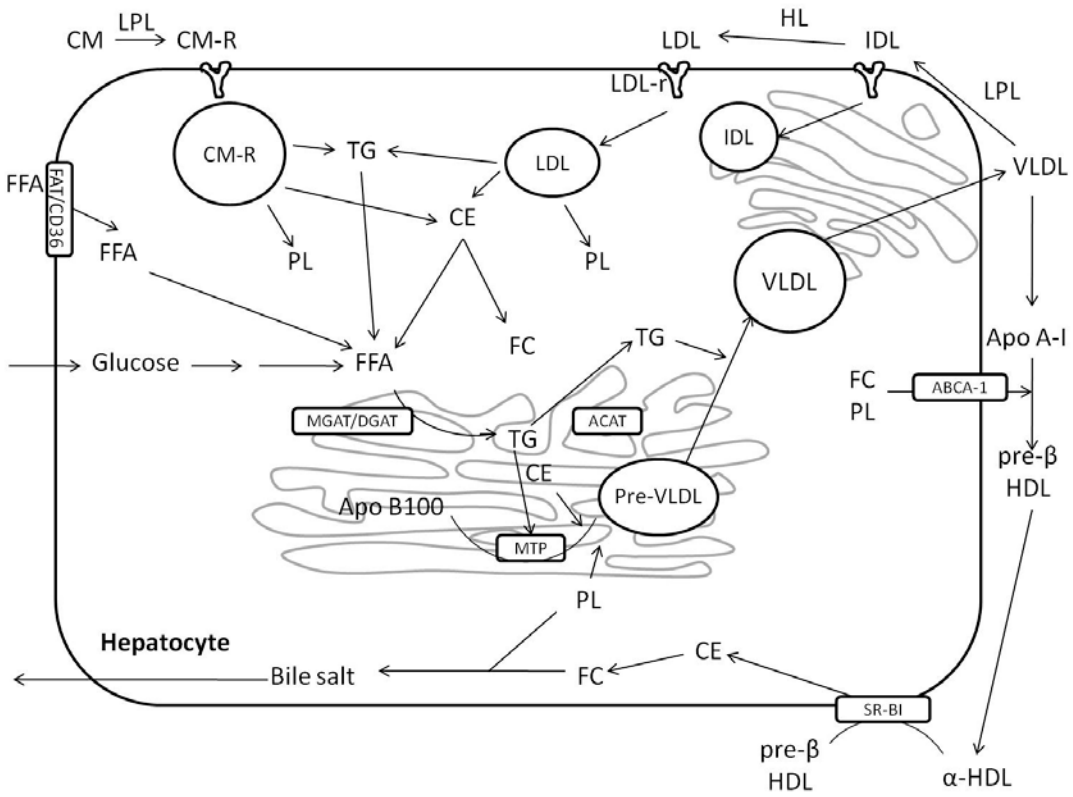

Figure 1-7. Schematic diagram of VLDL production in the liver.

1.4.3.4 Hepatic uptake of remnant VLDL

Peripheral lipoprotein lipase (LPL) hydrolysis of VLDL is the first step in the process of VLDL clearance (Sul *et al.* 2006). Gradually depleted with TG, VLDL becomes smaller and denser, transformed into IDL. The hydrolysis of TG by LPL only depletes the TG present on the surface of the particle. The equilibration between surface and core TG is critical in maintaining the persistent removal of TG from VLDL particles. LPL is robustly expressed in tissue parenchymal cells such as adipocytes and cardiomyocytes which require higher influx of FA for either energy or storage purpose. LPL is translocated to the lipid rafts at the endothelial cell surface of small capillaries of LPL secreting tissues, by interacting with the negatively-charged surface heparin sulfate proteoglycans (HSPG). HSPG also directly interacts with VLDL and facilitates LPL digestion, but this interaction might be interfered by apo C-III (Jong *et al.* 2000). The activity of LPL is regulated at different levels *in vivo*. During fasting, the expression of LPL in adipose tissue is down-regulated due to low circulating insulin

concentration, whereas in heart and other tissue, LPL expression is not affected in order to maintain consistent supply of energy. Apo C-II present on the surface of VLDL is considered an essential activator for LPL. However, an excess of apo CII may on the contrary inhibit TG hydrolysis. It is suggested that endothelial LPL in the heart has a higher affinity for lipoproteins than does LPL in the adipose tissue (Sul *et al.* 2006). This tissue-specific difference may provide explanation for the partitioning of fat between storage and oxidation. Humans with LPL deficiency have severe hypertriglyceridemia and are at higher risk for lipid disorders, pancreatitis and multiple organ failure (Fojo 1992). On the contrary, tissue-specific over-expression of LPL in liver and skeletal muscle led to elevated cellular uptake and storage of TG and insulin resistance may arise (Preiss-Landl 2002). In pancreatic beta cells, both deletion and over-expression of LPL leads to disrupted insulin secretion (Pappan *et al.* 2005). FFA released from VLDL by LPL is taken up locally by adipocytes and myocytes via plasma membrane FA transporters such as CD36. When the rate of FFA release by lypolysis exceeds the rate of tissue fatty acid uptake, some FFA may remain in the circulation as albumin-bound free fatty acids. Apo C-II gradually dissociates from the IDL surface, which is rapidly cleared by the liver, either via receptor-mediated uptake involving members of LDL-receptor (LDL-r) family, or by direct interaction with HSPG (Williams *et al.* 2008, Bishop *et al.* 2007).

Some of IDL particles, if not cleared from the blood in time, are subjected to further hydrolysis at hepatocytes surface by hepatic lipase (HL), producing LDL (Zambon *et al.* 2003). During this transition from IDL to LDL, apo E is transferred to HDL. LDL and other remnant lipoproteins have lost a substantial amount of TG but are still rich in cholesterol esters. LDL is internalized by peripheral tissue (e.g. endothelial cells) via LDL-r, which is an important pathway in acquiring cholesterol for normal cellular function. In humans, LDL transports over 70% of plasma cholesterol at any one time (Sul *et al.* 2006). After internalized into the cell, FC from IDL and LDL is released in endosomes, which is then esterified into CE by acyl-CoA cholesterol acyltransferase (ACAT) in the ER and stored in lipid droplets. ACAT is critical in regulating cellular FC concentration and the production of bile acids or steroid hormones in the liver and adrenal cortex,

respectively. ACAT is involved in foam cell formation from macrophages and also supplies CE for VLDL production (Leon *et al.* 2005).

The liver uptake of IDL and LDL is mediated either by high-affinity LDL-r (also called apo B100/apoE receptor), or by HSPG alone (Mahley *et al.* 1999). About 30 years ago, Goldstein and Brown demonstrated the ability of LDL-r in binding LDL via apo B100 and IDL via apo E, thus internalizing IDL/LDL into hepatocytes (Brown *et al.* 1986). It was later shown that CM-r could also bind to LDL-r via its surface protein apo E (Mahley *et al.* 1999). The LDL-r has an extracellular domain that contains apo B100/apo E binding site. The intracellular domain that directs the presentation of LDL-r at the plasma membrane region is called clathrin-coated pits. Upon binding with the remnant lipoprotein, the plasma-bound complex undergoes invagination within the coated pit to form a vesicle which then fuses with an endosome. In the early endosome, LDL-r dissociates from the remnant lipoprotein and is recycled back to cell surface in a transport vesicle. The endosome then fuses with a lysosome in the hepatocytes, allowing the lysosomal hydrolytic enzymes to digest the residual TG, CE and apolipoproteins into amino acids, free fatty acids and free cholesterol (Sul *et al.* 2006).

1.4.3.5 Involvement of liver in reverse cholesterol transport

HDL refers to a class of small, protein-rich lipoproteins that functions as a cholesterol scavenger via the reverse cholesterol transport pathway. It collects excess cholesterol deposition from endothelial cells and macrophages and thus is generally considered to be atheroprotective. A 4 mg/dL increase in HDL was associated with a 15% decrease in CVD end-points (Turner *et al.* 1998). Hypertriglyceridemia is often accompanied by low plasma HDL originated and disturbed reverse cholesterol transport, which exacerbates cholesterol accumulation and atherosclerotic plaque formation (Tato *et al.* 1997).

Approximately 95% of HDL in blood circulation is spherical α-HDL which consists of a hydrophobic lipid core and surface apolipoproteins including apo A-I and apo A-II. A small proportion of α-HDL also contain apo C-I, apo C-II, apo C-III and apo E, ready to be transferred to other apo B-containing lipoproteins. The remaining 5% of plasma HDL are the composed of apo A-I, phospholipids but no significant lipid core (Daniels *et al.* 2009). Apo A-I is the main apolipoproteins

associated with HDL and is secreted from the liver and small intestine. Humans and mice lacking apo A-I gene have extremely low HDL and develop severe atherosclerosis. Up-regulation of hepatic apo A-I expression significantly raises HDL concentrations and inhibits the progression or even promote the regression of atherosclerosis (Daniels *et al.* 2009).

Lipidation of preβ-HDL occurs primarily after its secretion and ATP-binding cassette transporter (ABC) A-1 in the liver is a critical participant of cholesterol efflux from the liver, responsible for the vast majority of the early lipidation (Daniels *et al.* 2009). Thereafter, preβ-HDL particles permeate through the vascular bed to the extravascular fluid of peripheral tissue where they are exceptionally active in accepting FC and PL effluxed from the parenchymal cells of extrahepatic tissues. The efflux of FC and PL is also dependent on cell membrane transporters ABCA-1, ABCG-1 and a cell surface receptor scavenger receptor class B type I (SR-BI) (Tall *et al.* 2008). During continuous lipidation, the small preβ-HDL becomes enlarged into disc-shaped discoidal HDL and re-enters into blood circulation via the lymph flow. Within the plasma, lecithin cholesterol acyl transferase (LCAT), an enzyme activated by apo A-I, catalyzes the formation of cholesteryl ester from free cholesterol and a sn-2 fatty acid donated by phosphatidylcholine. Newly synthesized cholesteryl esters may either sink into HDL core, transforming it into spherical α-HDL, or be transferred to apo B-containing lipoproteins by plasma cholesteryl ester transfer protein (CETP) in exchange of their TG. The TG, PL and CE content of α-HDL are selectively internalized via hepatic SR-BI. The α-HDL shrinks as its lipid core decreases, and apo A-I dissociates from the particle to regenerate preβ-HDL (Daniels *et al.* 2009).

Clinical treatment of hypertriglyceridemia by fenofibrate has been shown to not only substantially reduce plasma TG but also improve HDL and other atherogenic dyslipidaemia (Keating *et al.* 2007). Acting as a PPAR-□ activator, fenofibrate may function via increasing Apo A-I expression while reducing the activity of CETP, thus sustaining the circulating HDL and reverse cholesterol transport (Keating *et al.* 2007).

1.4.4 Intestinal lipid metabolism and cardiovascular disease

The primary role of intestine is to digest and absorb exogenous nutrients to meet daily requirement of the body. Intestinally-derived TG-rich CM particles function as vehicles delivering exogenous lipids to the peripheral tissue and the liver in order to maintain normal physiological functions of the body. The biosynthesis of CM follows similar pathways as VLDL packaging in the liver, but primarily relies on the monoacylglycerol pathway for TG synthesis using absorbed FFA, 2-MG and cholesterol during the fed state (Mansbach *et al.* 2010). The magnitude of intestine's influence on whole body lipid metabolism keeps expanding with emerging evidence suggesting an active involvement of intestine in maintaining cholesterol homeostasis. Novel findings suggest that the intestine is also involved in HDL formation by secreting apo A-I, although the relative contribution compared to the liver remains unknown (Lewis *et al.* 2006). Intestinally-derived apo A-I is lipidated upon secretion via ABCA1 and SR-BI at the basolateral membrane of the enterocytes. In addition to its role in facilitating reverse cholesterol transport, intestine is also capable of excreting excess cholesterol into the lumen (van der Velde *et al.* 2010). Cholesterol-rich lipoproteins (e.g. CM-r and LDL) are internalized from capillaries into enterocytes via LDL-r-mediated uptake at the basolateral membrane. The cholesterol released from remnant lipoproteins then approaches the apical membrane via the trans-intestinal movement and is eventually excreted by the ABCG5/G8 transporters (van der Velde *et al.* 2010).

With emerging research revealing the significant involvement in atherogenic process, the intestine has received increasing appreciation for its physiological importance, which challenges the long-standing recognition of liver being the most vital organ in lipid metabolism. Further understanding of how intestinally-derived CM particles are synthesized and metabolized in the body would allow clearer appreciation of its relationship with hypertriglyceridemia and post-prandial lipaemia.

1.4.4.1 Chylomicrons

Chylomicrons (CM) are intestinally-derived lipoproteins that transport dietary fat to the peripheral tissue and the liver where they are metabolized or stored as fuel. CM has the lowest density (<1.006 g/mL), the largest diameter (100-400 nm) and

the highest capacity of transporting TG (88% on average) per particle compared to other lipoprotein classes. The production and secretion of CM occur even in the absence of dietary fat at a basal rate. However, the ingestion of a fat-containing diet results in a stimulated increase in CM production, the composition of which normally reflects the dietary fat ingested (Cartwright *et al.* 1999).

CM can be differentiated from other lipoproteins by its unique structural component apo B48 (Kane *et al.* 1980). Apo B48 is the N-terminal 48% translation of the whole apoB gene (apo B100) and the only form of apoB synthesized in the adult human and rat intestines. The post-transcriptional action of apoB-editing catalytic component 1 (APOBEC1) deaminates cytosine to uracil, resulting in an in-frame stop codon UAA. The absence of C-terminal portion of apo B100 drastically changes the properties and biological functions of the protein (Chan *et al.* 1997). Apo B100 is a physiological ligand for LDL-r. The C-terminal portion and another motif within domain B of the full-length protein plays a vital role in the clearance of lipoproteins from the plasma, both of which are missing in apo B48 (Chan *et al.* 1997). As a result, apo B48 is unable to interact with the LDL-r and the clearance of CM is dependent on the presence of apo E. In addition, apoB48 is crucial in the lipidation of TG to pre-chylomicrons in the ER. In mice whose APOBEC1 gene was disrupted and only able to express apo B100 in the intestine, there was significantly less TG transported to lymph but more mucosal TG accumulation compared to wild type mice on a high-fat diet (Lo *et al.* 2008). There was also reduced apo B secretion in the knock-out mice during both fasting and fed state, but no change in microsomal triglyceride transfer protein (MTP, responsible for apo B lipidation) activity compared to wild-type mice. It was therefore suggested that apo B48 is preferred over apo B100 in chylomicron lipidation. Apo B48 is ideal for the determination of chylomicron kinetics as there is only one apo B48 molecule per CM particle; more importantly, it is essential for chylomicron assembly and not transferred to other lipoprotein during its metabolism (Phillips *et al.* 1997).

1.4.4.2 Chylomicron production, secretion and clearance

The assembly of CM particles in enterocytes follows the similar 2-step machinery as VLDL assembly in the liver and is summarized in Figure 1-8 (Mansbach *et al.*

2010). Firstly a pre-chylomicron particle containing a PL shell is formed with one molecule of apo B48 embedded on the surface, a hydrophobic core of CE and some TG transferred by MTP. The second step involves further lipidation of TG to the pre-chylomicron particle in the ER. One major difference between VLDL and CM production is the source of fatty acid used to synthesize TG in the ER. CM primarily uses dietary fat absorbed into enterocytes during the fed state, whereas VLDL incorporates fatty acids from hydrolysis of TG delivered in CM-r, free fatty acid released from the storage pool in adipose tissue, as well as the newly synthesized fatty acids using metabolites from dietary carbohydrates. Pre-chylomicrons are transported to Golgi in pre-chylomicron transport vesicles (PCTV). PCTV have an average diameter of 250 nm, large enough to accommodate pre-chylomicron-sized particles (Siddiqi *et al.* 2007). Interestingly, PCTV does not contain ER-resident proteins or fragmented ER membranes (Siddiqi *et al.* 2007), suggesting that it is not formed via the routine ER budding process. Instead, cytosolic L-FABP plays an essential role in generating PCTV which is supported by *in vitro* studies using recombinant L-FABP (Neeli *et al.* 2007). The major structural component shared by PCTV and the ER-derived protein transport vesicles is COP-II. Although COP-II is not required for PCTV synthesis, it is irreplaceable for PCTV-Golgi fusion. Electron microscopy studies show that PCTV acquires COP-II by recruiting its individual components directly from the cytosol (Siddiqi *et al.* 2007). In addition to COP-II, a vesicle-associated membrane protein (VAMP)-7 is also involved in the fusion of PCTV to *cis*-Golgi membrane. PCTV biogenesis is ATP dependent and involves a kinase and/or phosphorylation event. Inhibitors of protein kinase C substantially reduced PCTV formation from intestinal ER (Siddiqi *et al.* 2007). Chylomicrons are further processed in the Golgi where apo B48 is glycosylated and apo A-I is attached to the pre-chylomicron to form a mature CM that contains apo A-I, apo A-IV and apo B48 (Saddiqi *et al.* 2006). Upon exiting the enterocytes, apo A-I dissociated from nascent CM and HDL passes additional apolipoproteins to CM.

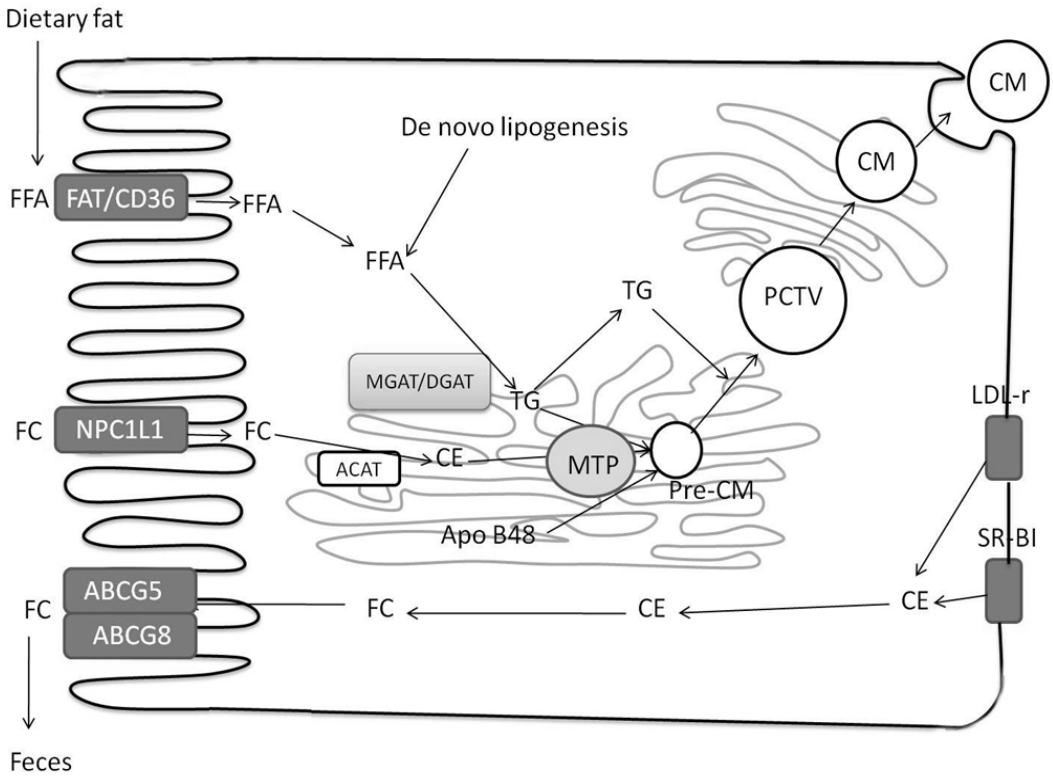

Figure 1-8. Schematic diagram of chylomicron synthesis in the enterocytes.

In the circulation, hydrolysis of CM-TG is also catalyzed by LPL present on the surface of peripheral capillary endothelial cells similar to VLDL catabolism in the circulation. As the CM-TG is hydrolyzed by LPL, the CM particle becomes smaller and denser. Apo C-II gradually dissociates from CM surface until approximately 80% of the initial TG has been lost, and the remaining apo C-II is insufficient to support LPL activity. The resultant CM-r is depleted of TG but rich in cholesterol. They are rapidly cleared by the liver, either via receptor-mediated uptake involving members of LDL-r family, or by direct interaction with HSPG (Williams *et al.* 2008, Bishop *et al.* 2007). The study of patients with familiar hypercholesterolemia provided new insights into possible alternative pathways in CM-r clearance. Researchers found that although LDL was accumulating in the plasma as a result of genetic defect in LDL-r, CM-r clearance was not affected in these patients. Follow-up studies revealed a distinctive mechanism of hepatic CM-r clearance which is not shared by LDL (Mahley *et al.* 1988). A LDL receptor-related protein (LRP) was identified on the surface of capillary epithelium that only recognizes apo E and not apo B100. A proposed model for this alternative

CM-r clearance involves the initial sequestering of CM-r by HSPG which not only docks LPL, but also facilitates the binding of apo E to LRP (Kowal *et al.* 1989).

Under normal conditions, more than 90% of remnant lipoproteins can be cleared via the hepatic LDL-r alone (Bowler *et al.* 1991). However, under conditions of reduced LDL-r expression, CM-r is inferior in the competition between CM-r and LDL for LDL-r because of its larger size. CM-r requires a cluster of four binding sites in order to be internalized whereas LDL only needs one. Since the expression of LDL-r is modulated by insulin, the disadvantage of CM-r in competing for LDL-r uptake severely weakens its clearance during insulin resistance. Since CM-r readily infiltrates vascular endothelium and fluxes through the tissue to the vaso-vasorum, more CM-r particles would be retained within the subcellular space and triggers atherosclerotic plaque formation during delayed CM-r clearance (Mamo *et al.* 1998).

1.4.4.3 Chylomicrons in atherogenesis

Results from a number of clinical trials have suggested that LDL is not the sole risk factor of atherogenesis. Up to 40% patients suffering from severe cardiovascular complications had normal LDL concentrations (Mediene-Benchekor *et al.* 2001, Colhoun *et al.* 2004). Fatal and non-fatal CHD events may still occur even after normalizing LDL by statin therapy in diabetic patients (Neeli *et al.* 2009). Indeed, there is growing recognition of the atherogenic potency of CM-r that is generated as a result of LPL digestion of TG-rich lipoproteins. Zilversmit was the first to challenge the traditional view of CVD risk by claiming that CM-r was potentially atherogenic (Zilversmit *et al.* 1979). Over the past two decades, it has been demonstrated that CM-r are capable of permeating the arterial wall and is preferentially retained in the subendothelial matrix, leading to the focal accumulation of cholesterol and the initiation of atherosclerosis (Karpe *et al.* 1994, Proctor *et al.*2004). Studies comparing the relative retention of apo B48 (i.e. CM-r) and apo B100-containing lipoproteins (i.e. LDL) and cholesterol deposition in the arterial wall revealed that, although there were fewer number of CM-r particles retained within the intima, the cholesterol deposition in the artery by CM-r was four fold greater compared to LDL (Proctor *et al.* 2002). These observations were supported by earlier human studies which

suggested that CM-r contained about 40 times more cholesterol per particle than apo B100-containing LDL particles (Proctor *et al.* 2002).

The mechanism for the preference of CM-r over LDL for arterial retention has been investigated. Earlier studies showed that atherosclerotic lesion development corresponds with the areas of lipoprotein retention and cholesterol deposition, which also corresponds with the expression of matrix proteoglycans (Skalen *et al.* 2002). Both LDL and CM-r are capable of binding to arterial proteoglycans; however, it appeared that the affinity of apo B48 for select proteoglycans is significantly greater than that of apo B48. Masking of epitope sites in the larger apo B100 molecules may explain the difference observed in the binding capacity (Flood *et al.* 2002). Moreover, it has been quantitatively shown that the rate of LDL efflux from the subendothelial matrix is 20 fold greater than that of CM-r, possible due to the smaller size of LDL (Proctor *et al.* 2004). During insulin resistance, there have been signs of vascular remodeling, resulting in increased sub-endothelial retention of cholesterol-rich lipoproteins. In addition, proliferation of smooth muscle cells in response to elevated circulating transforming growth factor (TGF)-β during insulin resistance would concomitantly increase the proteoglycan expression, leading to higher CM-r retention and cholesterol accumulation (Little *et al.* 2002).

1.4.5 Regulation of lipid metabolism by transcription factors

Genetic defect or environmental stimulation (e.g. life style) may interfere with key regulatory pathways of lipid metabolism, leading to reduced quality of life and in more severe cases, metabolic disorders such as diabetes and cardiovascular diseases. Several nuclear transcription factors have been shown to play a central role in modulating critical events in lipid metabolism (such as fatty acid synthesis and oxidation), thus providing a better understanding of the interaction between lipid metabolism and the etiology of certain metabolic disorders.

1.4.5.1 Sterol regulatory element binding proteins

SREBP is a group of membrane-bound transcription factors that regulates the expression of genes involved in cellular lipogenesis in the peripheral tissues, such as liver and intestine (Brown *et al.* 1997). The proteins have basic-helix-

loop-helix-leucine zipper (bHLH) domain and bind to sterol regulatory elements (SRE) in the promoter region of target genes. Two SREBP genes encode for three different proteins with different tissue specificity. SREBP-1a and SREBP-1c are produced from the same gene but using alternate promoters. SREBP-1a is a potent activator of all SREBP-responsive genes including those mediating FA and cholesterol synthesis, whereas SREBP-1c preferentially activates genes involved in fatty acid and lipoprotein synthesis (e.g. ACC, FAS and SCD). SREBP-2, on the other hand, regulates the expression of genes involved in cholesterol homeostasis such as HMG-CoA reductase and LDL-r (Horton *et al.* 2002). SREBP-1a is the primary isoform in the intestine and spleen, whereas SREBP-1c and SREBP-2 are predominant in most organs such as liver and adipose tissue. The expression of SREBP-1c in the liver is regulated by liver-X receptor (LXR)-□ , which upon activation by oxysterols dimerizes with retinoid X receptor (RXR) and binds to the promoter region of SREBP-1c (Wojcicka 2007). In addition, SREBP-1c mRNA is elevated during hyperinsulinemia and when cultured hepatocytes are exposed to insulin (Chen *et al.* 2004, Azzout-Marniche *et al.* 2000).

SREBP is synthesized and located on the ER membrane in its inactive form. The active N-terminal region of bHLH needs to undergo proteolytic cleavage and translocation to nucleus to exert transcriptional activity. The SREBP cleavage-activating protein (SCAP) has a sterol sensing domain (SSD) facing the cytoplasm. When the cellular cholesterol is depleted, SCAP dissociated from Insig1/2 and the SCAP-SREBP complex is escorted in COPII vesicles from ER to Golgi apparatus, where SREBP is cleaved in two steps by the site 1 protease (S1P) and site 2 proteases (S2P), releasing the active N-terminus into cytosol (nSREBP) (Brown *et al.* 2002). When cellular cholesterol is high, Insig 1/2 binds to SREBP-SCAP complex, changes SCAP conformation and inhibits its interaction with COPII proteins. As a result, SREBP-SCAP complex is trapped within the ER, unable to be activated in the Golgi (Espenshade *et al.* 2002). The cholesterol concentration in ER is relatively low, thus increasing the sensitivity to minor fluctuations in luminal cholesterol abundance. Both SREBP-1a and SREBP-2 activation are strictly controlled by the SCAP-Insig cleavage system in response to cellular cholesterol. However, SREBP-1c is less responsive to sterol

regulation (Sheng *et al.* 1995), but rather more sensitive to carbohydrate and fatty acids. The activation process of SREBP is summarized in Figure 1-9 (Shimano *et al.* 2008). Under conditions of over-nutrition, SREBP-1c expression and cleavage is stimulated by energy molecules (e.g. glucose and saturated fatty acids), resulting in elevated nuclear SREBP-1c protein and lipogenesis in the liver and adipose tissue. Starvation signals such as glucagon and cAMP, however, inhibit its expression (Shimano *et al.* 2008). The seemingly contradictory regulatory mechanism between SREBP-1c and the other two isoforms in fact reflect the physiological transcriptional regulation of energy storage in response to nutritional status. When energy source (i.e. glucose) is abundant, there is excess acetyl-CoA could be used as the substrate for fatty acid and cholesterol synthesis. This coexists with high expression of SREBP-1c. In contrast, in an energy-depleted state when SREBP-1c activity is suppressed, acetyl-CoA is spared for ATP production via citric acid cycle and oxidative phosphorylation (Shimano *et al.* 2008). Physiologically, the SREBP regulatory mechanism is essential for surviving starvation. However, chronic activation of SREBP due to over-nutrition exacerbates metabolic disturbances leading to diabetes, hyperlipidemia and MetS (Shimano *et al.* 2007).

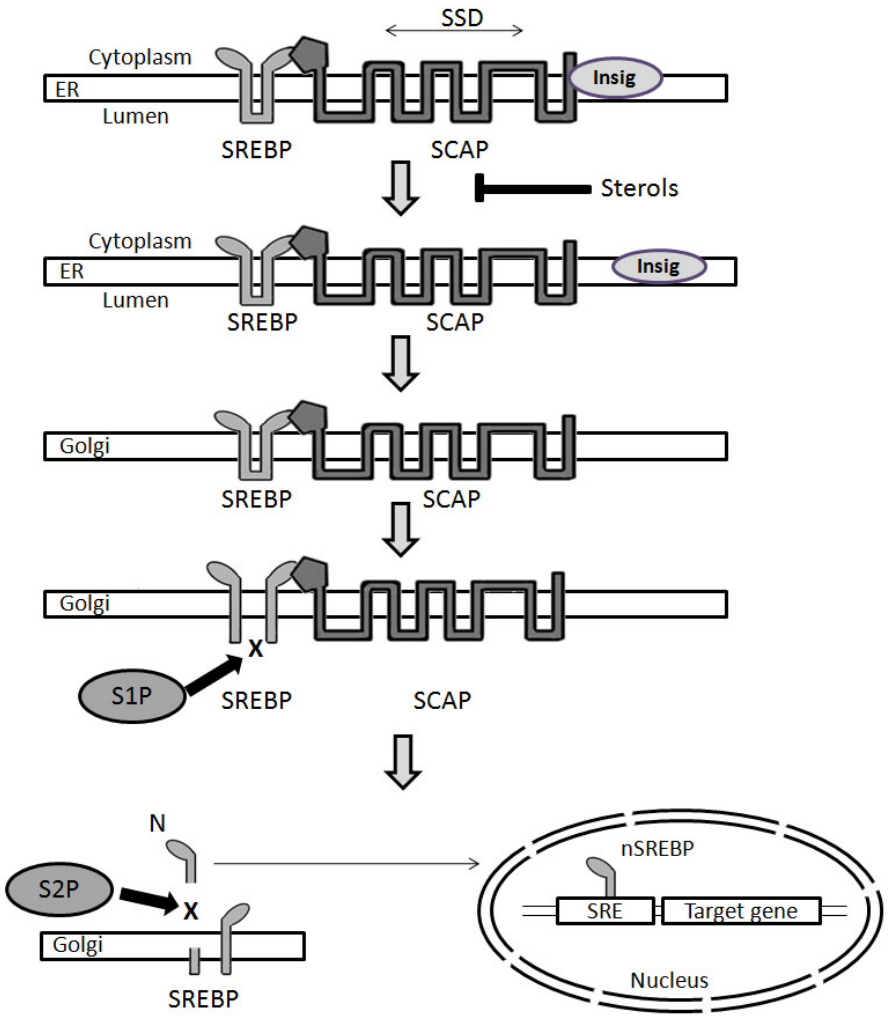

Figure 1-9. Schematic diagram of SREBP activation.

The impact of dietary fat on SREBP activity has been extensively studied. PUFA has been shown to reduce plasma TG concentration via down-regulating SREBP-1c and SREBP-1a mRNA and protein concentrations without affect SREBP-2 (Horton *et al.* 1998, Worgall *et al.* 1998). In addition to a direct inhibitory effect on LXR activation which in turn represses SREBP-1c transcription (Yoshikawa *et al.* 2002), PUFA may also enhance the ubiquitin-proteosome degradation of nSREBP and thus decrease their nuclear abundance (Botolin *et al.* 2006). Moreover, PUFA are natural agonists of several peroxisome proliferator-activated receptors (e.g. PPAR) which may compete with LXR for RXR heterodimerization (Yoshikawa *et al.* 2003).

1.4.5.2 Peroxisome proliferator-activated receptors

PPAR is a family of nuclear hormone receptors that are located in the cell nucleus and act as transcription factors under the stimulation of a diverse collection of natural and synthetic ligands (Brown *et al.* 2007). There are three forms, PPAR-α, PPAR-δ (also called PPAR-β) and PPAR-γ. Although three PPAR isoforms are encoded by separate genes, they share similar conserved domains: a ligand-binding domain (LBD) that binds directly with PPAR agonist; a transactivating domain (activation function 2, or AF2) which undergoes permissive conformational change upon ligand binding; and a DNA-binding domain that interacts with proxisomae proliferator response elements (PPRE) in the promoter region of PPAR targeting genes (Brown *et al.* 2007). PPAR activation is initiated by the binding of an agonist to LBD. A conformational change occurs in AF2 domain which releases co-repressors and recruits co-activators (e.g. cAMP response element-binding protein, PPAR gamma cofactor-1¤). These co-factors are critical determinants of PPAR's transcriptional response in that they directly induce chromatin remodeling and interact with basic transcriptional machinery (Rosenfeld *et al.* 2006). Ligand binding also allows the heterodimerization with RXR, another nuclear receptor required for PPAR activity. PPAR/RXR complex interacts with specific PPRE (direct repeat 1) and initiates target gene transcription (Figure 1-10). Each element in PPAR activation can subsequently influence biological and clinical outcome stimulated by a specific ligand. PPAR activation may not necessarily enhance the expression of target genes but depends on a complex interplay of a selective PPRE, ligand/cofactor availability, nuclear receptor isotypes and possibly other transcription factor binding in the vicinity of a specific PPRE. For example, one of PPAR-γ-mediated inflammatory regulations is achieved by stabilizing co-repressor and inhibiting TNF-α transcription (Pascual *et al.* 2005).

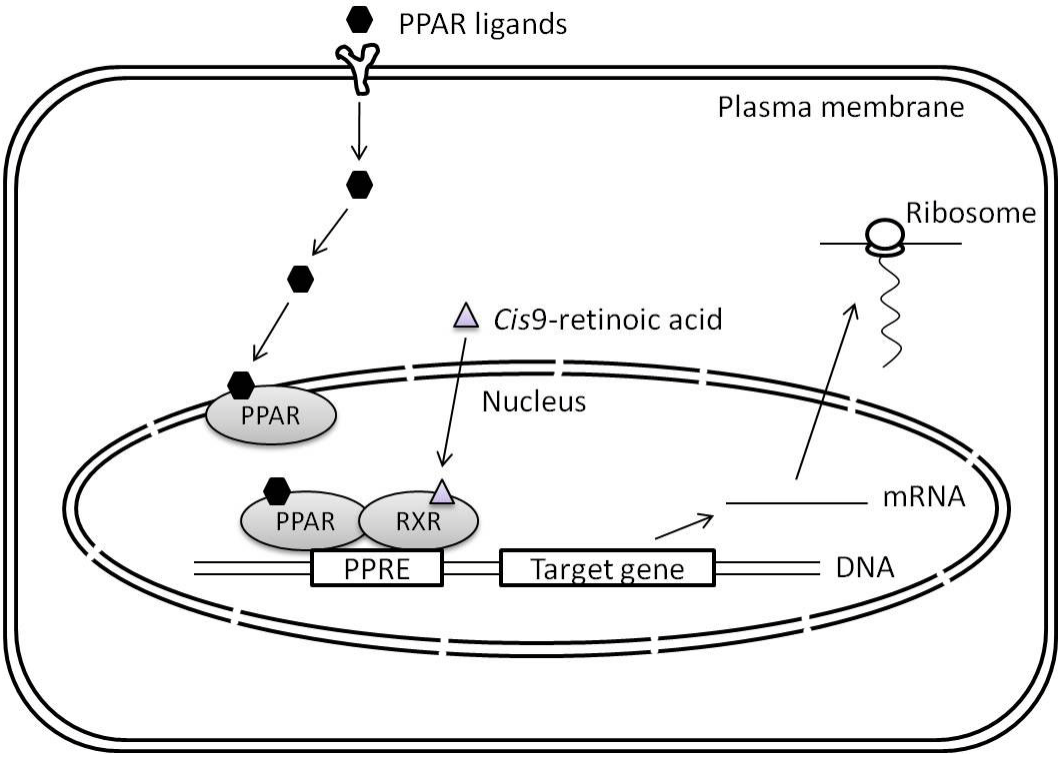

Figure 1-10. Schematic diagram of the activation of PPAR by ligands and the formation of PPAR/RXR heterodimer in regulating the transcription of downstream target genes.

PPAR-α is primarily expressed in tissues that are metabolically active and carry out significant catabolism of fatty acids, which include liver, heart, skeletal muscle, brown adipose tissue and kidney (Michalik *et al.* 2006). In the liver, PPAR targets at genes essential for fatty acid catabolism, gluconeogenesis, ketone body synthesis, lipoprotein assembly, heme synthesis and cholesterol catabolism (Michalik *et al.* 2006). Basic physiological changes in response to PPAR-α activation include increased fatty acid oxidation, lowered circulating TG, improved liver and muscle steatosis and insulin sensitivity (Table 1-3). In addition, PPAR-α agonists have been implicated in preventing atherogenesis. One proposed mechanism is the modulation of endothelial function by regulating nitric oxide signaling pathway and repressing nuclear factor-κB-mediated inflammatory responses such as vascular cell adhesion molecule (VCAM)-1 expression (Marx *et al.* 1999, Jackson *et al.* 1999). In human macrophages, PPAR-α also induces

cholesterol efflux from macrophages, suppresses IL-6 secretion by aortic smooth muscle cells, as well as limits pro-inflammatory signals such as interferon-γ and TNF-α expression in T lymphocytes (Brown *et al.* 2007). Collectively, the immune-regulatory effects of PPAR-α also play a key role in protective modulation of atherogenic pathways.

PPAR-δ has the broadest expression pattern. It is present in the skin, intestine, placenta, skeletal muscle, adipose tissue and brain, and regulates thermogenesis, fatty acid catabolism, and most importantly, cell proliferation and differentiation (Brown *et al.* 2007). Animal studies have linked PPAR-δ activation to fatty acid oxidation, adaptive thermogenesis in the adipose tissue and skeletal muscle with limited effect on lipid storage *per se* (Wang *et al.* 2003). Activation of PPAR-δ has also been implicated in inducing macrophage lipid efflux via increased expression of scavenger receptor class A and CD36 (Vosper *et al.* 2001). Macrophages from PPAR-δ knockout mice express lower levels of monocyte chemotactic protein (MMP)-1, MMP-9 and IL-1 (Lee *et al.* 2003). However, extensive expression of PPAR-δ across all cell types makes it difficult to use PPAR-δ-specific ligands in clinical research, thus limiting potential pharmaceutical application.

PPAR-γ has two isoforms, -γ1 and -γ2, identified by different N terminus and tissue distributions. PPAR-γ2 is highly expressed in the brown adipose tissue whereas PPAR- γ1 is expressed in the intestine, brain, vascular cells and immune cells (Dreyer *et al.* 1993). PPAR-γ is essential in maintaining specific adipocyte functions such as lipid storage in the white adipose tissue, energy dissipation in the brown adipose tissue and the survival of differentiated adipocytes (Table 1-3) (Rosen *et al.* 2000, Spiegelman *et al.* 1997). In addition to adipogenesis, it also modulates the interaction between carbohydrate and lipid metabolism which contributes to its insulin-sensitizing benefits (Lehrke *et al.* 2005). Potent anti-inflammatory effect on macrophage, endothelial and smooth muscle cells has also been discovered with PPAR-γ agonist treatment (Samaha *et al.* 2006, Joner *et al.* 2007).

The respective roles of these PPAR isoforms in inflammatory and atherosclerotic pathways have provided better understanding with regard to the integration of

metabolism and vasculature. Furthermore, pharmaceutical drugs targeting at these transcription factors have been developed to improve cardiovascular end-points. Lipid-lowering fibrates which activate PPAR-α and insulin-sensitizing thiazoloidinediones which activate PPAR-γ have been established as viable candidates, although their clinical significance is to be evaluated from large clinical trials (Touyz *et al.* 2006, Robinson *et al.* 2007). Naturally-occuring ligands such as dietary fatty acids have been shown to activate different PPAR isoforms to various degrees. PUFA (and their derivatives such as eicosanoids) tend to be more potent than other FA in activating all three isoforms (Kliewer *et al.* 1997). For instance, it has been reported that oleic acid (OA, 18:1), arachidonic acid (AA, 20:4), eicosapentaenoic acid (EPA, 22:5) and docosahexaenoic acid (DHA, 22:5) can effectively displace a potent PPAR-α ligand KRP-297 from recombinant human PPAR-α ligand binding domain, whereas linoleic acid (LA, 18:2) and SFA such as palmitic acid (16:0) are less potent activators (Murakami *et al.* 1999). In addition to free fatty acids, lysophospholipids and other phospholipid analogs have been shown to activate PPAR-γ and downstream targets in their intact form (McIntyre *et al.* 2003). Traditional gel filtration system, differential protease sensitivity, electrophoretic mobility shift assay, scintillation proximity assay as well as more commercialized competitive binding assay are the most common analytical techniques developed to assess the binding capacity between various ligands and ligand binding domains. However, the reported half maximal inhibitory concentration (IC_{50}) values for individual fatty acids may vary depending on the methods used, leaving the effective dose required for a meaningful activation and the connections between endogenous lipid metabolism and PPAR's response unresolved. Nevertheless, it has been suggested that circulating lipoproteins (e.g. VLDL) may function as reservoirs of potential PPAR ligands (Aarsland et al. 1998). LPL-mediated lipolysis was sufficient to boost FA uptake over the threshold, activating PPAR-α activity as well as its downstream responses, whilst stimulation from plasma albumin-bound FFA at physiological concentrations failed (Ziouzenkova et al. 2003, Doi et al. 2003). Other endogenous PPAR activators include 15d-prostaglandin J2, oxidized linoleic acid, leukotrienes and lysophosphatidic acid (Delerive et al. 2000, Devchand et al. 1996).

Table 1-3. Major metabolic pathways regulated by PPAR-α, PPAR-δ and PPAR-γ (adapted from Brown *et al.* 2007).

	Metabolic pathways	Target genes
PPAR-α	Fatty acid oxidation	↑Acyl-CoA oxidase, ↑[a]CPT-1, ↑FATP-4, ↑FAT/CD36 ↑Medium-chain acyl-CoA dehydrogenase
	Lipid metabolism	↑LPL, ↑Apo A-I, ↑ABCA-1, ↓Apo C-III ↑Angiopoietin-like protein 4
	Inflammation/vascular	↓ VCAM-1 ↓[b]COX-1, ↓IL-6
PPAR-◘	Fatty acid oxidation	↑Acyl-CoA oxidase, ↑FAT/CD36(in adipocyte)
	Glucose utilization	↑PGC-1α, PDK-2,3,4 (in muscle)
PPAR-γ	Adipogenesis	↑Adipocyte fatty acid binding protein-2, ↑Adiponectin ↑Adipsin
	Lipid metabolism	↑LPL, ↑ABCA1, ↑ABCG1, ↑FABP-4
	Glucose control	↑GLUT4, ↓Resistin
	Inflammation/vascular	↓Interferon-γ, ↓MMP-9, ↓TNF- α ↓Chemokines ↓Chemokine receptors

[a]CPT: carnitine palmitoyl transferase

[b]COX: cyclooxygenase

1.5 Insulin resistance and hypertriglyceridemia

The extensive interactions between hypertriglyceridemia and insulin resistance have been implied in numerous clinical studies (Rivellese AA *et al.* 2004, Riccardi *et al.* 2004, Haas *et al.* 2009). Pathophysiological changes during hypertriglyceridemia, such as TRL overproduction, elevated FFA released from TG hydrolysis, as well as increased uptake of FFA into endothelial cells, significantly contribute to the onset of insulin resistance. On the other hand however, insulin resistance and relative insulin deficiency could contribute to

hypertriglyceridemia through a series of mechanisms including overproduction of VLDL, decreased LPL activity, increased CETP activity and increased flux of FFA from adipose tissue to the liver, all of which further exacerbate VLDL production.

1.5.1 Physiological role of insulin

Insulin is a peptide hormone secreted by the β cells of the pancreatic islets of Langerhans. The biphasic insulin secretion begins to peak after 3-4 minutes after glucose stimulation and declines rapidly to a nadir at 8 minutes, followed by a gradually increased release to a plateau after a further 20-30 min (Straub *et al.* 2002). Insulin is the most potent anabolic hormone and plays a pivotal role in tissue development, growth and modulation of glucose and lipid homeostasis. When blood glucose is high, insulin decreases hepatic glucose output via suppressing gluconeogenesis and glycogenolysis. Glucose uptake in to peripheral tissue (primarily skeletal muscle and adipose tissue) is also stimulated via increased translocation of glucose transporters [e.g. glucose transporter (GLUT)-4] to cell surface. Insulin profoundly affects lipid metabolism by increasing lipid synthesis, fatty acid uptake and LPL activity, while attenuating hormone sensitivity lipase activity, TG hydrolysis and fatty acid release from the adipose tissue (Patti *et al.* 1999).

1.5.2 Tissue-specific actions of insulin

Skeletal muscle is the largest consumer of postprandial glucose. Insulin promotes glucose uptake by stimulating GLUT-4 translocation to the cell surface where they facilitate glucose uptake into the cells. It also promotes the storage of glucose as glycogen or gluconeogenic amino acids while inhibiting glycogenolysis (Dugani *et al.* 2005, Minokoshi *et al.* 2003).

In adipocytes, insulin not only stimulates glucose uptake through GLUT-4 translocation and the conversion of glucose metabolites to lipids, but also stimulate fatty acid uptake via increasing LPL section and translocation to cell surface (Foufelle *et al.* 1996). Lipogenic enzymes such as ACC and FAS, using substrates from glucose metabolism, are transcriptionally up-regulated by insulin (Foufelle *et al.* 1996). Elevated fatty acid availability promotes TG *de novo* synthesis, and meanwhile the hydrolysis of stored TG is inhibited through

inactivation of hormone sensitive lipase (HSL) activity. The overall consequence of insulin stimulation on adipose tissue is the significant expansion of TG storage in the adipocytes (Foufelle *et al.* 1996).

Glucose uptake in the liver is regulated in a different manner to that in skeletal muscle and adipose tissue, independent of insulin. Hepatocytes express the high-affinity, insulin-insensitive GLUT-2 glucose transporter (Burguera *et al.* 1995). Insulin plays a different role in hepatic glucose metabolism by increasing the production of glucokinase (Lynedjian *et al.* 2009). The enzyme converts glucose to glucose-6-phosphate which is the substrate for glycogen synthesis, pentose phosphate pathway as well as glycolytic pathway. The quick removal of cellular glucose by glucokinase is the driving force for more liver glucose uptake. Insulin also inhibits hepatic glucose output by down-regulating the key enzymes involved in gluconeogensis (such as phosphoenolpyruvate carboxykinase) and glycogenolysis (such as glycogen phosphorylase). The stimulatory effect on TG production by insulin is regulated in a similar mechanism to adipocytes possibly via SREBP-related pathways.

In addition, insulin has been associated with endothelial function by activating endothelial nitric oxide synthase (eNOS) in endothelial cells which generates the cardioprotective nitric oxide (Huang *et al.* 2009). Other tissues like the kidney and gonads also express insulin receptors, which mediate different metabolic responses.

1.5.3 Chylomicron overproduction during insulin resistance

Clinical studies have provided increasing evidence of a positive association between abnormal chylomicron metabolism and conditions such as obesity and insulin resistance. Couillard *et al.* examined the postprandial CM response following a high-fat meal and revealed that obesity was positively corrected with increased concentration of post-prandial TG-rich apo B48-containing lipoproteins (Couillard *et al.* 2002). Abdominal fat, rather than body weight, shows stronger correlation with plasma CM-r concentration (Mamo *et al.* 2001). Type-2 diabetic patients are four times more likely to develop CVD compared to non-diabetic subjects, and the production rate of CM has been shown to be significantly higher in those patients (Hogue *et al.* 2007, Curtin *et al.* 1996).

It was believed that increased circulating CM-r during insulin resistance was due to impaired CM-r hydrolysis attributed to a defective LPL activity as well as delayed clearance due to lower expression of hepatic LDL-r and LRP (Malmstrom *et al.* 1997). Moreover, the CM particles produced during insulin resistance have lower apoE content; thus the binding capacity with LDL-r may be severely impaired (Levy *et al.* 1985). Recent evidence have suggested an alternative underlying cause involving CM overproduction/secretion during insulin resistance (Duez *et al.* 2008, Lewis *et al.* 2005). Studies in human insulin resistant subjects revealed a significant association between apo B48 production and fasting insulin concentrations (Duez *et al.* 2006). Similarly, CM overproduction was evident in several insulin resistant animal studies (Vine *et al.* 2007, Duez *et al.* 2008, Lewis *et al.* 2005). In the JCR:LA-*cp* rat model of MetS, there was CM overproduction and severe post-prandial lipaemia following an oral fat challenge (Vine *et al.* 2007). The rate of CM production during the fasted state was one fold higher in insulin-resistant rats compared to normal rats, but was exacerbated when animals were challenged by an oral fat load (Vine *et al.* 2008). The elevation of CM secreted into the plasma directly contributes to the post-prandial hypertriglyceridemia. The underlying mechanisms of intestinal CM overproduction are yet to be elucidated. Nevertheless, disrupted insulin signaling pathway may lead to upregulation of lipogenesis, fatty acid uptake, apo B48 lipidation, and possibly prechylomicron transport vesicle formation (Duez *et al.* 2008, Lewis *et al.* 2008).

1.5.4 Underlying causes of insulin resistance

Insulin resistance refers to a state in which normal circulating levels of insulin fail to produce its expected physiological effects, possibly due to disrupted insulin signaling pathway. This results in a compensation of increased insulin production and secretion from pancreas in order to achieve the desirable metabolic regulation. Abnormally high circulating insulin may still be insufficient to trigger adequate regulatory signals in certain tissues, but may on the other hand increase the risk of excessive effect in other tissues. Insulin resistance is an essential feature of obesity, type-2 diabetes and MetS. Genetic disposition, caloric intake, age, body composition and physical inactivity are risk factors of

insulin resistance; however the strongest correlation exists between insulin resistance and obesity (Mehta *et al.* 2010).

Chronic caloric surplus and/or adipocyte hypertrophy cause macrophages and adipocytes within the adipose tissue to undergo transformation that ultimately alters their metabolism and secretion profile (Kirk *et al.* 2009). One distinctive feature is the release of lipids, hormones (e.g. leptin), inflammatory molecules (e.g. TNF-α, IL-6) from adipose tissue and the subsequent chronic low-grade inflammation which influence insulin sensitivity in other tissues (Kirk *et al.* 2009). Theories have been proposed trying to interpret the rather complicated cross-talk between obesity and insulin resistence, among which a feasible explanation for obesity-induced insulin resistance is lipotoxicity (Delarue *et al.* 2007, Gustafson *et al.* 2010). High circulating free fatty acids that efflux from adipocytes or from TRL hydrolysis are stored in non-adipose tissue such as the skeletal muscle and liver. The excess load of fatty acids, when exceeding the storage and oxidative capacity of the cell, lead to the formation of lipid metabolites (e.g. DG and ceramides) that antagonize insulin action and cause insulin resistance. Ceramide has been shown to interrupt insulin signaling pathway via blocking the activation of either insulin receptor substrate (IRS)-1 or Akt/protein kinase B (PKB) (Holland *et al.* 2007). Increased cellular oxidation of fatty acids may lead to ER stress and increased production of reactive oxygen species (ROS), both of which may lead to the development of insulin resistance (Hotamisligil *et al.* 2010).

1.6 Animal models of the metabolic syndrome

Most of the animal models used in mechanistic studies of CVD development are small and less expensive species like rodents. Genetic manipulation is also relatively easy with mice and rats specifically in studies targeting the metabolism of individual proteins. Several commonly used rodent models of insulin resistance and dyslipidemia have been characterized by genetic defect of either leptin or its receptors, which include the *ob/ob* mouse, the *db/db* mouse, the *fa/fa* Zucker rat and the JCR:LA-*corpulent* rat (Table 1-4) (Russell *et al.* 2006).

Leptin is a satiety signal hormone produced in the adipose tissue and regulates energy balance. It is released into the blood circulation in proportion to lipid storage, and acts on the leptin receptor in central nervous system to cease

eating behavior and increase energy expenditure. Either a lack of leptin production or irresponsiveness to leptin signal leads to overeating and positive energy balance (Bjørbaek *et al.* 2009). A common consequence of the absence of leptin receptor-mediated signaling pathway is the overproduction of leptin and subsequently high circulating leptin compared to normal phenotype. In addition to its role in maintain energy balance, leptin receptors have been documented to exist on the surface of immune cells such as macrophages, T cells and natural killer cells, suggesting potential immunoregulatory effect of leptin (Hillebrand *et al.* 2010). Some studies have also reported the effect of leptin in promoting fatty acid oxidation, which may contribute to the accumulation of visceral fat in some individuals with leptin resistance (Mathieu *et al.* 2008, Dyck *et al.* 2009, Al-Dokhi *et al.* 2009). Therefore rodent models with defects in leptin signaling pathway develops complications that better resemble the pathological changes in obese people, who normally have relatively high circulating leptin concentrations and relatively low leptin sensitivity compared to healthy volunteers (Hajer *et al.* 2008).

The obese Zucker rat is the very first genetic model of obesity. It is homozygous for the *fa* allele which bears a miss-sense mutation in the leptin receptor gene (ObR). The resulted receptor protein shows only 10% of the leptin binding affinity of the corresponding wide type (Russell *et al.* 2006). The Zucker rat develops a variety of metabolic disorders including obesity, hyperinsulinemia and hypertriglyceridemia. However, neither hyperglycemia nor further progression of type-2 diabetes or atherosclerosis develops in later life stages of obese Zucker rats, therefore less ideal for the studies of MetS. The *ob/ob* homozygous mice have no functioning leptin that can effectively bind to leptin receptors. It develops similar symptoms as the obese Zucker rat (e.g. obesity, insulin resistance, hypertriglyceridemia) and is a valuable model to study leptin functions (Russell *et al.* 2006). However, the *ob/ob* mice have only minor cardiac malfunction and no atherosclerotic lesions, making them less desirable for MetS research. The *db/db* mice carry a similar spontaneous mutation on the leptin ObR system and produce a leptin receptor with a premature termination in the intracellular domain and a defect in the ability of binding to leptin. In addition to the symptoms shown in obese Zucker rats, the *db/db* mice develop a severe hyperglycemia. Studies have shown endothelial and vascular dysfunction and glomerular sclerosis, but

no signs of atherosclerosis or end-stage CVD complications (Russell *et al.* 2006). As a brief summary, none of the mice models have been shown to spontaneously develop advanced CVD complications such as atherogenesis that resemble human disease. Rat models with normal genotypes are also resistant to atherosclerosis unless fed a high-fat/high-cholesterol diet (e.g. cholesterol-fed hamsters) (Russell *et al.* 2006). Of all the small animal models, only the JCR:LA-*cp* rat has been well documented to spontaneously develop clinical symptoms of MetS (obesity, hypertriglyceridemia and hyperinsulinemia) as well as pathological complications such as atherosclerotic lesions, and therefore best resembles the human populations bearing MetS.

The JCR:LA-*cp* strain, when homozygous recessive for the corpulent trait (*cp*), has a defect in leptin receptor expression and rapidly develops insulin resistance, hypertriglyceridemia, hypercholesterolemia and spontaneous ischemic lesions of the heart, which differentiate itself from the other insulin resistant models (Russell *et al.* 2006). Scanning electron microscopy and transmission electron microscopy have revealed extensive early intimal atherosclerotic lesions in major arterial vessels and small thrombi on the endothelial surface of middle-aged *cp/cp* male rats, which resemble the intimal atheroscelrosis commonly seen in patients with coronary artery diseases (Russell *et al.* 2006). Hyperinsulinemia seems to contribute significantly to hypertriglyceridemia and atherosclerotic lesion formation in *cp/cp* rats; however, they managed to maintain relative euglycemia even in postprandial state, possibly at the expense of considerably high circulating insulin concentration (Russell *et al.* 2006). The repeated spikes of insulin during postprandial phase exacerbate the adverse impact of insulin on lipid disorder, atherosclerosis and vascular damage. Overproduction of VLDL from the liver has been identified in the *cp/cp* rats, with upregulated lipogenesis from absorbed glucose (Elam *et al.* 2006). Recently the *cp/cp* rats have been shown to have significant postprandial lipaemia as measured by oral fat challenge experiments and apo B48 kinetic assessment (Vine *et al.* 2007). Overproduction of CM particles is also evident in the histological analysis of enterocytes as well as nascent lymph production (Hassanali *et al.* 2008). Thus, in the insulin resistant *cp/cp* rat, the presence of hypertriglyceridemia, insulin resistance and over-production and secretion of both VLDL and CM have

provided us with a valuable model to study the potential hypolipidemic and anti-atherogenic effect of dietary VA supplementation.

Table 1-4. Comparison between small animal models of obesity, dyslipidemia and MetS that have impaired leptin signaling pathway[1].

Animal model	Zucker rats (*fa/fa*)	JCR:LA-cp rats (*cp/cp*)	*db/db* mice	*ob/ob* mice
Defective gene	Leptin receptor	Leptin receptor	Leptin receptor	Leptin
Leptin signaling	Residual	None	Residual	None
Insulin resistance	YES	YES	YES	YES
Hyperglycemia	NO	NO	YES	NO
Hyperinsulinemia	YES	YES	YES	YES
Hypertriglyceridemia	YES	YES	YES	YES
Atherosclerotic lesions	NO	YES	NO	NO

[1]Adapted from Russell JC. Cardiovascular Pathology. 2006:318-30.

1.7 Conjugated linoleic acid

1.7.1 Introduction

CLA refers to a group of geometrical and positional isomers of linoleic acid (*cis9*,*cis*12-18:2), with *cis*9,*trans*11-CLA being the predominant isomer. When CLA is commercially made from vegetable oils, such as those found in retail supplements, it is usually comprised of a mixture of isomers, predominantly *cis*9,*trans*11- and *trans*10,*cis*12-CLA (Yu *et al.* 2003). In the absence of supplementation, one's intake of CLA is dependent almost exclusively on the amount of ruminant fat consumed. The average intake of CLA from food varies considerably, ranging from as low as 0.1 g/day to as high as 1.5 g/day (15-fold difference) due to distinctive dietary patterns and variations in the content of dairy products. For instance, the intake of the *cis*9,*trans*11 isomer by a group of healthy males in Western Canada was reported to be at the lower end of published reports (95 mg/d) (Ens *et al.* 2001), and similar to the estimated intake of Americans (Ritzenthaler *et al.* 2001); whereas the average daily intake of *cis*9,*trans*11 isomer in Australia has been reported to be as high as 1,500 mg (Parodi *et al.* 2003).

More recently, it has been recognized that the endogenous *in-vivo* synthesis of *cis*9,*trans*11-CLA from VA may be the greater contributor to whole body concentration of this isomer compared to luminal synthesis in ruminant animals (Palmquist *et al.* 2005). In humans, the conversion of VA to *cis*9,*trans*11-CLA has been estimated to range from 11-30% (Turpeinen *et al.* 2002). Due to the complex origins of this isomer, it is possible that the existing estimation of dietary intake of *cis*9,*trans*11-CLA alone, regardless of its accuracy, may not adequately represent the total pool *in-vivo*. Indeed, an increased dietary intake of VA would also add cumulatively to the putative abundance of *cis*9,*trans*11-CLA.

1.7.2 Effect of *cis*9,*trans*11-CLA on CVD risk factors

Over the past few decades, the health benefits of CLA on carcinogenesis, body fat redistribution, CVD risk factors and immune functions have been reported predominantly in animal models (Feitoza *et al.* 2009). Unfortunately results from human clinical trials have not been as consistent. It is noticeable that a great

number of those studies have used a mixture of CLA isomers rather than individual isomers, and this may confound the interpretation of results as different CLA isomers may have different biological effects (Bhattacharya *et al.* 2006). Albeit limited, animal studies using purified *cis*9,*trans*11-CLA seem to infer a consistent cardio-protective benefit (Table 1-5).

Feeding CLA, particularly the *cis*9,*trans*11 isomer, has been reported to improve the blood lipid profile at an average dose of 1.0% w/w (equivalent to 3-5% of energy) in several animal models. In hamsters, plasma TG, total cholesterol (TC) and LDL (especially small dense LDL) concentrations were shown to be lower when fed 0.5-1% w/w *cis*9,*trans*11-CLA for 12 weeks (Wilson *et al.* 2006). There have been additional reports that less cholesterol accumulates in the aortic arch and fewer fatty streak lesions develop in CLA-supplemented hamsters (Mitchell *et al.* 2005, Wilson *et al.* 2006). Compared to the cholesterol-fed hamster, the apoE-knockout mouse is arguably a better model to study the progression of fatty lesions due to its genetic susceptibility to atherosclerosis (Reddick *et al.* 1994). When fed 1% w/w *cis*9,*trans*11-CLA for 12 weeks, the development of atherosclerosis in apo $E^{-/-}$ mice was significantly reduced and associated with reductions in plasma cholesterol and free fatty acids as well as increased (beneficial) apoA-I concentration. There was also significantly less cross-sectional lesion area in the aortic root of treated mice (Arbones-Mainar *et al.* 2006). In addition to reduced progression, the regression of pre-established atherosclerotic lesions was also reported in the aorta of CLA-fed apo-$E^{-/-}$ mice (Toomey *et al.* 2003). Similar results have been reported in cholesterol-fed rabbits that had decreased scoring of atherosclerotic lesions in both the thoracic and arch regions of the aorta (Kritchevsky *et al.* 2004). A number of double-blind randomized clinical trials have been conducted in overweight but otherwise healthy subjects in an effort to elucidate the effect of *cis*9,*trans*11-CLA on risk markers of CVD (summarized in Table 1-5). A couple of studies concluded that *cis*9,*trans*11-CLA alone had neutral effect on biochemical parameters associated with atherosclerosis and MetS, whereas the CLA mixture (with various combinations of *cis*9,*trans*11- and *trans*10,*cis*12-CLA isomers), tend to be harmful to CVD risk markers. It is worthwhile to note that most of these trials were targeting healthy population with higher CVD risk rather than patients

diagnosed with cardiovascular complications *per se*, therefore the efficacy of CLA intervention remains largely limited.

1.7.3 *Cis*9,*trans*11-CLA activates PPAR pathways

CLA, more specifically the *cis*9,*trans*11 isomer, is one of the few known naturally occurring agonists of the PPAR pathway, which is proposed to be a primary mechanism via which CLA elicits pleiotropic effects (Banni *et al.* 2002, Brown *et al.* 2003). Up-regulation of PPAR-γ has been shown to normalize insulin sensitivity, improve lipid metabolism and the clearance of lipoproteins, as well as restore vascular contractility and endothelial function (Biscetti *et al.* 2009). Immunohistochemical staining of aortic vessels have shown an increased expression of PPAR-γ in *cis*9,*trans*11-CLA supplemented animals compared to controls (Toomey *et al.* 2003). Collectively, these changes may partially explain the reduced accumulation of cholesterol in arterial vessels and attenuated progression of atherosclerosis. Moreover, treatment with *cis*9,*trans*11-CLA *in-vitro* resulted in augmented acceptor-dependent cholesterol efflux from macrophage-derived foam cells and increased mRNA expression of genes involved including CD36, ABCA1, LXR-α, NPC1 and NPC2 (Ringsei 2008). CLA has also been shown to suppress the production of pro-inflammatory cytokines(such as PGE_2 and TNFα), as well as IFN-γ induced COX-2 activity in mouse and human macrophages; both of which are considered characteristic changes of a PPAR-agonist treatment (Yu *et al.* 2002, Iwakiri *et al.* 2002, Stachowska *et al.* 2009).

1.7.4 *Cis*9,*trans*11-CLA regulates lipoprotein clearance via LDL-r dependent pathways

In addition to the modulation of PPAR-γ expression, CLA has also been reported to increase the expression of the hepatic lipoprotein receptors (i.e. LDL-r) responsible for the clearance of cholesterol-rich lipoproteins, including LDL and remnant lipoproteins (Dane-Stewart *et al.* 2003, Valeille *et al.* 2004). Up-regulation of the hepatic LDL-r and the inhibition of HMG-CoA reductase are the primary mechanisms targeted by lipid lowering therapies, such as the statin compounds (Dane-Stewart *et al.* 2003). Valeille and colleagues recently reported that an increased mass of LDL-r in the liver of hamsters supplemented with either

the single *cis*9,*trans*11 isomer or mixed isomeric CLA preparations (Valeille *et al.* 2004). However, the increase in LDL-r mass was independent of the effects on HMG-CoA reductase in reducing cholesterol synthesis, suggesting that *cis*9,*trans*11-CLA may have direct regulatory effects on the molecular signaling/production pathways of LDL-r. Whilst the metabolic fate of *cis*9,*trans*11-CLA is extensively studied, VA, the predominant rTFA and the major precursor of *cis*9,*trans*11-CLA, received little scientific attention regarding its *in-vivo* bioactivity.

Table 1-5. Summary of animal and cell culture studies using *cis*9,*trans*11-CLA and human clinical trials using a mixture of CLA isomers with respect to the effect of dietary CLA supplementation on CVD risk factors.

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Animal feeding studies				
Toomey *et al.* 2003	Effect of CLA on COX and PPAR-γ pathways in ApoE$^{-/-}$ mice	ApoE$^{-/-}$ mice were fed for 16 weeks with 1% *cis*9,*trans*11-CLA or a COX-1 inhibitor	*Cis*9,*trans*11-CLA retarded atherosclerotic lesion development and induced arotic lesion regression, ↑ PPAR- γ expression at aortic root	*Cis*9,*trans*11-CLA benefits atherosclerosis as PPAR- γ agonist, not as COX-1 inhibitor
Kritchevsky *et al.* 2004	Effect of individual CLA isomers on the development and regression of cholesterol-induced atherosclerosis	Rabbits with atheromatous lesions were treated for 90 days with 0.5% *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or a mixture	↓ Atherosclerotic lesions in arch and thoracic area of aorta by *cis*9,*trans*11-CLA and CLA mixture. *Trans*10,*cis*12-CLA only ↓ lesions in aortic arch	CLA improves atherosclerotic lesions and the efficacy is higher in high cholesterol diet
Valeille *et al.* 2004	Efficacy of CLA and fish oil in modulating atherogenic risk markers	Cholesterol-fed hamsters were treated for 8 wks with 1) control diet 2)0.6% *cis*9,*trans*11-CLA 3)1.2% CLA mixture 4) 1.2% CLA mixture +1.2% fish oil 5) control diet + 1.2% fish oil	↑ LDL-r, scavenger receptor type B-1 by *cis*9,*trans*11-CLA	Part of the beneficial effects of CLA can be ascribed to *cis*9,*trans*11-CLA and are boosted by fish oil.
Mitchell *et al.* 2005	Effect of CLA on atherogenesis in hamsters	High fat high cholesterol-fed hamsters were treated for 12 weeks with 1% w/w *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or LA	↓Non HDL:HDL and aortic fatty streak lesion by *cis*9,*trans*11-CLA. No effect of *cis*9,*trans*11-CLA on plasma TG, LDL,	Individual CLA isomers beneficially changes lipoprotein profile and reduces atherosclerotic lesion development, but not different than LA

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Arbones-Mainar *et al.* 2006	Different atherogenic properties of *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA in ApoE$^{-/-}$ mice	ApoE$^{-/-}$ mice were treated for 12 weeks with 1% *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or LA.	*Cis*9,*trans*11-CLA ↓plasma cholesterol, free fatty acids, glucose, insulin and aortic lesions, ↑apo A-I; *Trans*10,*cis*12-CLA showed opposite effects	*cis*9,*trans*11-CLA impedes atherosclerosis development whereas *trans*10,*cis*12-CLA is pro-atherogenic
Wilson *et al.* 2006	Effect of CLA and linoleic acid (LA) on plasma lipids and aortic cholesterol in hamsters	Cholesterol-fed hamsters were treated for 12 weeks with 0.5% w/w *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or LA	↓ Plasma TC, HDL, non HDL, TG, cholesterol accumulation in aortic arch by *cis*9,*trans*11-CLA	*Cis*9,*trans*11-CLA improves CVD risk factors
LeDoux *et al*. 2007	Effect of individual CLA isomers on lipoprotein cholesterol in hamsters	Cholesterol-fed hamsters were treated for 12 weeks with 1% w/w of cis9t11-CLA, t10c12-CLA or CLA mixture.	↓ TC, HDL, LDL, small dense LDL by c9t11-CLA. No significant changes in the t10c12-CLA or the mixture group	*Cis*-9,*trans*-11 CLA beneficially affects lipoprotein profile in this model. T10c12-CLA and CLA mixture are less active.
Cell culture studies				
Stachowska *et al.* 2003	Effect of CLA isomers on COX-1 and COX-2 activity of macrophages	Macrophages were treated with *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or LA at 30 μM for 48 hours	Both CLA isomers reduced TXB_2, PGE_2 via inhibiting COX-1and COX-2 activity	CLA inhibits COX activities via competing with arachidonic acid
Ringseis *et al*. 2008	Effect of CLA on macrophage cholesterol accumulation as PPAR-α, γ agonists	Mouse macrophage-derived foam cells RAW264.7 were treated with *cis*9,*trans*11-CLA, *trans*10,*cis*12-CLA or LA at 50 μM for 24 hours	Both CLA isomers reduced cholesterol accumulation, stimulated cholesterol efflux and upregulated CD36, ABCA1, NPC-1, NPC-2 and LXR-α	CLA reduced macrophage cholesterol accumulation via PPAR-dependent pathways

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Randomized clinical trials				
Blankson *et al.* 2000	Effect of CLA on body fat mass	Overweight or obese subjects were treated with a CLA mixture (35:35) at doses from 1.7 to 6.8 g/day	CLA reduced fat mass but had no effect in lean body mass, BMI compared to control, reduced TC, HDL and LDL	CLA reduces body fat mass and improves plasma lipid profile
Benito *et al.* 2001	Effect of CLA on blood lipids, lipoproteins and tissue fatty acid composition	Healthy females were treated for 9 weeks with a CLA mixture containing 4 major isomers at a dose of 3.9 g/day	No effect on plasma cholesterol, LDL, HDL or TG. Tissue CLA increased with supplementation, prominently *cis*9,*trans*11-CLA	No adverse effect of CLA in this population
Mougios *et al.* 2001	Effect of CLA on body fat, serum parameters and CLA content in serum lipids	Non-obese subjects were treated with a CLA mixture (35:35) at 0.7 g/day for 4 weeks and 1.4 g/day for 4 weeks	CLA reduced skinfold thickness, percentage of body fat and fat mass during the 2nd period but not overall. Plasma HDL was reduced during 1st period, CLA content increased in FFA, TG, PL and CE; PL has highest content regardless of treatment	CLA modulates body fat and serum lipids and increases CLA content of serum lipids
Riserus *et al.* 2001	Effect of CLA on abdominal fat and CVD risk factors	Abdominally-obese men with metabolic disorders were treated for 4 weeks with a CLA mixture (37:37) or olive oil at a dose of 4.2g/day	CLA reduced abdominal diameter, but had no effect on overall obesity of CVD risk factors (LDL, TC, HDL, TG, FFA, glucose, insulin)	Larger sample size and longer duration might be required to show the effect of CLA
Smedman *et al.* 2001	Effect of CLA on anthropometric and metabolic variables and FA comp of serum lipids and thrombocytes	Healthy men and women were treated for 12 weeks with a CLA mixture (35:35) at a dose of 4.2 g/day	CLA reduced body fat, no change in BW, BMI, abdominal diameter, serum lipoproteins, FFA, plasma insulin, glucose, PAI-1. Increased stearic, DPA and decreased palmitic, oleic content. In lipids	CLA reduces percentage of body fat and affect fatty acid metabolism.

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Zambell *et al.* 2000 and 2001	Effect of CLA on body composition, energy xpenditure, fatty acid and glycerol kinetics	Healthy adult women were treated for 9 weeks with a CLA mixture (50:50) at a dose of 3 g/day	No change in fat mass, body weight and energy expenditure. No change in lypolytic rate, free fatty acid release or glycerol.	CLA treatment for 9 weeks at this dose has no significant effect on body composition and energy management
Noone *et al.* 2002	Effect of CLA on TG-rich lipoprotein metabolism and reverse cholesterol transport	Normalipidemic men were treated for 8 weeks with two CLA mixtures (50:50 or 80:20) at a dose of 3 g/day	The 50:50 CLA mixture reduced fasting plasma TG where as the 80:20 CLA misture reduced VLDL. No effect on LDL, HDL or reverse cholesterol transport, body weight, glucose or insulin concentrations.	CLA supplementation improves plasma TG and VLDL in healthy humans, confirming that some cardio-protective effects in animal studies are relevant to man
Petridou *et al.* 2003	Effect of CLA on body fat, serum leptin or CLA incorporation into serum lipid classes	Healthy non-obese sedentary women were treated for 45 days with a CLA mixture (35:35) at a dose of 2.1g/day	No effect on intake, body fat, serum leption, TG, TC, HDL. Increased CLA content in TG, PL and total lipids, no change in CE	CLA has no effect on body composition or lipid profile in this population
Malpuech-Brugère *et al.* 2004	Effect of CLA isomers on body composition	Overweight men and women were treated for 18 weeks with *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA at a dose of 1.5g or 3 g/d	No effect of CLA on body fat mass, lean body mass or dietary intake.	CLA isomers have no effect on body composition in this population
Moloney *et al.* 2004	Effect of CLA on glucose/insulin metabolism, lipoproteins and inflammatory markers of CVD	Patients with type 2 diabetes were treated for 8 weeks with a CLA mixture (50:50) a dose of 3.0 g/day	CLA increased plasma glucose and decreased insulin sensitivity. CLA increased HDL and reduced LDL:HDL ratio. No effect on CRP or IL-6 but reduced fibrinogen.	CLA should not be recommended for diabetic patients due to adverse effect on IR.

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Ramakers *et al.* 2005, Naumann *et al.* 2006	Effect of CLA isomers on immune function and LDL phenotype in subjects with a higher risk of CHD	Overweight men with LDL-phenotype B were treated for 13 weeks with oleic acid+sunflower oil, *cis*9,*trans*11-CLA or *trans*10,*cis*12-CLA at a dose of 3g/day	C*is*-9,*trans*-11CLA is more active in regulating cytokine expression and slightly reduced sdLDL. No effect of two isomers on plasma CRP or LPC-induced production of IL-6, IL-8 or TNFα by PBMC, or serum LDL, HDL, TG, glucose or insulin levels.	Two CLA isomers does not affect LPS-stimulated cytokine production or risk factors for CVD/diabetes, but may have enhanced immune function
Riserus *et al.* 2004	Effect of CLA on insulin sensitivity, lipid peroxidation and inflammation	Abdominally obese men were treated with *cis*9,*trans*11-CLA for 13 weeks at a dose of 3 g/day	CLA decreased insulin sensitivity by 15% and increased 8-iso-PGF2α and 15-keto-dihydro-PGF2α secretion by 50%	CLA negatively affects insulin resistance and lipid peroxidation
Tricon *et al.* 2004	Dose response of CLA isomers on body comp. blood lipid, IR markers and immune cell function	Healthy men were treated for 8 weeks with *cis*9,*trans*11-CLA at a dose of 0.63-2.52 g/d or *trans*10,*cis*12-CLA at a dose of 0.59-2.38 g/day	Plasma TG and LDL:HDL ratio were lower by *cis*9,*trans*11-CLA No effect on body composition or insulin sensitivity. Both isomers decreased mitogen-induced T lymphocyte activation in a dose-dependent manner. No effect on lymphocyte subpopulation, serum CRP or ex-vivo cytokine production.	Divergent effects of these two isomers. *Cis*9,*trans*11-CLA appears to be beneficial to blood lipids. T lymphocyte function was improved by both isomers
Smedman *et al.* 2004	Effect of CLA on lipid peroxidation and inflammation	Healthy men and women previously treated with COX-2 inhibitor, α-tocopherol or placebo for 6 wk were given a CLA mixture (50:50) (3.5 g/d) or *trans*10,*cis*12-CLA (4.0 g/d) for 4 weeks	Both CLA prep induced 8-iso-$PGF_{2\alpha}$ and 15-keto-dihydro-$PGF_{2\alpha}$, COX-2 inhibitor suppressed increase in 15-keto-dihydro-$PGF_{2\alpha}$ in *trans*10,*cis*12-CLA group only.	CLA induced production of $PGF_{2\alpha}$ is mediated by COX-2. Induced lipid peroxidation dependent on *trans*10,*cis*12-CLA

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Whigham *et al.* 2004	Safety of CLA product (Clarinol™)	Obese but healthy humans were treated for 12 months with a CLA mixture (35:35)[1] at a dose of 6 g/day	No change in body composition or other adverse effect.	This CLA product is safe for obese human for at least 1 year
Gaullier *et al.* 2005	Effect of CLA on body composition, bone mineral density and BMI	Healthy but obese men and women were treated for 24 months with a CLA mixture (50:50) at a dose of 4 g/day	CLA reduced plasma total and LDL, body weight and body fat mass, no change in bone mineral density, increased Lp(a) and thromocytes	CLA appears to improve lipid profile but shows no effect on body composition
Smedman *et al.* 2005	Effect of CLA on inflammation indicators	Healthy men and women were treated for 12 weeks with a CLA mixture (35:35) or olive oil at a dose of 4.2 g/day	CLA increased C-reactive protein, but had no effect on TNF-□ , TNF-□ receptor 1&2, VCAM-1	CLA had a weak effect on modulating inflammation
Laso *et al.* 2007	Effect of milk supplemented with CLA on body composition and biochemical parameters of the MetS	Healthy but overweight/obese men and women were treated for 12 weeks with milk (500 mL) alone or spiked with a CLA mixture (50:50) at a dose of 3 g/day	Total fat mass was decreased in overweight CLA-milk group. No effect on parameters of Met S or renal function	CLA-milk supplementation reduced body fat in overweight subjects, but not in obese subjects.
Mullen *et al.* 2007	Effect of CLA on indices of immunity relating to CVD	Healthy men were treated for 8 weeks with a CLA mixture (50:50) at a dose of 2.2 g/day or a fat blend of typical UK diet as the control	CLA reduced Con-A stimulated peripheral blood mononuclear cells IL-2 production. No effect on IL-6, CRP, fibrinogen, IL-10 or TNF-α production	CLA has weak effect on markers of human inflammatory response

Reference	Study objectives	Treatment	Health outcomes	Conclusion
Nazare *et al.* 2007	Effect of CLA enriched yogurt on body composition and related gene expression	Healthy men were treated for 96 days with yogurt supplemented with CLA mixture (35:35) as TG at a dose of 3.76 g/day	No effect on BW, fat mass, fat free mass. CLA increased basal energy expenditure and PPARγ mRNA expression, reduced HSL mRNA and did not affect UCP-2 and LPL expression	CLA mixture supplemented yogurt does not alter body composition but increases RMR and alters adipose gene expression.
Steck *et al.* 2007	Effect of CLA on body composition and clinical laboratory values	Healthy but obese women and men were treated with a CLA mixture (50:50) for 12 weeks at a dose of 3.2 g/d or 6.4 g/d, or safflower oil at a dose of 8 g/day	CLA at a dose of 6.4 g/day reduced serum HDL, haemoglobin, increased lean body mass, serum alkaline phosphatase, CRP, IL-6 and white blood cells	Whereas CLA increases lean body mass, it may also increases inflammatory markers in the short term
Syvertsen *et al.* 2007	Effect of CLA on insulin resistance	Healthy overweight men and women were treated for 6 months with a CLA mixture (37:37) at a dose of 3.4g/day	No effect on glucose uptake, HOMA score for treatment group.	CLA does not affect glucose metabolism or insulin sensitivity in this population
Raff *et al.* 2008	Effect of CLA on atherosclerosis, inflammation, type 2 diabetes and lipid peroxidation.	Non-obese healthy men were treated for 5 weeks with control low-CLA butter or additional CLA mixture (40:40) at a dose of 5.5 g/day	CLA diet resulted in increased 8-iso $PGF_{2\alpha}$ than control butter. No changes in plasma TG, HDL, LDL , TC, TC:HDL ratio or glucose/insulin metabolism.	CLA mixture increases lipid peroxidation but does not affect CVD risk factors
Wanders *et al.* 2010	Effect of CLA on lipoprotein profiles	Healthy men and women were treated for 9 weeks with oleic acid, iTFA and aCLA mixture (80:20) at a dose of 20g/day	Both CLA and iTFA groups had higher serum LDL and total-to-HDL ratio, lower HDL. No change in TG for CLA but increased with iTFA.	High intakes of CLA mixture raise total to HDL ratio, but the effect of CLA is less than that of iTFA.

[1] The ratio of *cis*9,*trans*11-CLA: *trans*10,*cis*12-CLA in each CLA mixture is indicated in brackets.

1.8 Vaccenic acid

1.8.1 Introduction

*Trans*11-vaccenic acid is the predominant *trans* monoene in ruminant fats (50-80% of total *trans* fat) (Lock *et al.* 2004). As mentioned earlier, VA is produced via the incomplete biohydrogenation of PUFA (e.g. linoleic and linolenic acid) by microorganisms in the rumen (Lock *et al.* 2004). It is also interesting that dietary VA can be desaturated to *cis*9,*trans*11-CLA in ruminants, rodents and humans (Santora *et al.* 2000,Turpeinen *et al.* 2002). There has been little research attention to this natural *trans* fat, despite the fact that it is the precursor for *cis*9,*trans*11-CLA, which is considered beneficial to cancer and CHD prevention. More recently, agricultural scientists have made efforts to increase the *cis*9,*trans*11-CLA content of animal fats, which has resulted in an elevated VA production up to 12% of total fat (Lynch *et al.* 2005). PHVO is another contributor and has been estimated to account for about 13-17% of total VA intake (Wolff *et al.* 2000). The presence of VA in industrial *trans* fats has raised the question regarding the safety of rTFA-enriched products as to whether VA elicits the same adverse health effects as industrially produced *trans* fats. However, the bioactivity of VA *per se* and how it could impact chronic disease remain unclear. Due to the lack of evidence, VA remains part of the *trans* fat content on food labels, whilst CLA has been exempted in Canada, the United States and Denmark.

Novel techniques have been developed most recently to synthesize highly-purified VA in large quantities, which endorses further understanding of VA's bioactivity and metabolism. The key step in the chemical synthesis of VA is a modified Wittig reaction of commercially available methyl ester yilid and heptanal under the phase transfer catalysis condition with an 89% yield (Mouloungui *et al.* 2009). The resulting mixture of methylated *trans*-18:1 and *cis*-18:1 (16:84) undergoes a *cis*-to-*trans* isomerization under strong alkaline conditions and a subsequent ester hydrolysis to release the carboxyl group of VA. The final step is fractional crystallization based on a 35 °C difference between the *cis* and *trans* isomers, which significantly increases the purity of VA to higher than 96% (Mouloungui *et al.* 2009).

1.8.2 Vaccenic acid and cardiovascular disease

A couple of epidemiological studies have attempted to but did not conclude any relationship between CHD risk and estimated dietary VA intake from natural sources (Aro *et al.* 2000, Voorrips *et al.* 2002,). There has been no clinical feeding study published so far using purified VA as the primary intervention, leaving the effect of this rTFA in human population largely unappreciated. The only limited evidence with regard to the effect of purified VA preparations on CVD risk factors came from an early animal study. Meijer *et al.* fed hamsters with diets containing VA, elaidic acid (*trans*9-18:1), oleic acid, palmitic acid, or a combination of medium-chain SFA for 4 weeks (10% of energy). The effect of VA on blood cholesterol profile and two lipid transferring enzymes (CETP and phospholipid transfer protein) was not significantly different compared to saturated fat or oleic acid (Meijer *et al.* 2001).

Due to the limited availability and high cost of pure VA, the dairy fat enriched with both VA and *cis*9,*trans*11-CLA has been used as an cost-effective alternative in several rodent models, addressing the effect of a mixture of rTFA on CVD risk (Table 1-6). Valeille *et al.* reported that feeding 20% butter fat led to increased reverse cholesterol transport potential, decreased aortic cholesterol-ester deposition, LDL-peroxidability index, as well as IL-1 mRNA abundance in hamster aorta (Valeille *et al.* 2005). In addition, when a VA/CLA-enriched butter was fed to rodents, serum cholesterol, TG, and the extent of atherosclerosis were reduced compared to regular butter (Lock *et al.* 2005). Two studies using a rabbit model have compared the effects of a VA/CLA-enriched dairy diet to either a *trans*10-18:1 (another major TFA in PHVO) diet or a control diet low in these fatty acids. These studies reported a neutral effect of VA/CLA butter on risk factors of atherogenesis as well as less aorta fatty streak development (Bauchart *et al.* 2007). However, the studies in rabbits also reported that the ratio of atherogenic lipoproteins [very low density lipoproteins (VLDL+LDL) and anti-atherogenic high density lipoproteins (HDL) was significantly lower in the butter group compared to the *trans*10-18:1 group (Roy *et al.* 2007). When VA/CLA-enriched dairy products were given to several groups of healthy volunteers, the lipid-lowering benefits appeared to be diminished, possibly due to relatively short intervention periods or, similar to previously discussed CLA trials, targeting

populations being relatively healthy. Nevertheless, animal feeding trials in rodent models with either induced or spontaneous dyslipidemia do not seem to support adverse health effect of VA. But without further appreciation of the metabolic fate of VA *per se*, it is difficult to conclude whether VA possesses any explicitly different healthy implication on CVD risk factors that distinguishes itself from other detrimental TFA.

Table 1-6. Summary of animal feeding studies and human clinical trials using pure VA or VA-enriched dairy fat with respect to the effect of dietary VA supplementation on CVD risk factors.

Reference	Study objectives	Treatment	Health outcomes	conclusion
Animal feeding studies				
Meijer *et al.* 2001	Differential impact of 2 TFA, vaccenic acid and elaidic acid (EA), on lipid and eicosanoid metabolism	Cholesterol-fed hamsters were fed for 5 wks with 1) MCFA (C8:0+C10:0) 2) SFA (C16:0) 3) MUFA (C18:1 9c) 4) EA 5) VA	↓LDL and TG content compared to SFA, no difference in LDL & VLDL cholesterol or TG, TC or free cholesterol compared to MUFA, EA or MCFA ↑LDL/HDL ratio compared to EA	Both EA and VA lowers CVD risk factors, but this study does not support that EA is more detrimental than VA
Valeille *et al.* 2005	Efficiency of CLA and fish oil in modulating atherogenic risks	Cholesterol-fed hamsters were fed for 12 wks with 1)20% butter fat (1% VA + 0.4% c9t11-CLA (B diet) 2) 20% butter fat + 1% c9t11-CLA(BR diet) 3) 20% butter fat+1% fish oil(BF diet)	BR diet had lowest aortic lipid deposition, reduced plasma non-HDL:HDL ratio, improved antioxidized LDL paraoxonase activity, downregulated expression of inflammatory-related genes (TNF-α, IL-1β, COX-2)	Milk fat rich in *cis*9,*trans*11-CLA reduces atherogenic process in hyperlipidemic hamsters
Lock *et al.* 2005	Effect of VA/CLA enriched butter on plasma lipoprotein and tissue fatty acid profile	Cholesterol-fed hamsters were given the following 3 diet for 4 wks 1) 20% standard butter 2)5% standard butter+15% VA/CLA butter 3)15% standard butter+5% PHVO	VA/CLA butter ↓ plasma cholesterol, VLDL, (VLDL+LDL):HDL ratio	VA/CLA butter improves plasma lipoprotein profile and reduces risk of atherosclerosis

Reference	Study objectives	Treatment	Health outcomes	conclusion
Bauchart *et al.* 2007 Roy *et al.* 2007	Impact of natural (milk) CLA, VA and trans10-18:1 on the risk of atherogenesis	Male New Zealand white rabbits fed one of three diets for 12 weeks: 1) *Trans*10-18:1 2) 0.8% VA+0.3% CLA 3) Low in all three fatty acids	↓ VLDL, plasma TC, phospholipids & apolipoprotein B. ↓ aortic lipid infiltration vs. trans10-18:1, but not control diet. ↑ liver TG vs. other diet groups.	VA/CLA butter has neutral effect towards risk of atherogenesis, whereas *trans*10-18:1 is detrimental
Blewett *et al.* 2009	Effect of VA on inflammatory regulation in rats with dyslipidemia and MetS	Lean and obese JCR:LA-*cp* rats were fed either a control "Western" diet or 1% VA for 3 weeks	↓IL-2, TNF-α production by splenocytes	VA has reduced pro-inflammatory cytokine production under dyslipidemic conditions
Ruth *et al.* 2009	Effect of VA on inflammatory regulation in rats with dyslipidemia and MetS	Obese JCR:LA-*cp* rats were fed either a control "Western" diet or 1% VA for 16 weeks	↓Proinflammatory cytokine production by splenocytes Improves T lymphocyte function	VA improves inflammatory regulation under conditions of dyslipidemia and the metabolic syndrome
Tyburczy *et al.* 2009	Effect of EA and VA on CHD risk in cholesterol-fed hamsters	Cholesterol-fed hamsters were fed either a control "Western" diet or 2% supplementation of PHVO, VA or EA for 4 weeks	VA and EA reduced plasma total:HDL and non HDL:HDL ratios while PHVO increased these values	Hypercholesterolemic effect of PHVO are not dependent on EA or VA
Bassett *et al.* 2010	Effect of commercially hydrogenated shortening rich in elaidic acid and butter rich in VA on atherosclerosis	LDL-r knockout mice wre fed for 14 weeks with regular fat, elaidic shortening, regular butter, VA butter, or an atherogenic diet containing 2% cholesterol with each of the above 4 types of fat	VA butter spiked with cholesterol ↓ plasma cholesterol and TG compared to control butter with cholesterol, ↓atherosclerotic plaque formation compared with other diets containing cholesterol.	VA-rich butter protects against atherosclerosis whereas elaidic acid-rich diet stimulates atherosclerosis.

Reference	Study objectives	Treatment	Health outcomes	conclusion
Human clinical trials				
Desroches *et al.* 2005	Effect of VA/CLA enriched butter on plasma lipoproteins and body composition	Healthy but overweight/obese men were treated with control butter (0.2 g CLA+0.7 g VA /day) or CLA/VA butter (2.2 g CLA+4.7 g VA/day) for 4 weeks	CLA/VA butter reduced plasma TC and total:HDL ratio. No difference on body composition was observed.	CLA/VA-enriched butter does not induce great beneficial metabolic effects in this population.
Tricon *et al.* 2006	Effect of VA/CLA enriched dairy products on blood lipid profile, insulin resistance and inflammation	Healthy men were given ultra-heat-treated milk, butter and cheese for 4 weeks at a dose of 1.4g *cis*9,*trans*11-CLA+7g VA per day	No effect on enriched dairy on BW, inflammatory markers, insulin, glucose, TG, TC, LDL or HDL, but a small increase in LDL:HDL ratio.	Full-fat dairy product enriched with VA and CLA does not affect changes in CVD risk variables.
Chardigny *et al.* 2008	Compare the effect of TFA from industrially produced and natural sources on CVD risk factors	Healthy subjects were treated for 3 weeks with enriched dairy/day from two sources (1.66g VA+0.53g CLA).	TFA from natural sources increase HDL and LDL in women only, as confirmed by apo A1 and apo B. Only large HDL and LDL are modified by natural TFA, not by iTFA	TFA from industrial and natural sources have different effect on CVD risk factors in women. The HDL-lowering effect is specific to iTFA.
Motard-Belanger *et al.* 2008	Effect of iTFA and rTFA on plasma LDL and CVD risk factors	Healthy men received a high or low doses of either rTFA (10.2 g/d, 4.2 g/d) or iTFA (10.2 g/d, 2.2 g/d) for 4 weeks	Both high rTFA and iTFA diets resulted in higher plasma LDL and lower HDL than moderate rTFA diet. No difference between moderate rTFA and low iTFA diet (control)	Moderate intake of rTFA has neutral effects on plasma lipids and CVD risk factors while high intake might be detrimental.
Fonolla *et al.* 2009	Effect of enriched milk and skim milk/semi-skimmed milk on CVD risk factors	Healthy volunteers received 500 mL milk (supplemented with EPA, DHA, OA, vitamins, etc), semi-skimmed or skimmed milk for 1 year	Enriched milk increased serum folate and HDL, decreased plasma TG, TC, LDL. No effect on glucose, homocysteine or CRP.	Daily intake of enriched milk improves nutritional status and CVD risk markers while skimmed milk does not.

Reference	Study objectives	Treatment	Health outcomes	conclusion
Tardy *et al.* 2009	Compare the effect of iTFA and rTFA on insulin resistance	Overweight women with normal insulin sensitivity received a diet with low-TFA (0.54 g/d), rTFA (4.86 g/d, 1.5g CLA+ 2.2gVA) or iTFA (5.58 g/d) for 4 weeks	No change in fasting glycemia, insulinemia or insulin sensitivity in either group	rTFA or iTFA at nutritional levels do not impair peripheral insulin sensitivity among healthy population.

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Chapter 2 Rationale: Objectives and Hypotheses

2.1 Rationale

2.1.1 Introduction

Cardiovascular disease (CVD) remains the leading cause of death in North America and is responsible for an estimated 28% of total mortalities globally every year (Lloyd-Jones *et al.* 2010). Over the past few decades, there has been a growing body of evidence from epidemiological, clinical and animal studies, indicating that dietary intake of *trans* fatty acids (TFA) increases the risk of coronary heart disease (CHD) (Uauy *et al.* 2009). *Trans* fat consumption is positively associated with cardiovascular disease risk factors such as insulin resistance, dyslipidemia and inflammation (Teegala *et al.* 2009). Consequently, health organizations worldwide such as World Health Organization, US Food and Drug Administration and Health Canada have recommended elimination and/or reduction of *trans* fats in foods. Food labeling regulations have also been approved by Canada and the US government to promote a public health "*trans* fat-free" food choice (Eckel *et al.* 2007). However, legislatorial regulations often oversimplify the complex origin, natural or industrial-hydrogenated, of various TFA isomers. Given the emerging evidence suggesting a neutral or beneficial role of natural ruminant TFA-enriched dairy fat on blood lipid profiles, this 'broad approach' of eliminating all dietary TFA may not be necessary. Clearly it is now critical for industry, research scientists and the consumers to resolve the health implications of the different types of TFA associated with possible development or exacerbation of CVD risk factors.

2.1.2 Health implications of trans fatty acids

The majority of TFA isomers are formed during the partial hydrogenation of vegetable oils, a process to make commercial oils (e.g. margarines, shortenings). Major industrially-produced TFA (iTFA) isomers include up to 20 types of different fatty acids such as *trans*9-18:1 and *trans*10-18:1, whose relative abundance in partially hydrogenated vegetable oils (PHVO) may vary drastically depending on the manufacturing techniques and the types of vegetable oils (Allison *et al.* 1995). TFA also occur naturally in ruminant food products including dairy and ruminant meat. Compared to iTFA, the composition of ruminant sourced TFA (rTFA) is relatively stable, primarily composed of *trans*11-18:1 (vaccenic acid or VA) and *cis*9,*trans*11-18:2 (conjugated

linoleic acid, or CLA) (Allison *et al.* 1995). Both of these major rTFA are synthesized in the lumen of ruminant animals via bacterial fermentation of linoleic acid and α-linolenic acid (Palmquist *et al.* 2005). CLA can also be synthesized from VA *in vivo* by stearoyl-CoA desaturase, acting as the primary contributor to the CLA pool in ruminant animals (Palmquist *et al.* 2005). There has been growing evidence to support a beneficial role of CLA in carcinogenesis and dyslipidemia (Bhattacharya *et al.* 2006). As a natural agonist of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ, *cis*9,*trans*11-CLA can modulate a wide range of PPAR-related metabolic pathways (Lampen *et al.* 2005, Moya-Camarena *et al.* 1999). Convinced by these CLA findings, several countries including Canada have made amendments to the food labeling definitions and now exclude fatty acids with conjugated *trans* double bonds from the total *trans* fat classification. While this was met with approval worldwide in the food industry, it has not unilaterally addressed the issue of the differences between natural (VA) and industrially-produced TFA sources in the food supply. Intriguingly, while CLA is likely the most-studied rTFA, it is not the most abundant rTFA as VA can occur in concentrations that are 3-4 folds greater than CLA (Lock *et al.* 2004).

Prompted by the newly-discovered health benefits of CLA, modification to agricultural practices has been developed to enhance the content of *cis*9,*trans*11-CLA, such as supplementing feed with oil seeds or genomic screening for cattle strains with high CLA productivity (Lock *et al.* 2004, Kgwatalala *et al.* 2009). As a consequence, it is now a regular occurrence that rTFA can be as high as 12% of total fat in dairy products [which mediates additional reductions in saturated fatty acids (SFA)] (Mendis *et al.* 2008). The increase in the proportion of rTFA in dairy-derived products, with an initial aim of increasing CLA content, has confounded the premise for minimizing total dietary TFA and has sparked a critical need to better understand the bioactivity of specific rTFA isomers (e.g. VA), particularly in the context of CVD risk.

2.1.3 Dyslipidemia and cardiovascular disease

Dyslipidemia is routinely characterized by elevated plasma triglyceride (TG), low density lipoprotein cholesterol (LDL), as well as decreased high-density lipoprotein cholesterol (HDL) concentrations. The liver plays a central role in maintaining the balance in lipid metabolism. Overproduction of very low density lipoproteins (VLDL) by the liver is causally associated with increased plasma pool of TG and LDL. LDL has been regarded

as a traditional CVD risk factor due to its proposed role in the initiation and progression of atherosclerosis (Genest *et al.* 2003). Interestingly, clinical research has revealed that up to 40% of subjects diagnosed with CHD have normal plasma LDL (Mediene-Benchekor *et al.* 2001). Further, significant findings from prospective cohort studies suggested that both fasting and non-fasting hypertriglyceridemia are independently associated with the early pathogenesis of CVD (Bansal *et al.* 2007, Langsted *et al.* 2008). Most recently, the clinical significance of postprandial hypertriglyceridemia was further vitalized by several large-scale cohorts confirming the strong association with end-stage myocardial infarction, ischemic heart disease and related death (Nordestgaard *et al.* 2007, Freiberg *et al.* 2008). Complementary mechanistic studies have demonstrated that hypertriglyceridemia is associated with overproduction and/or delayed clearance of TG-rich lipoproteins, symptoms that are often observed in patients with type-2 diabetes (Ginsberg *et al.* 2005, Ooi *et al.* 2001). More intriguingly, hypertriglyceridemia might also be involved in the progression of impaired insulin sensitivity and associated signaling pathway, which may provide a much earlier window of identifying high-risk populations before the onset of more severe lipid disorders, compared to other traditional risk indicators (Ginsberg *et al.* 2001). Indeed, elevated plasma TG rather than LDL has been regarded as a necessary risk factor in the latest diagnostic criteria for metabolic syndrome (MetS) (defined by International Diabetes Federation) which represents an earlier stage in the development of CVD and strongly predicts the onset of type-2 diabetes (Kendall *et al.* 2002).

The contribution of intestine in regulating whole body lipid metabolism has become greatly appreciated and there is accumulating evidence that demonstrates its involvement in maintaining cholesterol homeostasis (Hui *et al.* 2008, Vine *et al.* 2008). Chylomicrons (CM), TG-rich lipoproteins that are synthesized in the enterocytes, as well as the resulting remnants after CM-TG hydrolysis, contribute to the plasma TG and cholesterol pool especially during the post-prandial phase. Fasting and post-prandial CM concentrations (as measured by apolipoprotein B48) are elevated in subjects with obesity, insulin resistance and MetS (Chan *et al.* 2002, Chen *et al.* 1993, Cohn *et al.* 2006). Well established findings from our group have shown that cholesterol dense chylomicron remnants (CM-r) permeate and accumulate in the subendothelial and extracellular matrix of arterial tissue (Proctor *et al.* 2002). Given CM-r deliver a greater amount of cholesterol per particle (Proctor *et al.* 2004), they are considered to have

significant atherogenicity, if not greater than LDL. The preferential delivery of cholesterol to arterial vessels via CM-r may have significant consequence in individuals with increased production and/or delayed clearance of CM-r in the post-prandial state. The contribution of CM over-production to post-prandial hypertriglyceridemia and the significant involvement of CM-r in atherogenesis underlie the strong associateion between imparired CM metabolism and CVD development.

2.1.4 Vaccenic acid and cardiovascular disease

*Trans*11-VA is the predominant *trans* monoene in ruminant fats (50-80% of total *trans* fat) (Lock *et al.* 2004). Dietary VA can be desaturated to *cis*9,*trans*11-CLA in ruminants, rodents and humans, which in the latter is considered beneficial in cancer and CHD prevention (Santora *et al.* 2000,Turpeinen *et al.* 2002, McLeod *et al.* 2004). However, with no direct scientific evidence, VA was assumed to have similar detrimental effects on CVD risk factors compared to other iTFA present in PHVO. Consequently by default, it was recommended that it be removed from the food supply with other TFA. VA is included in the *trans* fat content on food labels, whilst CLA is exempted from the TFA definition in several countries. Nevertheless, it has remained unclear what the bioactivity (if any) of VA is and whether VA mediates any physiological effects on insulin sensitivity, inflammatory regulation, lipid metabolism under either physiological or pathophysiological conditions. Many rodent models have dyslipidemia and impaired leptin metabolism, however the JCR:LA-*cp* rodent is unique in that it is a well established model of MetS and spontaneously develops macro- and micro-vascular complications, resembling early human CVD (Russell *et al.* 2007). The *corpulent* phenotype has increased plasma TG and cholesterol, and increased production of hepatic VLDL and intestinal CM in the fasting state and in the post-prandial phase, thus provides an opportunity to study the effect of VA on dyslipidemia and CVD risk factors (Vine *et al.* 2008).

2.2 Primary Thesis Aim

The primary aim of this thesis was to assess the effect of dietary supplementation of purified VA on CVD risk factors in a rodent model of dyslipidemia and MetS—the JCR:LA-*cp* rodent. To achieve this aim, the investigation of intestinal absorption and bioavailability of VA was first required in order to determine the relative contribution of dietary VA to the whole body lipid pool. Subsequently a short-term feeding study and a

corresponding long-term feeding study were undertaken to investigate the effect of VA on biochemical parameters, inflammation and whole body lipid metabolism, with specific focus on hepatic and intestinal lipogenesis. Finally, to explore mechanisms of VA action the expression of genes involved in hepatic and intestinal lipid metabolism, and the efficacy of VA in mediating PPAR and other regulatory proteins was investigated.

2.3 General working hypothesis

The working hypothesis for this thesis is that dietary supplementation of purified VA has beneficial effects on lipogenic and inflammatory pathways associated with conditions of dyslipidemia and MetS.

2.4 Objectives and hypotheses

In order to test the working hypothesis, the following general and specific objectives were employed:

General Objective 1. Determine the direct intestinal lymphatic absorption and bioavailability of VA from different food matrices including synthetic purified VA oil, a meal containing purified VA oil or beef fat enriched with VA; and to assess the *in-vivo* conversion of VA to *cis9,trans11-CLA* in JCR:LA-*cp* rodent model of MetS (Chapter 3).

Hypothesis: VA is directly absorbed by the intestine and is bioavailable from either natural or synthetic sources, and the intestine is a minor contributor to the conversion of VA to *cis*9,*trans*11-CLA.

Specific objectives:

1(i) Assess the intestinal bioavailability of synthetic VA in a lipid emulsion, synthetic VA in a mixed meal, and a mixed meal containing VA/CLA-enriched beef fat in the JCR:LA-cp rodent model.

1(ii) Determine the intestinal conversion of synthetic VA to cis9,trans11-CLA in the JCR:LA-cp rodent model.

General Objective 2. Investigate the acute effect of purified VA dietary supplementation (3 weeks) on food intake, body weight, insulin sensitivity, lipid metabolism and inflammatory markers in the JCR:LA-*cp* rodent model of MetS (Chapter 4).

Hypothesis: Short term feeding of VA has no detrimental impact on the biochemical parameters measured in lean rats. Nor does it exacerbate biochemical CVD and pre-diabetes risk factors in the JCR:LA-*cp* rodent model of MetS.

Specific Objectives:

2(i) Evaluate the effect of acute VA supplementation on body weight, food intake and glucose/insulin metabolism under conditions of both normolipidemia and MetS.

2(ii) Assess the effect of acute VA supplementation on fasting lipid profile and inflammatory markers under conditions of both normolipidemia and MetS.

General Objective 3. Assess the effects of prolonged dietary VA supplementation (16 weeks) on CVD and pre-diabetes risk factors, in particular fasting and post-prandial plasma lipid profile, as well as key metabolic pathways in the liver and intestine of the JCR:LA-*cp* rodent model of MetS (Chapter 5).

Hypothesis: Chronic VA supplementation results in consistent and substantial improvement in both fasting and postprandial lipid profile as well as modulates hepatic and intestinal lipogenic pathways in the JCR:LA-*cp* rodent model of MetS.

Specific Objectives:

3(i): *Assess the effect of chronic VA supplementation on fasting plasma lipid profile and postprandial lipaemia in the JCR:LA-cp rodent model of MetS.*

3(ii): *Determine the effect of chronic VA dietary supplementation on hepatic steatosis and fatty acid de novo synthesis in the JCR:LA-cp rodent model of MetS.*

3(iii): *Measure the direct effect of chronic VA dietary supplementation on intestinal chylomicron production and secretion from nascent mesenteric lymph collected from the JCR:LA-cp rodent model of MetS.*

General Objective 4. To assess the effect of VA on PPAR associated lipid metabolism in the JCR:LA-*cp* rodent model of MetS (Chapter 6).

Hypothesis: VA activates PPAR-α activity and modulates the gene expression of the key factors involved in regulating intestinal lipid metabolism.

Specific Objectives:

4 (i) Assess the efficacy of VA in activating PPAR-α via in vitro competitive binding assay as compared to several known fatty acid ligands.

4 (ii) Evaluate the effect of chronic VA dietary supplementation on protein expression of PPAR-α and PPAR-γ in primary enterocytes isolated from the JCR:LA-cp rodent model of MetS.

4 (iii) Determine the effect of chronic VA dietary supplementation on the expression of PPAR-related and other genes involved in lipid transport, lipogenesis, chylomicron production and key regulatory transcription factors in the intestinal jejuna mucosa isolated from the JCR:LA-cp rodent model of MetS.

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Chapter 3 Intestinal bioavailability of vaccenic acid in JCR:LA-*cp* rats

3.1 Introduction

Obesity-related CVD has become the leading cause of death worldwide and accounts for approximately one third of deaths in the United States (Lloyd-Jones *et al.* 2010) and 31% in Canada (Statistics Canada 2005). Epidemiological evidence suggests that CVD prevalence is strongly associated with the modern sedentary lifestyle and an unhealthy Western dietary pattern high in total fat and cholesterol (Uauy *et al.* 2009). Accumulating evidence from prospective cohort and case-control studies have revealed that increased intake of *trans* fat especially from PHVO has been positively associated with the incidence of CHD (Micha *et al.* 2009). As a result, WHO recommended that *trans* fat intake be limited to less than 1% of overall energy consumption (WHO 2002). Unfortunately the public health campaign of eliminating dietary *trans* fatty acids has not discriminated between the origin of *trans* fatty acids. Emerging research findings suggest a neutral to beneficial role of certain *trans* fatty acids from natural ruminant products (e.g. butter, beef) (Field *et al.* 2009, Aro *et al.* 2001, Bhattacharya *et al.* 2006), therefore the differentiation between natural and industrial hydrogenated TFA's needs to be addressed by food regulatory bodies. *Cis*9,*trans*11-CLA, a rTFA, has been reported to have anti-carcinogenic and anti-diabetic benefits in many experimental models, which has fostered investment by industry to develop CLA-enriched functional food products. Subsequently, agricultural scientists have reported supplementing cow feed with sunflower seeds which significantly increases the abundance of *cis*9,*trans*11-CLA, and leads to a concomitant rise in the precursor TFA—VA (Cruz-Hernandez *et al.* 2007).

VA (*trans*11-18:1) is the predominant *trans* isomer in ruminant fat (50%-80% of total trans content). Both VA and CLA are produced via the biohydrogenation of dietary PUFA (e.g. from sunflower seeds) by the luminal bacteria of ruminant animals. In addition, VA is the major precursor to endogenous synthesis of *cis*9, *trans*11-18:2 (conjugated linoleic acid or CLA) in humans and animals via the enzyme stearoyl-CoA desaturase (SCD)-1 (Bauman *et al.* 2003). High SCD-1 activity has been associated with insulin resistance and future development of metabolic syndrome (Vessby *et al.* 2002, Warensjo *et al.* 2005).

The majority of dietary VA is sourced from natural dairy food and ruminant meats (e.g. beef, lamb); however, approximately 10% of total VA intake is derived from PHVO,

which has been shown to contribute to CVD risk (Wolff *et al.* 2000). The enrichment of VA/CLA in dairy and beef products by means of modifications to cow feed and bioengineering techniques further increases dietary intake of VA (Lock *et al.* 2004). Unfortunately there has been a void in the literature addressing the direct metabolic bioactivity of VA *per se*. Therefore interpreting whether VA present in CLA-enriched functional foods and/or other dairy/meat products has beneficial or undesirable health implications has not been established. In addition there is a lack of knowledge on the intestinal bioavailability of VA and/bioconversion to CLA in the body which may interplay with the proposed health benefits of CLA. Bioavailabile VA refers to the proportion of dietary VA that is absorbed through the intestine, incorporated into intestinal CM. CM particles then enter the lymphatic and systemic circulation, hwre VA can be utilized by the body. The lack of research progress thus far may be attributed to the application of appropriate study designs and analytical techniques (e.g. mesenteric lymph collection) for assessing VA bioavailability. Also the limited availability and high cost of pure VA oil and or dairy/beef products enriched in VA/CLA has limited research on the direct health and metabolic effects of VA compared to mixed isomer/CLA cocktails available. Our group managed to gain access to highly purified VA oil and to beef meat enriched with VA (5.8% of total fat) and CLA (0.8% of total fat). To assess the direct potential health benefits of VA, it is essential to first determine the intestinal bioavailability of VA, which may be altered at several pathways: VA absorption across the brush boarder membrane into the enterocyte, in-vivo conversion of VA to CLA inside the enterocyte, re-esterification of VA for incorporation into CM-TG, as well as CM secretion into the lymphatic circulation. It is unknown whether the bioavailability of VA from synthetic oil sources would be different from that present in naturally enriched foods. Other dietary components (carbohydrate, protein and other lipids) may also pose influence on the bioavailability of VA when consumed as part of a balanced meal. Dietary lipid is absorbed and incorporated into intestinal chylomicrons, which are then secreted into the lymphatic circulation. Therefore using a model with established techniques to sample mesenteric lymph is necessary to directly measure VA bioavailability from different food sources. Furthermore, given the target population for the potential health benefits of VA is the obese, metabolic syndrome (MetS) phenotype, quantitating bioavailability in an animal model, such as the JCR:LA-*cp* rodent of MetS, may be the most appropriate and effective tool to study the bioactivity of VA. The aims of this study were to firstly assess the intestinal bioavailability of VA from different sources including synthetic VA, synthetic

VA in a mixed meal, and a mixed meal containing VA/CLA-enriched beef fat. The second aim was to determine the intestinal conversion of VA to *cis*9, *trans*11-CLA from synthetic VA in the JCR:LA-*cp* rodent model.

3.2 Materials and Methods

3.2.1 VA sources

Pure VA was synthesized by Dr. Reaney (University of Saskatchewan) using the Wittig reaction of methyl ester yilid and heptanal under phase transfer catalysis conditions (Mouloungui *et al.* 2009). The resulting mixture of methylated *trans* 18:1 and *cis* 18:1 undergoes a *cis*-to-*trans* isomerization and ester hydrolysis to release the carboxyl group of VA. The final step is fractional crystallization which increases the purity of VA to higher than 96% as verified by GC. The beef fat used in preparation of the mixed meals was pooled from several preparations of meat fat to give a high VA content of 5.8% of total fat, and was kindly provided by Dr. Michael E.R. Dugan (Meat Lipid Science, Agriculture and Agri-Food Canada). The fatty acid profile of CLA/VA-enriched beef fat is shown in Table 3-1.

Table 3-1. Fatty acid composition of the VA/CLA enriched beef fat.

	% of total fat
14:0	2.6
16:0	22.2
*Cis*9-16:1	3.2
18:0	19.2
VA	5.8
*Cis*9-18:1	39.2
*Cis*11-18:1	1.7
20:0	2.3
*Cis*9,*trans*11-CLA	0.8

3.2.2 Animal model and experiment protocol

Animals: Male rats of the JCR:LA-*cp* strain, both obese (*cp*/*cp*) and lean (*+/?*), were raised in the established breeding colony at the University of Alberta, as previously

described (Russell *et al.* 1995). Rat care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee. In order to reflect western dietary fat intake and intestine lipid exposure for bioavailability studies, rats were fed a semi-purified basal diet (Table 3-2) containing 15% lipids (w/w) for 3 weeks before the scheduled lymphatic/gastronomy surgery for bioavailability studies.

Table 3-2. Composition of the basal diet and the mixed meals containing synthetic VA and beef fat[1]

	g/kg
Casein	266.7
L-Methionine	2.4
Dextrose, Monohydrate	231.3
Corn Starch	221.8
Cellulose	49.4
Sodium Selenite	0.4
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	0.3
Mineral Mix, Bemhart-Tomarelli (170750)	50.2
Vitamin Mix, A.O.A.C. (No. 40055)	9.9
Inositol	6.2
Choline Chloride	1.3
Cholesterol	10.0
Fat blend	15

Lipid-meal emulsions: The following lipid or meal emulsions were prepared 1) control triolein, 500 µL triolein; 2) VA-triolein, 7.5 mg synthetic VA oil in 500 µL triolein; 3) VA-meal, semi-purified rodent chow containing 1.0% (w/w) VA; 4) Beef fat-meal, semi-purified rodent chow containing beef fat high in VA/CLA (Table 3-1). The semi-purified rodent diet used in each meal emulsion was lipid balanced to contain 1% cholesterol, 43.1% carbohydrate, 28% protein, 8% fiber and 15% lipid (w/w) with a PUFA:SFA ratio

of 0.6 and (n-6):(n-3) PUFA ratio of 10. The fatty acid composition of meal emulsions was verified by GC (Table 3-3). For VA-triolein emulsions, VA oil (7.5 mg) was dissolved in 100 µL chloroform and mixed with 500 µL triolein (Cat# T7140, Sigma, USA). The chloroform was then evaporated under a constant stream of N_2. The vial was flushed with N_2 and sealed with parafilm. For meal emulsions, the semi-purified diet powder (40 g) was dissolved in 50 mL of water containing soy lecithin (0.5 g/mL) as an emulsifier. The mixture was then homogenized (Polytron) for 5 minute. All meal emulsions were freshly prepared and wrapped in foil to prevent light exposure.

Lymph cannulation procedure: At 9 weeks of age, the gastroduodenal axis and the superior mesenteric lymph duct of each rat was cannulated in order to collect nascent lymph, as previously described (Vine *et al.* 1997). In brief, following abdominal incision, the lower gastrointestinal tract was moved aside to expose superior mesenteric lymph duct. The canula was then inserted into the lymph duct to sample nascent lymph. A gastrostomy was performed at the upper duodenum to which a canulae was inserted and fixed for emulsion infusion. After surgery and recovery, obese (n=15) rats were randomized to receive the lipid or meals (1-4) via the gastric cannula. Lean rats (n=5) received the VA-triolein emulsion. Each emulsion was warmed to approximately 37 °C and infused into the gastric cannula. The cannula was then flushed with 1 mL saline to ensure all the emulsion was infused. After 2 hours, saline (4% glucose) infusion was commenced via the gastric cannula and continued to the end of the experiment to ensure hydration of rats. The emulsion container, syringe and needle were weighed before and after infusion to accurately calculate the amount of emulsion delivered. Lymph was collected for 10 hours into EDTA-coated vacutainers and was centrifuged at the end of the experiment at 3000 rpm for 10 minutes to remove cell debris.

Fatty acid identification and quantification in lymph: Total lipid extraction of lymph was performed using adapted methods (Folch *et al.* 1956) and the fatty acid composition of TG extracted from diet emulsions and lymph samples was assessed by GC as previously described (Cruz-Hernandez *et al.* 2004) (Figure 3-3). In brief, lipid was isolated from lymph in a 4:1 mixture of $CaCl_2$ to $CHCl_3$:CH_3OH (2:1). Different lipid classes were separated on silica G plates, visualized with 8-anilino-1-naphthalenesulfonic acid under UV light and compared to the appropriate standards (Layne *et al.* 1996). Fatty acid methyl esters were prepared from the TG scraped silica

band using the base-catalyzed method with sodium methoxide. Prepared FAME were flushed with N_2 and stored at -80°C until analysis by GC. Fatty acids were separated by automated GC (Varian 3800,Varian Instruments) using a 100-m CP-Sil 88 fused capillary column (Varian Instruments) as previously described (Cruz-Hernandez *et al.* 2004).

Table 3-3. Major fatty acid composition of the fat blend of the fat blend used in the basal diet and the mixed diet containing synthetic VA or beef fat[1]

	Basal diet	VA-meal	Beef fat-meal
14:0	0.1	0.2	2.4
16:0	12.9	8.9	20.3
Cis9-16:1	0.1	0.2	3.0
18:0	35.5	44.9	15.7
*Cis*9-18:1	20.9	9.9	34.6
*Cis*11-18:1	0.1	0.4	1.2
VA	0.4	6.3	3.2
*Cis*9,*cis*12-18:2n6	26.0	22.7	2.4
*Cis*9,*trans*11-18:2n6	0	0	0.8
18:3n3	2.6	1.7	0.3

[1]Values expressed as percentage of total fat

3.2.3 Calculation of fatty acid bioavailability

The percentage intestinal bioavailability of VA was calculated by the equation:

$$\% \text{ Intestinal bioavailability of VA} = \frac{\text{Amount of VA in 10-hr lymph collection (mg)}}{\text{Amount of VA in the meal/lipid emulsion (mg)}} \times 100\%$$

The bioavailability of other major dietary fatty acids was calculated using similar equations. Relative percentage of intestinal bioavailability of each fatty acid was assessed by normalizing the % intestinal bioavailability to the total lipids extracted from mesenteric lymph [an indicator of the total bioavailability of lipids absorbed, predominantly TG) absorbed following infusion of emulsions].

3.2.3 Statistical analysis

All results are expressed as Mean±SEM. Data were tested for normal distribution and differences in intestinal bioavailability and bioconversion were analyzed using 1-way ANOVA followed by Tukey's post hoc tests. The level of significance was set at $p<0.05$ (Graph Pad Prism 5.0).

3.3 Results

3.3.1 Total lipid mass and fatty acid composition in mesenteric lymph

The total lipids mass extracted from mesenteric lymph was compared to the amount of lipids given in the lipid/meal infusion into the duodenum, which is shown in Table 3-4. In general, the total lipids extracted from the 10-hour mesenteric lymph collection following infusion of the lipid/meal emulsions was less than 5% of that present in the emulsions for all four groups. Lymph collected from obese rats had 4-fold greater lipid content compared to lean rats when infused with VA-triolein emulsions ($p<0.05$), suggesting that obese rats may have greater capacity for lipid absorption, higher enterocytic lipid synthesis and/or increased chylomicron secretion compared to lean rats. In addition, there were a 3-fold higher total proportion of lipids in the lymphatic circulation in obese rats infused with lipid-only emulsions (VA-triolein 3.1%) compared to those infused with meal emulsions (1.2%). The higher total lymphatic lipids in rats fed the beef fat-meal as compared to those fed VA-meal might be associated with the slightly different lipid content in their respective meal emulsions, however percentage of dietary lipid absorbed in the lymph was the same (1.2%).

Table 3-4 The weight of lipids in lipid/meal and mesenteric lymph in lean and obese JCR:LA-*cp* rats. Values are Mean±SEM. Means without a common letter differ, $p<0.05$.

	Lean VA-triolein	Obese VA-triolein	Obese VA-meal	Obese Beef fat-meal
Lipid/meal (mg)	347±41.9 [b]	359±27.2 [b]	1517±47.8 [a]	1713±29.9 [a]
Lymph (mg)	2.3±0.9 [c]	11.4±6.2 [b]	18.2±8.4 [b]	20.7±10.2 [a]
Percentage of dietary lipids delivered in lymph	0.7% [c]	3.1% [a]	1.2% [b]	1.2% [b]

The fatty acid composition of mesenteric lymph is shown in Table 3-5. When infused with triolein emulsions, there was no difference in the proportion of VA absorbed, but there was greater myristic acid, palmitoleic acid, oleic acid (OA), eicosapentaenoic acid and docosahaexanoic acid ($p<0.05$), and lower arachidonic acid ($p<0.05$) in obese rats compared to lean rats. Since only VA and OA wre provided in the VA-triolein emulsion, other fatty acids incorporated into lymph TG may be derived either from cellular storage or via intracellular elongation and desaturation of available precursor fatty acids.

The fatty acid compositions of mesenteric lymph from meal emulsion groups were reflective of the respective dietary fatty acid composition, with the beef fat-meal group having higher OA and lower linoleic acid (LA), α-linolenic acid (ALA). Interestingly, the percentage of VA in the lymph was comparable between two meal emulsion groups despite of a higher content of VA in the synthetic VA-meal emulsion, which suggests a higher bioavailability of VA from the natural beef fat source.

Table 3-5. Fatty acid composition in mesenteric lymph TG after lipid/meal emulsion infusion in lean and obese JCR:LA-*cp* rats. Values are means±SEM expressed as percentage of total fatty acids. Means without a common letter differ, $p<0.05$.

	Lean VA-triolein	Obese VA-triolein	Obese VA-meal	Obese Beef fat-meal
14:0	0.3±0.05 ^{d}	0.9±0.06 ^{b}	0.6±0.05 ^{c}	2.1±0.04 ^{a}
16:0	16.6±1.7 ^{ab}	16.3±2.3 ^{ab}	13.9±0.6 ^{b}	19.1±0.3 ^{a}
*Cis*9-16:1	0.4±0.04 ^{d}	2.4±0.2 ^{b}	1.1±0.2 ^{c}	3.1±0.04 ^{a}
18:0	14.1±1.7 ^{b}	13.5±0.9 ^{b}	17.6±0.7 ^{a}	14.4±0.3 ^{b}
VA	0.3±0.09 ^{b}	0.4±0.06 ^{b}	3.4±0.3^{a}	3.4±0.1^{a}
*Cis*9-18:1(OA)	24.7±7.7 ^{ab}	27.9±5.2 ^{ab}	16.9±0.5 ^{b}	34.4±0.7 ^{a}
*Cis*11-18:1	1.4±0.03 ^{a}	1.7±0.2 ^{a}	1.6±0.2 ^{a}	1.2±0.3 ^{a}
*Cis*9,*cis*12-18:2 (LA)	25.8±3.0 ^{b}	21.5±1.7 ^{b}	32.0±0.8 ^{a}	10.3±0.4 ^{c}
20:0	0.3±0.03 ^{ab}	0.3±0.02 ^{b}	0.3±0.01 ^{a}	0.2±0.005 ^{c}
*cis*9,*cis*12,*cis*15-18:3 (ALA)	0.7±0.1 ^{c}	1.2±0.1 ^{b}	1.9±0.07 ^{a}	0.8±0.03 ^{c}
*Cis*9,*trans*11-CLA	ND[1]	0.2±0.06 ^{a}	0.3±0.06 ^{a}	0.5±0.02 ^{b}
20:4n6	10.0±1.0 ^{a}	5.2±0.6 ^{b}	4.0±0.2 ^{b}	2.2±0.1 ^{c}
20:5n3	0.2±0.03 ^{b}	0.5±0.06 ^{a}	0.5±0.04 ^{a}	0.4±0.03 ^{a}
22:6n3	1.3±0.1 ^{bc}	1.8±0.2 ^{a}	1.4±0.1 ^{ab}	0.9±0.05 ^{c}

[1]ND: not detected

3.3.2 Intestinal bioavailability of VA from different sources

The bioavailability of VA was not significantly different between the VA sources (Figure 3-1), although there was a trend for a higher bioavailability of VA from the beef fat-meal (1.42±0.34%) compared to the VA-triolein (1.11±0.43%) and the VA-meal (0.71±0.18%). There was a relatively large range of fluctuation for lymphatic VA abundance in each rat, indicative of individual variation in lipid absorption efficiency, which is typical for bioavailability studies. As a result, further adjustment was performed to eliminate this confounding factor by normalizing the individual lymphatic abundance of VA to the respective total lipids extracted from mesenteric lymph (Figure 3-2). The bioavailability of VA was significantly greater in obese rats infused with VA-triolein emulsion compared to those infused with the VA-meal emulsion ($p<0.001$), implying that when VA was provided

with other dietary components in the meal emulsions it may have decreased bioavailability. Interestingly, VA from naturally enriched beef fat was more effectively absorbed compared to the synthetic VA-triolein ($p<0.05$).

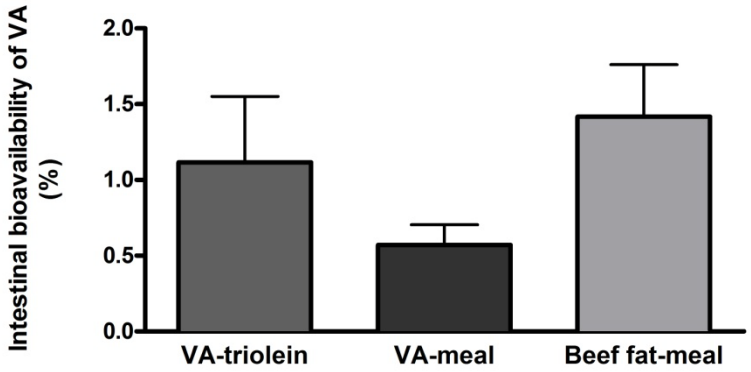


Figure 3-1. Percentage of intestinal bioavailability of VA from different sources in obese JCR:LA-*cp* rats. Values are Means±SEM.

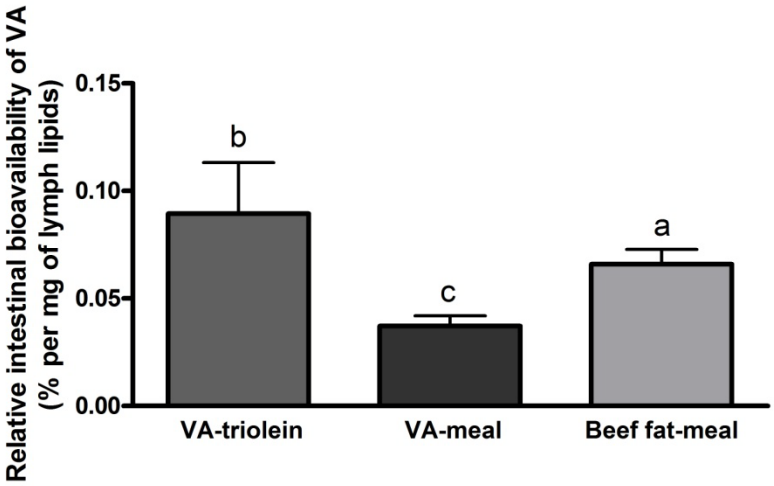


Figure 3-2. Relative percentage of intestinal bioavailability of VA from different sources in obese JCR:LA-*cp* rats. Values are Means±SEM. Means without a common letter differ, $p<0.01$.

In addition, synthetic VA-triolein showed significantly higher bioavailability in obese rats compared to lean rats with or without adjusting for the total lipids extracted from lymphatic ($p<0.05$ before and $p<0.01$ after normalization, Figure 3-3 and 3-4). These findings may be indicative of intestinal villus hypertrophy observed in the obese

phenotype of the JCR:LA-*cp* rodent model which may subsequently incur greater capacity for lipid absorption (Hassanali *et al.* 2008).

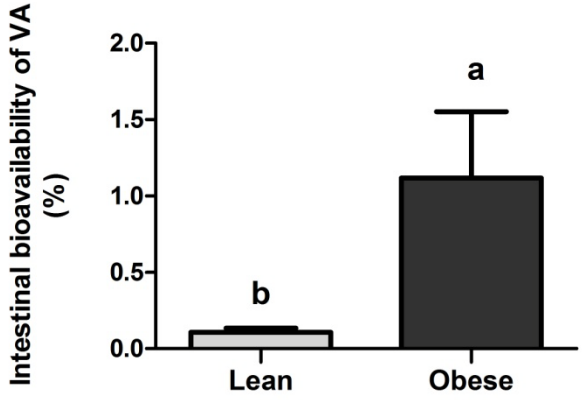


Figure 3-3. Percentage of intestinal bioavailability of VA from triolein emulsions in both lean and obese JCR:LA-*cp* rats. Values are Means±SEM. Means without a common letter differ, $p<0.05$.

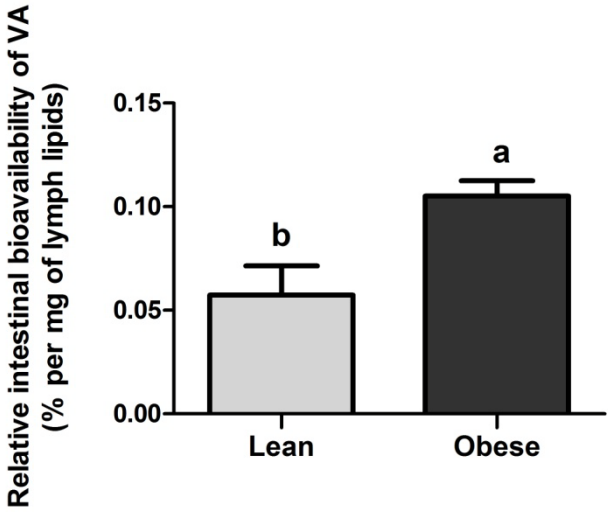


Figure 3-4. Relative percentage of intestinal bioavailability of VA from triolein emulsions in both lean and obese JCR:LA-*cp* rats. Values are Means±SEM, Means without a common letter differ, $p<0.01$.

3.3.3 Intestinal bioconversion of VA to cis9, trans11 CLA

Intestinal synthesis of *cis*9, *trans*11-CLA from VA was assessed by the ratio of *cis*9, *trans*11-CLA to VA in the lymph following the infusion of either the VA-triolein or VA-

meal emulsion which did not contain *cis*9, *trans*11 CLA. The conversion of VA to *cis*9, *trans*11-CLA was 3-fold higher in the VA-triolein compared to the VA-meal ($p<0.001$, Figure 3-5), indicating that other dietary components, such as fatty acids, protein, carbohydrate, present in the VA-meal may influence the bioconversion of VA to CLA. Lymphatic *cis*9, *trans*11 CLA was not detectable in lean rats given the VA-triolein preparation, which is likely due to either a relatively lower absorption of VA as the substrate or lower intestinal SCD activity compared to obese rats.

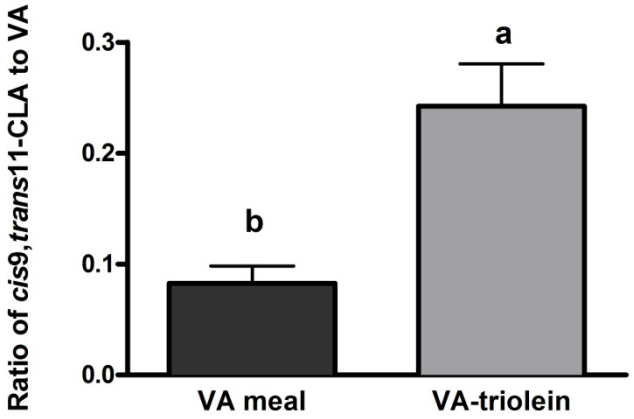


Figure 3-5. Intestinal bioconversion of VA to *cis*9, *trans*11-CLA in obese JCR:LA-*cp* rats. Values are Means±SEM, Means without a common letter differ, $p<0.001$.

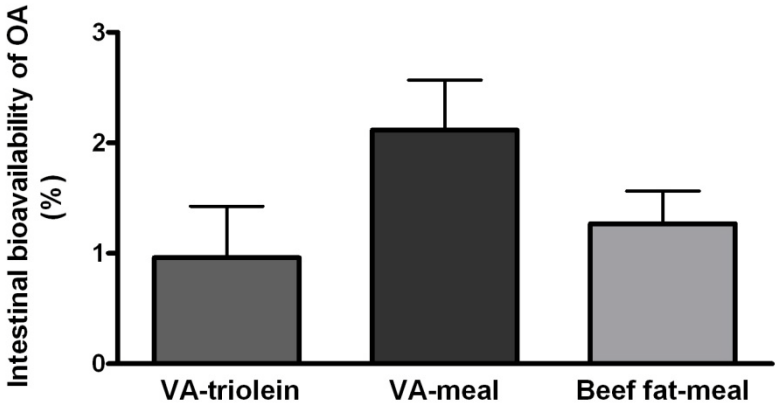
3.3.4 Intestinal bioavailability of other dietary fatty acids

OA is the major monounsaturated fatty acid present in all the lipid/meal emulsions used in this study. No statistical difference in the bioavailability of OA was detected between different emulsions given to obese rats (Figure 3-6A), but the relative intestinal bioavailability of OA tended to be lower from beef fat compared to the VA-meal (Figure 3-6B, $p<0.01$). In obese rats, there was a greater relative intestinal bioavailability of triolein-OA compared to the lean rats (Figure 3-7B, $p<0.01$).

Myristic acid (14:0) and palmitic acid (16:0) from beef fat appeared to have a lower intestinal bioavailability compared to these fatty acids in VA-meal emulsion ($p<0.01$, Table 3-6). Searic acid (18:0) showed higher bioavailability from the beef fat-meal emulsion ($P<0.01$, Table 3-6). Interestingly, other cis-MUFA (*cis*9-16:1 and *cis*11-18:1) showed a similar pattern to oleic acid to as there were less bioavailable from the beef fat-meal emulsion compared to the VA-meal emulsion($P<0.01$, Table 3-6). LA

(*cis*9,*cis*12-18:2) and ALA (*cis*9,*cis*12,*cis*15-18:3) were the major dietary poly-unsaturated fatty acids present in the VA-meal and beef fat-meal emulsions. In addition, both are considered essential fatty acids critical to maintain normal physiological functions. Interestingly, both LA and ALA showed similar high bioavailability from the beef fat-meal emulsion compared to the VA-meal emulsion, despite of a lower abundance in the beef fat-meal ($p<0.01$, Table 3-6).

A)



B)

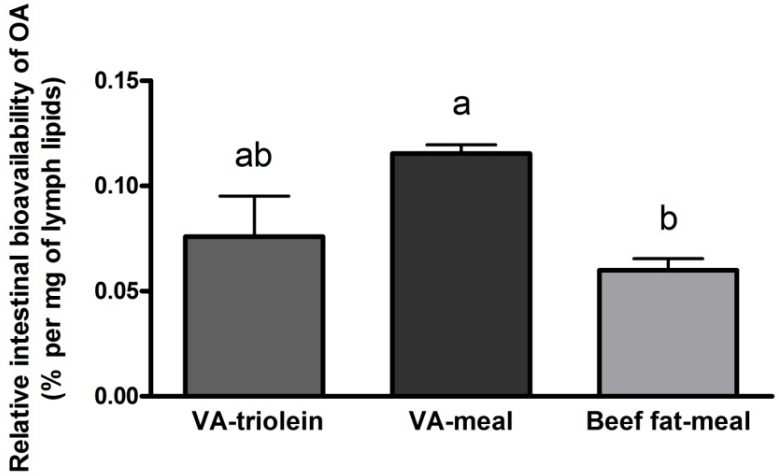
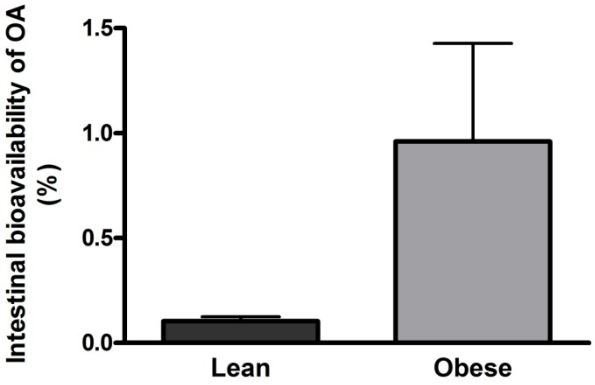


Figure 3-6. A) Intestinal and B) relative intestinal bioavailability of OA from different sources in obese JCR:LA-*cp* rats. Values are Means±SEM. Means without a common letter differ, $p<0.01$.

A)



B)

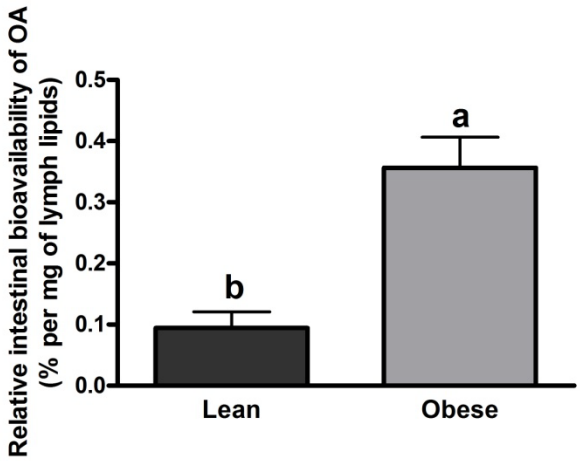


Figure 3-7. A) Intestinal and B) relative intestinal bioavailability of OA from triolein emulsions in lean and obese JCR:LA-*cp* rats. Values are Means±SEM, Means without a common letter differ, $p<0.01$.

Table 3-6. Intestinal bioavailability and relative percentage of intestinal bioavailability of fatty acids from VA-meal emulsion and beef fat-meal emulsion in obese JCR:LA-*cp* rats. Values are Means±SEM. Means without a common letter differ for each parameter, $p<0.01$.

	% bioavailability		Relative % bioavailability	
	VA-meal	Beef fat-meal	VA-meal	Beef fat-meal
14:0	3.3±0.4[a]	1.1±0.2[b]	0.2±0.02[a]	0.06±0.003[b]
16:0	1.8±0.2[a]	1.3±0.3[b]	0.1±0.008[a]	0.06±0.003[b]
*Cis*9-16:1	7.1±0.8[a]	1.3±0.3[b]	0.5±0.09[a]	0.06±0.006[b]
18:0	0.5±0.1[b]	1.2±0.2[a]	0.04±0.002[b]	0.06±0.003[a]
*Cis*11-18:1	4.4±0.6[a]	1.2±0.4[b]	0.3±0.04[a]	0.06±0.002[b]
*Cis*9,*cis*12-18:2	1.7±0.4[b]	5.4±1.2[a]	0.09±0.004[b]	0.3±0.02[a]
20:0	0.8±0.1[b]	1.5±0.3[a]	0.05±0.003[b]	0.07±0.006[a]
*cis*9,*cis*12,*cis*15-18:3	1.3±0.3[b]	2.7±0.6[a]	0.07±0.006[b]	0.1±0.01[a]

3.4 Discussion

The aim of this study was to assess the intestinal bioavailability of VA from different dietary sources and the endogenous bioconversion of VA to *cis*9, *trans*11-CLA from these sources in the intestine. The results from this study are unique in providing the first data on direct intestinal bioavailability of VA and incorporation into lymphatic CM. The data shows that VA from either synthetic or natural sources is absorbed, but the bioavailability varies with regard to different sources and the composition of the meal or emulsion matrix. Despite of a higher abundance in the VA-meal emulsions, chemically-synthesized purified VA (in the form of free fatty acids) appeared to be less abundant in mesenteric lymph when compared to naturally sourced VA (esterified to beef fat TG) (Table 3-3). In enterocytes, dietary fatty acid incorporation into chylomicron particles is dependent on the rate of re-esterification of the fatty acids into TG. As a result, CM synthesis can be increased in the fed state due to a sufficient supply of FA and sn-2 monoacylglycerol (or glycerol-3-phosphate) from dietary TG as compared to the fasted state. It has bee suggested that VA is present in the sn-2 position of beef fat TG, which directly enters the enterocytes in this monoacylglycerol ester form and is then rapidly re-esterified into TG for lipidation of CM particles (Redgrave *et al.* 2008). On the contrary,

when VA is delivered in the form of free fatty acids rather than beef fat TG, there is a shortage of exogenous sn-2-monoacylglycerol and/or a slower supply of glycerol-3-phosphate originating from glycolysis, which may lower the rate of VA re-esterification and TG synthesis. Collectively, this may lower incorporation of VA into CM-TG and lower immediate bioavailability. VA that was absorbed into the enterocytes but did not get re-esterified and enter theTG synthetic pathway may be re-esterified to TG at a later stage, dehydrogenated into *cis*9,*trans*11-CLA, or metabolized in the mitochondria for fuel. Similar to VA, the position of *cis*9,*trans*11-CLA on TG also has been shown to affect its bioavailability in rats, with CLA esterified to the sn-2 position of TG having better bioavailability and higher concentrations fould in rat carcass (Chardigny *et al.* 2003)

In addition to the rate of TG synthesis, the structure of naturally-synthesized TG and the ratio of other fatty acids in beef fat may act to enhance VA bioavailability. Beef fat contains a greater proportion of short-chain and medium-chain fatty acids compared to the VA-triolein or VA-meal emulsion, which can diffuse across the basolaterol membrane into mesenteric circulation bypassing CM incorporation and the lymphatic pathway. Given the VA-meal emusion and beef-fat emulsion had the same proportion of lipids absorbed, the presence of more short- and medium-chain fatty acids in beef fat may allow VA to have less competition as a substrate for enterocytic TG synthesis.

On the other hand, the bioavailability of synthetic VA with or without co-administration of a mixed meal was directly compared in this study and VA infused with triolein appeared to be more bioavailable than that present in the meal emulsions. It is proposed that a more complicated food matrix (such as the presence of fiber in the VA-meal emulsion) may be a contributing factor which interfers with overall absorption of dietary lipids (Table 3-4). Although fecal output of lipids was not monitored in this study, the presence of dietary fiber in the meal may have accelerated gastric emptying, interfering with micelle formation in the intestinal lumen which may have possibly inhibited the absorption of VA and other fatty acids (Borel *et al.* 2003).

In this study, direct evidence of intestinal synthesis of *cis*9, *trans*11-CLA from VA was shown in obese rats infused with VA-triolein and VA-meal emulsions, with higher conversion rate appearing in the VA-triolein group. The addition of a Δ9 double bond to VA is catalyzed by SCD, which is also is the primary enzyme involved in the biosynthesis of palmitoleic acid (*cis*9-16:1) and OA (*cis*9-18:1). The overall conversion

rate of VA to CLA has been estimated to be 11-30% in human and 5-12% in animal models (Turpeinen *et al.* 2002). Although it is highly expressed in the liver and adipose tissue, SCD is also present in the intestinal epithelium, catalyzing the synthesis of *cis*-9, *trans*-11 CLA (Reynolds *et al.* 2008). It is possible that the competition for the catalytic site of SCD at substrate level (i.e. OA, VA and palmitic acid from the VA-meal emulsion) may have affected the endogenous synthesis of *cis*9, *trans*11-CLA *per se*, but on the other hand, enhanced the bioavailability of VA in the body. Further, the gene expression of SCD might be inhibited by PUFA present in the mixed meal, which is also potent enough to override the stimulatory effect of dietary carbohydrates (Ntambi *et al.* 2004). The intestinal synthesis of *cis*9, *trans*11-CLA was negligible in lean rats, consistent with the finding of lower SCD activity in lean subjects (Hulver *et al.* 2005), but could also merely be an indication of lower lipid absorption and substrate availability for SCD in lean rats.

3.5 Conclusion

In conclusion, VA from either synthetic or natural sources is bioavailable in both lean and obese JCR:LA-*cp* rats and provides rationale for using this animal model to assess the bioactivity of synthetic VA under healthy and metabolic syndrome conditions. In addition, the bioavailability of VA originated from natural ruminant sources may be more readily available than the synthetic form.

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Chapter 4 *Trans*-11 vaccenic acid induces hypolipidemic effects in JCR:LA-*cp* rats

4.1 Introduction

*Trans*11-vaccenic acid [VA, *trans*11-18:1(n-9)] is the predominant *trans* monoene in ruminant fat, which is produced naturally during the partial bio-hydrogenation of linoleic acid (LA) [18:2(n-6)] and alpha-linolenic acid (ALA) [18:3(n-3)] (Adolf *et al.* 2000, Lock *et al.* 2004). VA acts as a precursor for the endogenous synthesis of *cis9*,*trans*11-conjugated linoleic acid (CLA) via the action of the △9-desaturase enzyme in both humans and animals (Adlof *et al.* 2000, Turpeinen *et al.* 2002). The rate of the conversion of VA to *cis9*,*trans*11-CLA has been estimated to range from 5-12% in rodents to 19%-30% in humans (Turpeinen *et al.* 2002). Recent nutritional studies have provided insight into the beneficial health effects of dietary-derived *cis9*,*trans*11-CLA in redistributing visceral fat stores (in both animals and humans), protecting against several types of cancer, as well as improving dyslipidemia (Chardigny *et al.* 2006, McLeod *et al.* 2004, Brown *et al.* 2003, Bhattacharya *et al.* 2006). Interestingly, while the dairy industry has made efforts to increase the content of *cis9*,*trans*11-CLA in foods in order to take advantage of these beneficial properties, recent reports have shown that these production processes also increase VA by up to 4-fold more than the *cis9*,*trans*11-CLA (Cruz-Hernandez *et al.* 2007).

Nutritional recommendations in North America have further highlighted that *trans* fatty acids (particularly from commercial hydrogenated vegetable oils, e.g., elaidic acid) are linked to increased risk of CVD (Lichtenstein *et al.* 2006, Woodside *et al.* 2005, Fernandes *et al.* 2002, Sommerfeld *et al.* 1983). The existing literature relates industrial *trans* fatty acids to decreased insulin sensitivity in adipose tissue (Saravanan *et al.* 2005), increased total- and low density lipoprotein-cholesterol (LDL) concentrations, systemic inflammation, and endothelial dysfunction (Lichtenstein *et al.* 1998, Judd *et al.* 1994, Mozaffarian *et al.* 2006). Unfortunately, the literature to date has not differentiated between the detrimental effect of industrial-hydrogenated *trans*-fat vegetable oils and the effect of naturally occurring *trans* fats, including *cis9*,*trans*11-CLA and VA. In addition, very little was known about the potential bioactivity of VA on blood lipids or inflammation. The availability and/or expense of purified VA have been primary factors limiting the characterization of VA's potential metabolic actions. However, it is interesting to note

that there is some evidence (albeit limited) supporting the hypothesis that VA *per se* may not be detrimental to health (Meijer *et al.* 2001, Tholstrup *et al.* 2006, Raff *et al.* 2006, Bauchart *et al.* 2007, Lock *et al.* 2005).

To address the void in the current literature, an established animal model of metabolic syndrome (MetS)— JCR:LA-*cp* rodent was chosen in order to assess the health benefits of increased dietary VA (Brindley *et al.* 2002). The JCR:LA-*cp* rat is a unique strain that has a complete absence of the leptin receptor in the plasma membrane (Brindley *et al.* 2002, Russell *et al.* 1986). It spontaneously develops symptoms associated with MetS and the pre-diabetic state in humans, including obesity, insulin resistance, hyperlipidemia and inflammatory dysregulation (Elam *et al.* 2001, Russell *et al.* 2006). Thus the objective of this study was to investigate the potential for dietary VA supplementation to improve dyslipidemia, insulin resistance and/or inflammatory status associated with MetS in the JCR:LA-*cp* rat.

4.2 Materials and Methods

4.2.1 Animal model and experimental protocol

Male rats of the JCR:LA-*cp* strain, both obese (*cp/cp*) (n=20) and lean (*+/?*) (n=20), were raised in the established breeding colony at the University of Alberta, as previously described (Russell *et al.* 1995). At 3 weeks of age, rats were transferred from the isolated breeding colony areas to a state-of-the-art individually ventilated caging environment (Tecniplast™, Exton PA, USA). At 6 weeks of age, rats had access to a standard rat non-purified diet #5001(PMI Nutrition International, Brentwood, MO) for 2 weeks. Rats of the same genotype were randomly divided into two groups and were fed either a lipid balanced control diet or an isocaloric lipid balanced control diet containing 1.5% (w/w) purified VA (Sigma, Cat#693-72-1). Animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee. Food consumption and body weight were recorded throughout the study. At 11 weeks of age, rats were fasted overnight and killed the following morning under isofluorane anesthesia. Blood was collected from the left ventricle and centrifuged at 3,000 rpm, 4 °C for 10 mintues. Plasma was collected and stored at -80 °C. The epididymal fat pads were removed and snap frozen until analyzed for lipids.

4.2.2 Diet preparation

A lipid-balanced control diet (control) was designed to resemble the Western diet as previously described (Robinson *et al.* 1998). The control diet was composed (w/w) of 1% cholesterol, 43.1% carbohydrate, 28% protein, 8% fiber and 15% lipid (w/w) with a PUFA/SFA ratio of 0.6 and n-6/n-3 PUFA acid ratio of 10 (Tables 1 and 2). The VA diet was prepared by substituting a proportion of oleic acid to provide 1.5% (w/w) of VA while maintaining total fat content as well as PUFA/SFA and n-6/n-3 PUFA ratios (Tables 4-1 and 4-2). The amount of VA in the diet was chosen based on previously published studies allowing for metabolic sufficiency while maintaining normal dietary fatty acid proportion (Tholstrup *et al.* 2006, Raff *et al.* 2006). The diet mixture was extruded into pellets, dried at room temperature and stored at 4 ^{o}C in air-tight containers. Automated GC analysis was performed on fat blend samples to confirm fatty acid composition (Table 4-2).

4.2.3 Meal tolerance test (MTT)

At 10 weeks of age, blood glucose and insulin concentrations were measured in the conscious, unrestrained rat in order to mimic a clinical oral tolerance test in humans (Russell *et al.* 1987). After fasting overnight, rats (n=4, randomly chosen from each group) were kept warm on a heated table to ensure vasodilatation of the tails, and 0.5 mL of blood was taken from the tip of the tail as T=0 min. Rats were then placed in their cages and given 5 g of the diet pellet 30 min after the beginning of the dark phase (Russell *et al.* 1987). The diet pellet used corresponded to the diet group each rat was assigned to. Timing was started when 50% of the diet pellet had been consumed, and three additional samples of blood were taken at time points T=30 min and T=60 min following the initial consumption of the food pellet meal. Area under the curve (AUC) analysis was used to calculate the total postprandial excursion of both glucose and insulin (GraphPad Prism 4.0).

Table 4-1. Compositions of the control diet and the VA diet.

Ingredients	Control Diet	VA Diet
	g/kg	*g/kg*
Casein	266.7	266.7
L-Methionine	2.4	2.4
Dextrose, Monohydrate	231.3	231.3
Corn Starch	221.8	221.8
Cellulose	49.4	49.4
Sodium Selenite	0.4	0.4
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	0.3	0.3
Mineral Mix, Bemhart-Tomarelli (170750)	50.2	50.2
Vitamin Mix, A.O.A.C. (No. 40055)	9.9	9.9
Inositol	6.2	6.2
Choline Chloride	1.3	1.3
Cholesterol	10.0	10.0
Sunflower Oil	65.0	65.7
Flaxseed Oil	6.1	6.4
Soy Tallow	58.6	62.9
Olive Oil	20.3	0.0
*Trans*11-vaccenic acid	0.0	15.0

Table 4-2. Fatty acid composition of control and VA diets were verified by GC after the oil mixture was prepared. Data are percentage of total fatty acids.

Fatty Acid	Control Diet	VA Diet
12:0	0.03	0.0
14:0	0.1	0.1
16:0	12.9	10.7
16:1	0.1	0.0
17:1	0.0	0.1
18:0	35.5	37.0
cis9-18:1	20.9	11.8
*trans*11-18:1, VA	0.4	9.1
18:2 (n-6), LA	26.0	28.2
18:3 (n-3), ALA	2.6	2.0
20:0	0.3	0.4
20:1	0.7	0.0
20:2	0.04	0.0
22:0	0.2	0.3
22:2	0.0	0.08
22:5 (n-3), DPA	0.0	0.2
24:0	0.04	0.0

4.2.4 Adipose fatty acids

Lipid was isolated from epididymal fat pad tissue in a 4:1 mixture of $CaCl_2$ to $CHCl_3$:CH_3OH (2:1) as previously described (Folch *et al.* 1957). Total triglycerides were separated on silica G plates and visualized with 8-anilino-1-naphthalenesulfonic acid under ultraviolet light and compared to the appropriate standards (Layne *et al.* 1996). Triglyceride fatty acid methyl esters (FAMES) were prepared from the scraped silica band using the base-catalyzed method with sodium methoxide. Prepared FAMES were flushed with N_2 and stored at -35 ºC until analysis by GC. Fatty acids were separated by automated gas liquid chromatography (Varian 3800, Varian Instruments, Mississauga, ON) using a 100m CP-Sil 88 fused capillary column (Varian Instruments) (Cruz-Hernandez *et al.* 2004).

4.2.5 Plasma biochemical components and serum cytokines

The concentration of select biochemical parameters in either fasting and/or postprandial plasma from lean and obese groups were assessed using commercially available homogenous, enzymatic colorimetric assays. Triglyceride (TG) (Wako Pure Chemical Industries Ltd., Cat#998-40391, 1.0 mg/dL minimum), total cholesterol (TC) (Wako Pure Chemical Industries Ltd., Cat#993-00404, 0.1 mg/dL minimum), LDL (Wako Pure Chemical Industries Ltd., Cat#993-00404, 1-400 mg/dL) and high density lipoprotein (HDL) (Diagnostic Chemical Ltd., Canada, Cat#258-20, 2-150 mg/dL) were measured using direct colorimetric chemical enzymatic reactions. Haptoglobin was assayed by an enzymatic procedure (Tridelta Development Ltd., Maynooth, Ireland, TP 801). Plasma glucose was measured as per the glucose oxidase method (Diagnostic Chemical Ltd., Cat#220-32, 0.6-600 mg/dL). Plasma insulin (Ultrasensitive Rat Insulin ELISA, Mercodia AB, Uppsala, Sweden, Cat# 80-INSRTU-E01, 0.15-5.5 ug/L) was determined using commercially available enzymatic immunoassays for rodents. Serum interleukin (IL)-6 and IL-10 was measured using BD OptEIA enzyme-linked immunoabsorbant assay kits [BD Biosciences, PharMingen, Mississauga, ON, Canada, 550319(IL-6) and 555134 (IL-10), 4 pg/mL minimum]. Samples were analyzed using assay kits from a single lot and performed in one batch. Samples were measured in triplicates, except cytokines which were measured in duplicate, with an intra-assay co-efficient of variance at < 5%.

4.2.6 Statistical analysis

All results are expressed as mean±SEM. Data was tested for normal distribution and differences between lean and obese groups as well as between control and VA treatment groups were analyzed using two-way ANOVA followed by Bonferroni post-hoc tests (GraphPad Prism 4.0).

4.3 Results

4.3.1 Food intake and body weight gain

At the end of the 3-week study, food consumption and body weight gain were significantly lower in lean rats (17.0±0.7 g/day and 54±2.3 g, respectively) relative to obese JCR:LA-*cp* rats (36.7±1.3 g/day and 119.9±3.9 g, respectively) ($p<0.0001$). Increased dietary VA did not significantly affect food intake or weight gain of rats irrespective of genotype.

4.3.2 Fatty acid in epididymal adipose tissue

The amount of *cis*9, *trans*11-CLA in adipose tissue TG was elevated by 6.5 fold in both lean and obese rats fed VA compared to control diet groups (from 0.04±0.01 to 0.3±0.04 and from 0.04±0.02 to 0.3±0.01, respectively) (Table 4-3, values are expressed as percentage of total fatty acids). Moreover, lean rats showed a higher concentration of VA in adipose tissue (8 fold increase from 0.4±0.01 to 3.6±0.2) than obese rats (1.5 fold increase from 0.4±0.02 to 1.0±0.3) when fed the VA diet. Thus, the ratio of VA: *cis9*,*trans*11-CLA in TG from adipose tissue was identical (10.0) for both lean and obese rats from each of the control diet groups. However, the VA diet resulted in a lower ratio of VA: *cis9*,*trans*11-CLA in triglycerides from adipose tissue in obese rats relative to lean rats (3.3 and 12.0, respectively). Adipose tissue from lean control rats had a lower proportion of oleic acid ($p<0.001$) and a higher proportion of both LA ($p<0.001$) and ALA ($p<0.05$), compared to adipose tissue from obese control rats. Further, the proportion of oleic acid in adipose tissue from lean rats fed the VA-diet was lower ($p<0.01$), while LA was higher ($p<0.001$) when compared to the lean rats fed the control diet.

Table 4-3. Fatty acid composition (% w/w) of adipose tissue TG in JCR:LA-*cp* rats treated with control or VA diet. Values are means±SEM, n=10. Means in the same row without a common letter differ, $p<0.05$.

	Lean control	Lean VA	Obese control	Obese VA
*Trans*11-18:1	0.4±0.01^{c}	3.6±0.2^{a}	0.4±0.02^{c}	1.0±0.3^{b}
*Cis*9-18:1	31.6±0.3^{b}	26.3±0.4^{c}	35.3±0.2^{a}	33.7±0.4^{b}
18:2(n-6)	29.4±0.2^{b}	32.6±0.4^{a}	16.5±0.4^{c}	16.2±0.5^{c}
18:3(n-3)	2.0±0.1^{a}	2.3±0.1^{a}	1.4±0.3^{b}	1.4±0.04^{b}
*Cis*9,*trans*11-18:2	0.04±0.01^{b}	0.3±0.04^{a}	0.04±0.02^{b}	0.3±0.01^{a}

4.3.3 Fasting plasma lipid and serum inflammatory markers

Fasting plasma triglyceride concentration in obese rats (*cp/cp*) supplemented with 1.5% VA was lower by approximately 40% compared to obese rats fed the control diet (Table 4-4, $p<0.05$). No significant change was observed in plasma lipid parameters in lean rats

supplemented with VA relative to lean rats fed the control diet. Serum IL-6 concentration did not differ between the VA-treated and control dietary groups, while serum IL-10 concentration was lower with VA treatment regardless of genotype ($p<0.05$). Haptoglobin concentration was significantly higher in obese rats compared to lean rats ($p<0.05$). Dietary VA supplementation did not improve the haptoglobin concentration relative to the control diet.

4.3.4 Glucose and insulin metabolism

Dietary VA did not significantly affect fasting plasma glucose or insulin concentrations (Table 4-4). The metabolism of glucose or insulin was not altered between dietary groups, as measured by AUC throughout the MTT. Euglycemia was maintained with no change in insulin postprandial excursion.

Table 4-4. Fasting plasma lipid concentrations, serum inflammatory markers as well as glucose and insulin area under the curve (AUC, n=4) after MTT in JCR:LA-*cp* rats treated with control or VA diet. Data are means ±SEM, n=10. Means in the same row without a common letter differ, $p<0.05$.

	Lean control	Lean VA	Obese control	Obese VA
Triglyceride, *mmol/L*	0.5±0.04^{c}	0.6±0.07^{c}	4.1±0.6^{a}	2.7±0.3^{b}
TC, *mmol/L*	2.4±0.1^{b}	2.4±0.1^{b}	5.8±0.3^{a}	5.1±0.2^{a}
LDL, *mmol/L*	0.4±0.03^{b}	0.6±0.08^{b}	1.1±0.1^{a}	0.9±0.1^{a}
IL-6, *nmol/L*	2.8±0.6^{a}	2.0±0.6^{a}	2.3±0.4^{a}	2.6±0.4^{a}
IL-10, *nmol/L*	0.4±0.09^{ab}	0.4±0.07^{ab}	0.3±0.08^{a}	0.1±0.05^{b}
Haptoglobin, *pmol/L*	8.4±1.1^{b}	8.1±0.5^{b}	21.0±4.7^{a}	17.0±3.1^{a}
Glucose, *mmol/L*	8.1±1.2^{a}	8.3±0.9^{a}	7.3±0.9^{a}	7.9±0.7^{a}
Insulin, *pmol/L*	117.9±10.1^{b}	163.9±42.9^{b}	2941.3±724.3^{a}	2652.4±495.8^{a}
Glucose, *AUC*	114.6±6.4^{a}	123.9±11.3^{a}	129.2±5.2^{a}	134.8±11.1^{a}
Insulin, *AUC*	11320±1973^{b}	11107±766.2^{b}	335381±58081^{a}	344827±72513^{a}

4.4 Discussion

Increased intake of *trans* fatty acids from hydrogenated vegetable oils (e.g., elaidic acid) has consistently been shown to be related to increased coronary heart disease risk, incidence of myocardial infarction, elevated LDL concentration and small-dense HDL particles (Judd *et al.* 1994, Lichtenstein *et al.* 1998). However, few studies have drawn a consistent conclusion on the potential health effects of naturally-derived *trans* fats, in particular VA, and its relationship with CVD risk. Bauchart and colleagues have reported that VA/CLA-enriched butter had a neutral effect on the risk of atherogenesis related to plasma lipoprotein profile in hamsters, whereas *trans*10-18:1 butter enhanced pro-atherogenicity (Bauchart *et al.* 2007). However, it is feasible that the VA diet described by Bauchart *et al.* may have been confounded by differences in either *cis9*,*trans*11-CLA and/or the amounts of hydrolyzed fat used. It is also curious that Meijer *et al.* ascribed no beneficial effect to blood lipids in hamsters, when comparing diets that contained 10% of energy originating from either VA or elaidic acid (Meijer *et al.* 2001). In contrast, several other animal or human studies have indicated that feeding VA-enriched dairy products may have neutral health effects or may even improve plasma lipid profile related to reducing atherosclerotic risk. Lock *et al.* showed that an increased intake of VA (15% of total fat) was associated with a reduced risk of atherosclerosis by improving plasma lipoprotein profile in cholesterol-fed hamsters (Lock *et al.* 2005). Further, Tholstrup *et al.* showed that feeding butter with a higher content of VA significantly lowered TC in healthy men (Tholstrup *et al.* 2006). In very recent studies, two independent research groups both suggest that *trans* fatty acids from natural sources had neutral to beneficial effects on risk factors of CVD with modest consumption in humans (Motard-Belanger *et al.* 2008, Chardigny *et al.* 2008). While the evidence from these important clinical studies provided proof-of-concept, they may also be limited due to the potential discrepancy of other dietary bioactive fatty acids such as saturated fatty acids, *cis9*,*trans*11-CLA and oleic acid. It is also noteworthy that many of the clinical VA-related studies have included subjects with relatively normal blood lipid profile, which may have limited the potential of VA to improve these parameters.

In the current study, it is shown that distinct from industrially-produced *trans* fatty acid, a diet containing 1.5% (w/w) purified VA (10% of total fat) did not result in any adverse effects on body weight, food consumption and/or inflammatory status under either normolipidemic or hyperlipidemic conditions in the JCR:LA-*cp* rat. Fasting levels of

insulin or glucose, or corresponding postprandial metabolism in response to a MTT was not affected by VA, consistent with recent findings by Tardy et al (Tardy *et al.* 2008). Interestingly, it has been shown that VA significantly reduced blood triglycerides in the JCR:LA-*cp* rat, supporting a beneficial effect on CVD risk. While total and LDL concentrations tended to be lower in rats fed the VA-diet, this did not reach significance in this 3-week feeding study. It is speculated that longer term feeding of VA may have a greater potential to influence cholesterol metabolism in this model. It is also noteworthy that the VA-diet in this study contained 50% less oleic acid compared to the control diet (due to the substitution for VA). Consequently, the improvement in lipid profile could not be attributed to the presence of oleic acid alone, which has been previously shown to improve postprandial glucose response and lipid profiles (Paniagua *et al.* 2007). Similarly, the discrepancy between the amount of oleic acid in epididymal fat pads from the control and VA groups could be the result of differential dietary oleic acid as opposed to an effect of VA *per se.*

The bioconversion of VA to *cis9*,*trans*11-CLA has been calculated previously in different animal species. Early reports have estimated that approximately 5-12% of VA is converted to *cis9*,*trans*11-CLA in rodents (Turpeinen *et al.* 2002). In humans, conversion has been estimated to be within the range of 19% to 30% (Turpeinen *et al.* 2002). It has been previously shown in chapter 3 that a small portion of VA was converted into *cis9*,*trans*11-CLA in the intestine of obese rats, but not lean rats, in the postprandial phase. In this study, feeding VA for 3 weeks led to an 8.0 and 1.5 fold increase in VA incorporation into adipose tissue in lean and obese rats respectively. In addition, a 6.5 fold increase was observed in *cis9*,*trans*11-CLA in triglycerides from adipose tissue compared to the control group. Interestingly, lean rats had a greater ratio of VA to *cis9*,*trans*11-CLA compared to the obese rats after 3-week feeding, consistent with earlier observation of a lower conversion to *cis9*,*trans*11-CLA in the lean phenotype. One of the limitations of this study was that both VA and *cis9*,*trans*11-CLA concentrations were not monitored continuously throughout the study, which would have enabled us to evaluate the incorporation and conversion rate of VA (Turpeinen *et al.* 2002). While it is plausible that indirect endogenous production of *cis9*,*trans*11-CLA from dietary VA may have mediated the hypolipidemic effects observed in this study, no corresponding change was evident in body weight, food intake or insulin sensitivity, which are distinctive biological effects of dietary CLA supplementation in this animal

model and others (Proctor *et al.* 2007, Salas-Salvado *et al.* 2006). Therefore, it is proposed that the substantial hypolipidemic benefits of VA treatment were not caused by indirect bio-conversion to *cis9*,*trans*11-CLA but rather by the direct dietary supplementation of VA.

Since obesity and diabetes are associated with impaired inflammatory regulation, the concentrations of serum cytokines (e.g., IL-6, IL-10) and acute phase proteins such as haptoglobin are regarded as important pro-inflammatory biomarkers (Guzik *et al.* 2006, Zulet *et al.* 2007). Indeed, others have shown that *cis9*,*trans*11-CLA has anti-inflammatory effects (Zulet *et al.* 2005, Butz *et al.* 2007, Moloney *et al.* 2007). In this study, feeding VA resulted in reduced serum IL-10 concentration, indicating a potential direct anti-inflammatory effect of VA on inflammatory regulation, for which the mechanisms remain unclear (Giugliano *et al.* 2006).

On considering the potential mechanistic properties of VA, it is important to note that VA acts as a precursor for the endogenous synthesis of *cis9*,*trans*11-CLA in animals and humans. It is plausible that both these fatty acids may regulate similar hepatic or intestinal lipogenic pathways to mediate hypolipidemic effects. There is emerging evidence that *cis9*,*trans*11-CLA is one of the few known naturally-occurring agonists of both peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ (Brown *et al.* 2003, Wargent *et al.* 2005, Lampen *et al.* 2005, Selberg *et al.* 2005). Therefore, it would seem reasonable to speculate that VA may play a role in regulating PPAR-α. Additionally, there is evidence to suggest that VA could inhibit the activity of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), or both, and these are the subject of ongoing studies (Clarke *et al.* 1990, Jayan *et al.* 2000).

4.5 Conclusion

Short term feeding of 1.5% (w/w) VA did not result in any detrimental health effects in either lean or obese rats, and thus, distinguishes this natural *trans* fatty acid from commercially-hydrogenated sources of *trans* fats. Further, it has been shown for the first time that dietary VA supplementation leads to a significant decrease in circulating plasma triglyceride concentration in JCR:LA-*cp* rats, an established rodent model of MetS. Consequently, it is proposed that VA may have substantial hypotriglyceridemic benefits under conditions of dyslipidemia. These observations contribute to the

hypothesis that industrial and ruminant *trans* fatty acids have differential bioactivity that warrants further investigation.

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Chapter 5 Trans-11 Vaccenic Acid Reduces Hepatic Lipogenesis and Improves Post-prandial Lipaemia in JCR:LA-cp rats

5.1 Introduction

The potential deleterious cardiovascular properties of *trans* fats (from partially hydrogenated vegetable oils [PHVO]) have received considerable attention over the past few years (Lichtenstein *et al.* 1999, Mozaffarian *et al.* 2006). However there has been a void in the understanding of the different metabolic properties of naturally-derived *trans* fats such vaccenic acid [*trans*11-18:1, VA] compared to those *trans* fats derived from PHVO and produced by industrial means. VA is a monounsaturated fatty acid (MUFA) and the predominant isomer accounting for approximately 70% of the total *trans* fatty acids (TFA) found in ruminant-derived lipids (Lock *et al.* 2004, Cruz-Hernandez *et al.* 2007). VA is also the primary precursor for endogenous synthesis of *cis*9, *trans*11-conjugated linoleic acid (CLA) in animals and humans (Palmquist *et al.* 2005, Lock *et al.* 2004, Turpeinen *et al.* 2002). Clinical evidence indicates that relative to industrial-derived TFA, natural sources of TFA (such as VA-enriched butter) in the diet can elicit either neutral or beneficial health effects on blood lipid variables among healthy individuals (Motard-Belanger *et al.* 2008, Chardigny *et al.* 2008). More specifically, it has been reported that feeding VA-enriched dairy foods lowers atherogenic lipoproteins and inhibits adipose tissue lipogenesis in several animal models (Lock *et al.* 2005, Faulconnier *et al.* 2006, Bauchart *et al.* 2007, Tyburczy *et al.* 2009). It has recently been reported that novel plasma triglyceride (TG)-lowering properties of pure VA supplementation in hyperlipidemic JCR:LA-*cp* rats (Chapter 4). However, the hypolipidemic benefit of VA supplementation has not been demonstrated under chronic feeding conditions, nor have the corresponding putative mechanisms been elucidated. Consequently, in this study it was hypothesized that long term supplementation of VA in JCR:LA-*cp* rats would result in a persistent improvement in dyslipidemia, associated with effects on lipogenic pathways involved in whole body lipid homeostasis.

JCR:LA-*cp* rats, when homozygous for the *cp* trait (*cp/cp*), develop leptin receptor deficiency (Koletsky *et al.* 1973). Rats from this strain exhibit symptoms of metabolic syndrome (MetS) and pre-diabetes, which include obesity, insulin resistance, hepatic steatosis, hypertriglyceridemia, as well as exacerbated production of hepatic VLDL and intestinal chylomicrons (CM) (Russell *et al.* 1998, Vine *et al.* 2008). Hepatic lipogenic

enzymes including acetyl-CoA carboxylase-1 (ACC-1) and fatty acid synthase (FAS) are two key components involved in the production of long-chain fatty acids, which are subsequently used for TG synthesis and VLDL assembly. The transcriptional factor sterol regulatory element binding protein-1 (SREBP-1) regulates associated lipogenic pathways. The abundance of SREBP-1, ACC-1 and FAS mRNA in JCR:LA-*cp* rats is elevated (Elam *et al.* 2001) and thus represent a potential regulatory mechanism by which VA supplementation may improve hepatic fatty acid and TG *de novo* synthesis. Complimentary studies have also demonstrated post-prandial dyslipidemia and intestinal CM over-secretion in JCR:LA-*cp* rats (Vine *et al.* 2008). Therefore it was proposed that VA may improve circulating TG concentration in these rats by lowering hepatic and intestinal *de novo* lipogenesis, and/or intestinal CM secretion.

5.2 Materials and methods

5.2.1 Animal model and experimental protocol

Male rats of the corpulent JCR:LA-*cp* strain, both obese (*cp*/*cp*) (n=16) and lean (*+/?*) (n=8), were raised in the established breeding colony at the University of Alberta, as previously described (Russell *et al.* 1995). At 8 weeks of age, obese rats were randomized into two groups and were fed for 16 weeks either a lipid balanced control diet or an isocaloric lipid balanced diet containing 1.0% (w/w) purified VA. Lean littermates were fed the control diet for the same period of time as normolipidemic controls. Rat care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Care and Use Committee. Food consumption and body weight were recorded throughout the study. At 23 weeks of age, an oral fat challenge test, as previously described (Vine *et al.* 2007), was performed in four randomly chosen rats from each obese group. At 24 weeks of age, rats were fasted overnight and killed the following morning under isofluorane anesthesia. Plasma and sera were collected from the left ventricle. Liver as well as peri-renal, inguinal and epididymal fat pads were weighed and snap frozen in liquid N_2 at 80^{o}C for further analysis. Jejunum was rinsed with PBS for mucosal enterocyte collection. Adipose fatty acid composition was measured on the epididymal fat pad as previously described (Chapter 4).

5.2.2 Diet preparation

A lipid-balanced control diet was designed to resemble the Western diet as previously described for similar studies exploring the effects of fatty acid bioactivity (Robinson 1998). The control diet was composed (w/w) of 1% cholesterol, 43.1% carbohydrate, 28% protein, 8% fiber and 15% lipid (w/w) with a PUFA/SFA ratio of 0.6 and (n-6)/(n-3) PUFA ratio of 10 (Table 5-1). The VA diet was prepared by adjusting the lipid composition of the control diet to provide 1.0% (w/w) of VA while maintaining the PUFA/SFA and (n-6)/(n-3) PUFA ratio. Purified VA was produced by chemical alkali isomerization from linoleic acid-rich vegetable oil (Mouloungui *et al.* 2009). The amount of VA in the diet was chosen based on previous findings (Chapter 4) whilst still maintaining normal dietary fatty acid ratios. The diet mixture was extruded into pellets, dried at room temperature and stored at 4°C in air-tight containers. GC analysis was performed on lipid blend samples to confirm fatty acid composition (Table 5-2).

Table 5-1. Composition of the control and the VA diet.

Ingredient	Control diet	VA diet
	g/kg	*g/kg*
Casein	266.7	266.7
L-Methionine	2.4	2.4
Dextrose, Monohydrate	231.3	231.3
Corn Starch	221.8	221.8
Cellulose	49.4	49.4
Sodium Selenite	0.4	0.4
Manganese Sulfate ($MnSO_4 \cdot H_2O$)	0.3	0.3
Mineral Mix, Bemhart-Tomarelli (170750) [1]	50.2	50.2
Vitamin Mix, A.O.A.C. (No. 40055) [1]	9.9	9.9
Inositol	6.2	6.2
Choline Chloride	1.3	1.3
Cholesterol	10.0	10.0
Sunflower Oil	55.0	56.0
Flaxseed Oil	4.5	4.5
Soy Tallow	76.2	76.5
Olive Oil	14.3	0.0
VA oil	0.0	13.0

[1]Compositions have been previously reported in Clandinin MT, Yamashiro S. J Nutr;110:1197-1203.

Table 5-2. Fatty acid composition of control and VA diets[1]

Fatty Acid	Control diet	VA diet
	g/100g total fatty acids	*g/100g total fatty acids*
16:0	9.1	8.9
16:1	0.1	0.1
18:0	47.0	47.1
*Cis*9-18:1	17.3	9.0
*Trans*11-18:1, VA	0.0	6.0
18:2 (n-6), LA	23.4	24.7
*Cis*9,*trans*11-18:2, CLA	0.0	0.0
18:3 (n-3), ALA	1.6	1.9
20:0	0.4	0.3
20:2 (n-6)	0.1	0.1
SFA	57.6	57.3
MUFA	17.4	16.1
PUFA	25.0	26.7
P/S ratio	0.43	0.46
(n-6)/(n-3) PUFA ratio	14.9	13.4

[1]Verified by GC after the oil mixture were prepared.

5.2.3 Plasma biochemical components and serum cytokines

The concentration of select biochemical variables in plasma from lean and obese rats obtained in the food-deprived and post-prandial state were assessed using commercially available direct colorimetric assays including plasma TG, total cholesterol (TC), low density lipoprotein-cholesterol (LDL) as well as serum inflammatory markers [haptoglobin and interleukin (IL)-10] as previously described (Chapter 4). Plasma free fatty acids (FFA) were measured using an enzymatic colorimetric assay (HR Series FFA-HR, Cat# 999-34691, Wako Diagnostics, Richmond, VA, USA). Samples were analyzed using assay kits from a single lot and performed in one batch. Samples were measured in triplicate, except cytokines which were measured in duplicate, with an intra-assay CV at < 5%. Apo B48 protein abundance (representing chylomicron particle number) was quantified by western blot coupled with enhanced chemiluminescence on plasma obtained from rats in the food-deprived and post-prandial state (Vine *et al.* 2007).

5.2.4 Liver homogenization, hepatic TG and relative protein abundance of lipogenic factors

Rat liver (0.5 g) was homogenized in 200 uL lysis buffer [PBS (pH 7.4) with 1.5% v/v Triton X-100 and 1% protease inhibitor cocktail (Sigma, Cat# P8340)]. The concentration of TG was measured by a direct colorimetric assay as previously described in Chapter 4, using an aliquot of the whole homogenate and adjusted by the protein concentration of the homogenate. The remainder of the homogenate was centrifuged at 700 *g* for 15 min to separate the homogenate from debris. The supernatant was collected and stored at -80 °C. Hepatic ACC-1, FAS and SREBP-1 proteins were determined by western blot analysis as previously described (Qin *et al.* 2009). In brief, liver proteins were separated using SDS-PAGE based on the molecular weight of each protein and transferred to polyvinylidene fluoride membranes at constant voltage (150 V) for 90 minutes. Membranes were blocked overnight at 4 °C with 2% ECL Advanced™ blocking agent (Cat# RPN418, GE Health, Amersham, UK) diluted in Tris-buffered saline with 1% Tween X-100). Membranes are then incubated for 2 hours at room temperature with primary antibodies raised against ACC-1 (1:10000 dilution, Cat# 3662, Cell Signaling, Danvers, MA, USA), FAS (1:10000 dilution, Cat# sc-55580, Santa Cruz Biotechnology, Santa Cruz, CA, USA), SREBP-1 (1:10000 dilution, Cat# sc-366, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and beta-actin (internal control) (1:20000 dilution,

Cat# A5441, Sigma-Aldrich, St. Louis, MO, USA). Membranes are washed thoroughly with TBST and incubated for 1 hour at room temperature with secondary antibodies required for specific primary antibodies. In the end, membranes were washed thoroughly with TBST and adherent antibodies were visualized by chemiluminescence using ECL Advance™ Western Blotting Detection Kit (Cat# RPN2135, GE Health, Amersham, UK). Protein bands were quantified using Image J (version 1.41) software developed by NIH. The final value of mature SREBP-1, FAS and ACC-1 relative protein abundance was normalized based on the respective beta-actin protein mass.

5.2.5 Mucosal enterocyte collection, protein extraction, and relative protein abundance of lipogenic factors

The jejunum was excised and enterocyte fractions (1–10) were collected using the Weiser method (Weiser *et al.* 1973). In brief, rat jejunum was rinse with phosphate-buffered saline (PBS) and incubated in 1mM dithiothreitol (DTT) diluted in PBS for timed serial removal of cellular fractions. Enterocytes were then sequentially removed with fraction 1 representing villus tip enterocytes and fraction 10 representing those at the villus crypt. Samples (50 mL) from each fraction were pooled and SREBP-1, ACC-1, and FAS relative protein abundances were assessed on the pooled enterocyte protein extract as described above for liver homogenates.

5.2.6 Mesenteric lymph duct cannulation procedure

To determine the direct effect of VA on the secretion of CM in mesenteric lymph, the superior mesenteric lymph duct of 9-week old obese JCR:LA-*cp* rats (n=8) were cannulated following consumption of the control diet for 3 weeks (Vine *et al.* 1997). In brief, following abdominal incision, A canula was inserted into the superior mesenteric lymph duct to sample nascent lymph. A gastrostomy was performed and a canula was inserted into the duodenum for emulsion infusion. Following recovery from anesthesia, rats were randomized to receive either a pure triolein emulsion or a triolein emulsion containing VA.

5.2.7 Triolein emulsion preparation and nascent lymph collection

VA oil (7.5 µg) was dissolved in 100 µL chloroform and vortexed with 500 µL glyceryl trioleate (Sigma, Cat# T7140) and the chloroform evaporated under N_2 as described previously in Chapter 3. The emulsion was flushed with N_2, sealed with parafilm and

protected from exposure to light. The prepared emulsion was then infused to rats via the gastric cannula, which was flushed just prior with saline (1mL). The triolein emulsion was prepared identical to that described for the VA emulsion with the exception of VA. Mesenteric lymph was collected into EDTA-coated vacutainers for 5 hours following the emulsion infusion. TG, total cholesterol and apoB48 concentrations were all measured as described above for plasma.

5.2.8 Statistical analysis

All results are expressed as mean±SEM. Differences among lean control, obese control and obese VA groups for most variables and were analyzed using one-way ANOVA followed by Tukey's post hoc tests. Post-prandial lipemia was assessed by area under the curve (AUC) and incremental AUC analysis. Differences in post-prandial and lymphatic lipid profile between groups were analyzed using an unpaired t-test. The level of significance was set at $P<0.05$ (Graph Pad Prism 5.0).

5.3 Results

5.3.1 Food intake, body weight and tssue/organ weights

At the end of the dietary intervention (16 weeks), food consumption and body weight did not differ between obese rats fed the control or VA diet (Table 5-3). Epididymal, inguinal and peri-renal adipose tissue weight, and jejunum weight were not different between the obese groups. However, VA-fed obese rats had a lower liver weight compared to obese control rats ($p<0.05$).

Table 5-3. Food intake, body weight and tissue weight in lean and obese JCR:LA-*cp* rats fed a control or VA supplemented diet for 16 weeks. Values are means±SEM, n=8. Means in the same row without a common letter differ, p<0.05.

	Lean control	Obese control	Obese VA
		g	
Food intake	19.7±0.3[b]	32.4±0.5[a]	33.2±1.0 [a]
Body weight	383.9±8.2[b]	646.0±9.3[a]	659.4±10.9[a]
Liver weight	9.1±0.4[c]	23.1±0.7[a]	18.9±1.0[b]
Jejunum weight	3.4±0.1[a]	4.7±1.0[a]	5.6±0.84[a]
Epididymal fat pad weight	2.0±0.2[b]	11.5±0.3[a]	11.9±0.3[a]
Pararenal fat pad weight	1.3±0.1[b]	7.4±0.4[a]	7.1±0.4[a]
Inguinal fat pad weight	1.4±0.2[b]	17.9±1.1[a]	18.6±1.0[a]

5.3.2 Fatty acid composition of adipose tissue

The composition of total SFA, MUFA and PUFA in epididymal adipose tissue did not differ between the obese groups (Table 5-4). However, there was an 80-fold increase in the amount of VA (p<0.001) in the VA-fed obese rats compared to obese control rats. The amount of *cis*9,*trans*11-CLA was also elevated 20-fold in VA-fed obese rats compared to obese rats fed the control diet (p<0.001), likely due to the *in-vivo* conversion from VA. In addition, obese rats fed the VA diet had lower proportions of myristic acid (14:0), α-linolenic acid [18:3(n-3)], and arachidonic acid [20:4(n-6)], and a higher proportion of stearic acid (18:0) in adipose tissue compared to obese control rats.

Table 5-4. Fatty acid composition of adipose tissue TG in lean and obese JCR:LA-*cp* rats fed a control or VA supplemented diet for 16 weeks. Values are means±SEM, n=8. Means in the same row without a common letter differ, p<0.05.

	Lean control	Obese control	Obese VA
		g/100g fatty acids	
14:0	0.7±0.04[c]	1.4±0.03[a]	1.3±0.01[b]
16:0	13.0±0.4[b]	23.4±0.1[a]	23.2±0.2[a]
16:1(n-9)	0.7±0.1[b]	3.8±0.1[a]	3.8±0.07[a]
18:0	14.4±0.6[a]	6.4±0.1[c]	7.3±0.1[b]
VA	0.06±0.01[b]	0.02±0.01[c]	1.6±0.04[a]
*Cis*9-18:1	29.1±0.2[b]	37.9±0.6[a]	34.4±2.3[a]
Cis11-18:1	1.7±0.05[b]	2.2±0.03[a]	2.2±0.02[a]
18:2(n-6)	36.0±0.2[b]	20.5±0.2[a]	20.0±0.3[a]
18:3(n-3)	1.3±0.03[a]	0.9±0.01[b]	0.8±0.02[c]
*Cis*9,*trans*11-18:2	0.03±0.01[c]	0.04±0.01[b]	0.8±0.02[a]
20:4(n-6)	0.5±0.02[a]	0.4±0.02[b]	0.3±0.01[c]
22:5(n-3)	0.2±0.04[a]	0.1±0.01[a]	0.1±0.01[a]
22:6(n-3)	0.3±0.07[a]	0.1±0.01[a]	0.1±0.01[a]
SFA	28.4±0.3[b]	31.4±0.3[a]	32.0±0.3[a]
MUFA	31.6±0.3[b]	44.4±0.5[a]	42.4±2.4[a]
PUFA	38.8±0.1[a]	22.8±0.2[b]	22.2±0.3[b]

5.3.3 Plasma lipids and serum inflammatory markers

Obese rats fed the control diet had higher plasma TG, TC, LDL and FFA concentrations compared to lean rats fed the control diet (Table 5-5; $p<0.05$). Dietary VA supplementation reduced plasma TG ($p<0.001$), TC ($p<0.001$), LDL ($p<0.01$) and FFA ($p<0.05$) concentrations compared to obese controls (Table 5-5). Surprisingly, the LDL concentration did not differ between lean rats fed the control diet and obese rats fed the VA-diet, suggesting a normalizing effect of dietary VA. Chronic dietary supplementation of VA also normalized the serum concentration of haptoglobin in obese rats to levels comparable with lean rats fed the control diet (Table 5-5; $p<0.001$). Supplementation of VA in obese rats did not affect the concentration of IL-10 (Table 5-5).

Table 5-5. Plasma lipid concentrations, serum inflammatory markers, and hepatic TG in lean and obese JCR:LA-*cp* rats fed a control or VA supplemented diet for 16 weeks. Values are means±SEM, n=8. Means in the same row without a common letter differ, $p<0.05$.

	Lean control	Obese control	Obese VA
TG, *mmol/L*	0.5±0.03[c]	3.3±0.4[a]	1.6±0.2[b]
TC, *mmol/L*	2.3±0.06[a]	5.8±0.3[b]	4.0±0.2[c]
LDL, *mmol/L*	1.0±0.08[b]	1.8±0.1[a]	1.3±0.2[b]
FFA, *mmol*/L	0.3±0.02[c]	0.5±0.02[a]	0.4±0.03[b]
IL-10, *nmol/L*	1.2±0.2[a]	1.0±0.2[b]	0.7±0.1[b]
Haptoglobin, *pmol/L*	4.7±0.6[b]	10.8±0.9[a]	5.2±0.6[b]
Liver TG, *mmol/g protein*	5.1±0.3[c]	20.5±1.9[a]	10.5±0.7[b]

5.3.4 Hepatic TG and lipogenic factors

Hepatic TG (as shown in Table 5-5) was higher in obese rats fed the control diet compared to lean rats fed the control diet ($p<0.001$). Further, the relative protein abundance of both FAS (Figure 5-1 panels, A and B) and ACC-1 (Figure 5-1, panels A and C) was higher in obese rats fed the control diet ($p<0.001$) relative to lean rats fed the control diet. In contrast, VA supplementation in obese rats resulted in a lower (49%) hepatic TG concentration compared to obese rats fed the control diet ($p<0.001$). In addition, VA supplementation reduced the relative abundance of FAS and ACC-1 enzymes by approximately 40% ($p<0.05$) compared to obese rats fed the control diet. The relative protein mass of mature SREBP-1 in liver did not differ among groups.

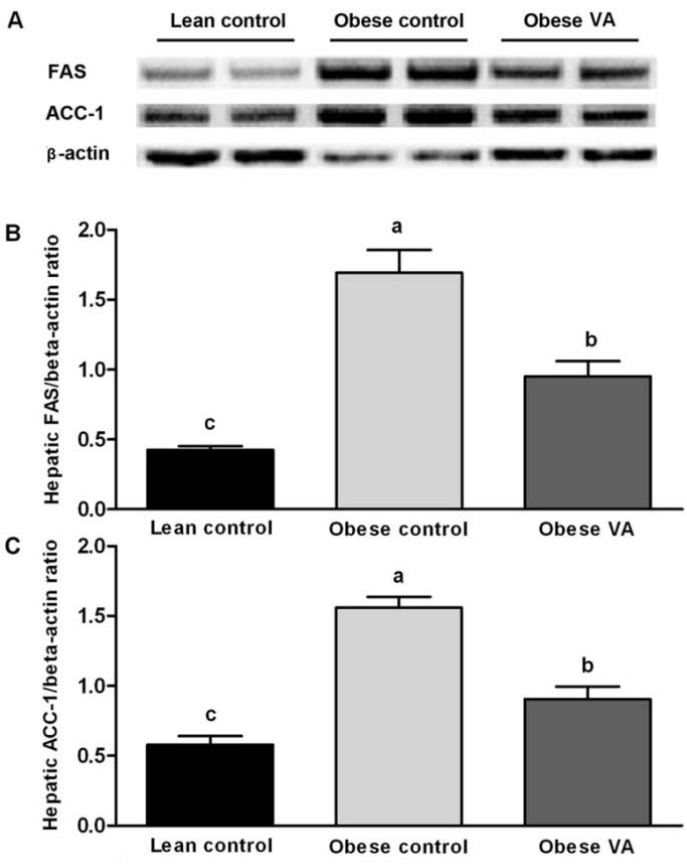


Figure 5-1. Western blots of hepatic lipogenic enzymes (A) and relative protein abundance of ACC-1 (B) and FAS (C) in the lean and obese JCR:LA-cp rats fed the control or VA diet for 16 weeks. Values are means±SEM, n=8. Means without a common letter differ; $p<0.05$.

5.3.5 Postprandial assessment of plasma TG and apo B48

Obese rats supplemented with VA had a significantly lower post-prandial TG (Figure 5-2, panel A) and apoB48 (Figure 5-2, panel B) response following an oral lipid load compared to obese rats fed the control diet. Area under the curve (AUC) analysis of post-prandial plasma TG ($p<0.05$) and apoB48 ($p<0.05$) indicated improvements to post-prandial dyslipidemia in obese rats fed dietary VA compared to those fed the control diet. Incremental AUC (iAUC) was also assessed by substracting plasma TG and apoB48 concentrations at T=0 from each time point (Table 5-6). No difference was observed for TG iAUC between VA-fed obese rats and obese controls, suggesting the improved post-prandial TG response may be primarily attributed to a significant reduction in fasting total

plasma TG concentration. Interestingly, iAUC for apoB48 was significantly lower in VA-fed obese rats compared to obese controls ($p<0.05$), implying an overall reduction in CM synthesis and accumulation during the post-prandial phase and potentially accelerated clearance of CM particles in response to prolonged VA supplementation.

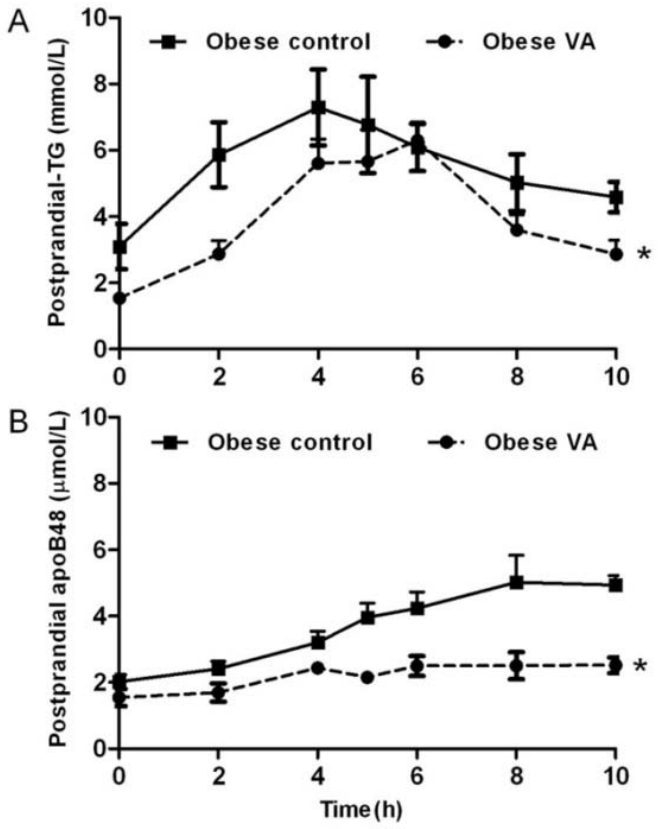


Figure 5-2. Postprandial response following an oral fat challenge in obese JCR:LA-*cp* rats fed the control or VA diet for 16 weeks. Panel A: plasma TG response; Panel B: plasma apoB48 response. Values are means ± SEM, n=4. *$p<0.05$ for AUC.

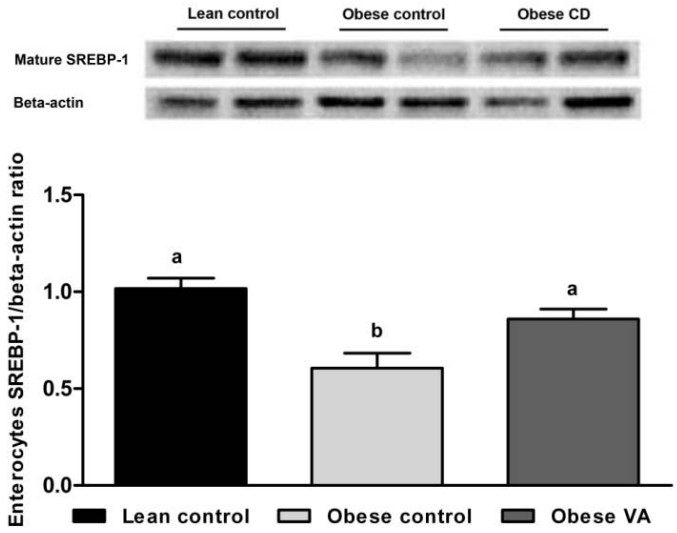
Table 5-6. Area under the curve (AUC) and incremental AUC (iAUC) for post-prandial TG and apoB48 response following an oral fat challenge in obese JCR:LA-*cp* rats fed the control or VA diet for 16 weeks. Values are Means±SEM. Means without a common letter differ, $p<0.05$.

	Obese control	Obese VA
TG, AUC*, mmol/(L*h)*	63.3±10.5[a]	40.9±8.2[b]
TG, iAUC*, mmol/(L*h)*	25.4±2.4[a]	25.4±4.7[a]
ApoB48, AUC*, µmol/(L*h)*	37.0±7.7[a]	22.0±3.3[b]
ApoB48, iAUC*, µmol/(L*h)*	16.8±3.0[a]	7.0±2.6[b]

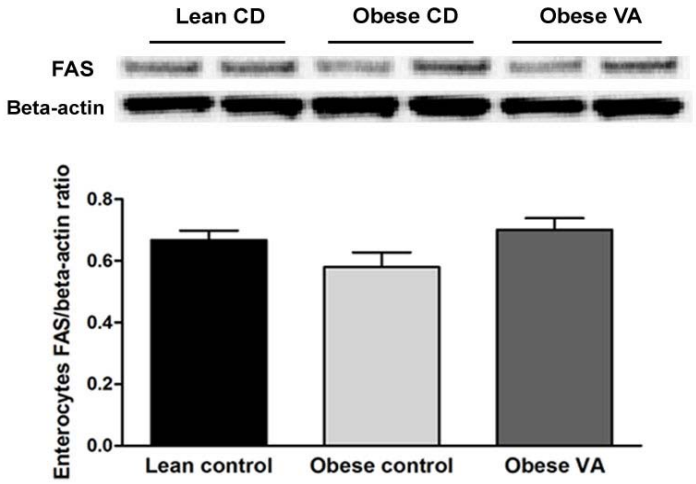
5.3.6 Intestinal lipogenic factors

The abundance of mature SREBP-1 protein was lower in enterocytes isolated from obese rats fed the control diet relative to lean controls (Figure 5-3A, $p<0.01$).Interestingly, VA supplementation in obese rats resulted ina small, yet significant increase (25%) in mature SREBP-1 compared with obese rats fed the control diet ($p<0.05$) to an extent not different from lean control rats (Figure 5-3A). The relative abundance of FAS and ACC-1 protein in enterocytes from obese rats fed VA did not differ from obese rats fed the control diet (Figure 5-3B and 3C).

A)


Lean control
Obese control
Obese CD
Mature SREBP-1
Beta-actin
Enterocytes SREBP-1/beta-actin ratio
1.5
1.0
0.5
0.0
a
b
a
Lean control
Obese control
Obese VA

B)


Lean CD
Obese CD
Obese VA
FAS
Beta-actin
Enterocytes FAS/beta-actin ratio
0.8
0.6
0.4
0.2
0.0
Lean control
Obese control
Obese VA

C)

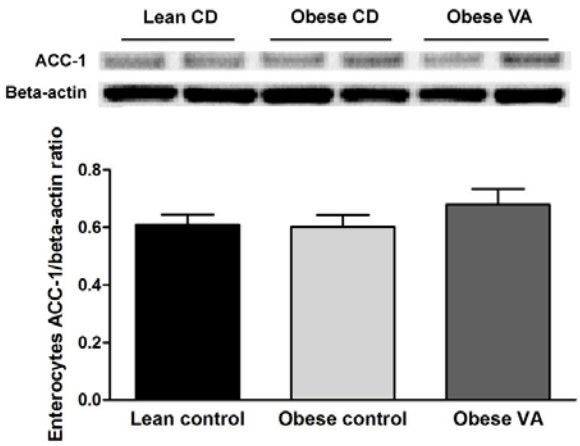


Figure 5-3. Relative protein abundance of intestinal A) SREBP-1, B) FAS and C) ACC-1 in the lean and obese JCR:LA-*cp* rats fed the control or VA diet for 16 weeks. Values are means ± SEM, n=8. Means without a common letter differ, $p<0.05$.

5.3.7 Mesenteric lymphatic chylomicron secretion

Infusion of the VA-containing triolein emulsion in obese rats fed the control resulted in a 40% reduction in the TG concentration of lymph ($p<0.05$) and the number of chylomicron particles (determined by apoB48 concentration) was reduced by 30% ($p<0.01$), compared to those infused with the triolein-only emulsion (Table 5-6). The concentration of lymph TC did not differ between rats infused with triolein-VA or triolein alone. The lipid composition of chylomicron particles was also assessed. The percentage of TG or TC per particle (indicated by the ratio of TG or TC to apo B48) and CM particle size did not differ significantly between groups (Table 5-7).

Table 5-7. Mesenteric lymph concentrations of TG, TC and apoB48 following gastrointestinal infusion of an emulsion containing triolein-alone or triolein with VA in obese JCR:LA-*cp* rats previously fed the control diet for 3 weeks. Values are means±SEM, n=4. Asterisks indicate different from the triolein-infused group: $^{*}p<0.05$, $^{**}p<0.01$

	Triolein	VA-triolein
TG, *mmol/L*	40.0±8.0	24.4±8.6*
TC, *mmol/L*	5.1±0.8	3.9±0.9
apoB48, *µmol/L*	54.2±2.2	37.3±5.9**
TC/apoB48 ratio	100.8±9.8 [a]	106.3±8.3 [a]
TG/TC ratio	7.8±0.6 [a]	6.7±0.6 [a]
TG/apoB48 ratio	796.1±123.0 [a]	723.1±103.8 [a]

5.4 Discussion

Hypertriglyceridemia and hypercholesterolemia are risk factors of MetS and cardiovascular diseases (CVD) (Falko *et al.* 2005, Rana *et al.* 2007). Limited evidence to date has shown that feeding VA or VA-enriched dairy products may have neutral or even beneficial health effects in animal models of these two diseases (Meijer *et al.* 2001, Lock *et al.* 2005, Faulconnier *et al.* 2006, Bauchart *et al.* 2007). Several research groups have reported the potential for (acute) benefits of feeding ruminant *trans* fats on CVD risk markers in healthy human populations (Tholstrup *et al.* 2006, Motard-Belanger *et al.* 2008, Chardigny *et al.* 2008). Three-week feeding of a diet enriched with purified VA reduces plasma TG by approximately 40% without any concomitant change in TC, LDL or glucose/insulin metabolism in dyslipidemic JCR:LA-*cp* rats (Chapter 4). These studies suggested differential properties of the natural ruminant derived *trans* fatty acid-VA, compared to industrially produced *trans* fats. More recently, Tyburczy *et al.* reported similar hypocholesterolemic effects following 4-week dietary supplementation with VA in hamsters, compared to a diet containing partially-hydrogenated vegetable oil (Tyburczy *et al.* 2009).

The current study is consistent with previous results from a shorter-term study in JCR:LA-*cp* rats (Chapter 4), in that chronic (16-week) VA feeding did not significantly

influence food intake, body weight or glucose/insulin metabolism. Rather, chronic supplementation of VA, resulted in a striking reduction in plasma TG by 50%, in addition to reductions in plasma TC, LDL and FFA concentrations in JCR:LA-*cp* rats. Moreover, improvements in post-prandial dyslipidemia, TG and apoB48 were observed with longer-term VA dietary supplementation. These results support the hypothesis that chronic VA intake at a level comparable to 6.0 g of synthetic VA per day for human population may modulate lipogenic pathways under conditions of dyslipidemia.

Elevated concentrations of circulating acute phase proteins (such as C-reactive protein, fibrinogen and haptoglobin) have emerged as biomarkers of CVD risk and/or progression (Maresca *et al.* 1999, Libby *et al.* 2002, Engstrom *et al.* 2002). Interestingly, Balestrieri *et al* and Cigliano *et al* have both reported that haptoglobin may inhibit the activation of lecithin-cholesterol acyltransferase (LCAT) activity by binding with apoA-I, and may attenuate HDL maturation (Balestrieri *et al.* 2001, Cigliano *et al.* 2008). In this study, serum haptoglobin concentration was reduced by 50% in obese rats fed VA, suggesting a mechanism by which VA may improve both the inflammatory regulation and reverse cholesterol transport during conditions of dyslipidemia.

It is well established that feeding PUFA, such as the (n-3) PUFA docosahexanoic acid (DHA) confers TG lowing properties, in part by down-regulating genes involved in lipid biosynthesis including ACC-1 and FAS (Jump *et al.* 2008). In this study, lower hepatic ACC-1 and FAS protein in VA-fed obese rats suggests that VA may also regulate endogenous fatty acid synthesis during hypertriglyceridemia. Elam *et al.* has previously shown that hepatic SREBP-1 mRNA was higher in the obese JCR:LA-*cp* rat (Elam *et al.* 2001). However, in this study it was shown that the relative protein abundance of mature hepatic SREBP-1 did not differ among groups, suggesting possible post-transcriptional and/or post-translational regulation of lipidogenic pathways following VA supplementation. Although VLDL production was not directly measured in this study, it is speculated that reduced hepatic fatty acid synthesis could explain the reduced total hepatic TG observed and imply a reduced VLDL contribution to circulating plasma TG and LDL.

Nascent intestinal CM and hepatic VLDL are two predominant contributors to fasting and post-prandial plasma TG. During insulin resistance, the regulation of post-prandial lipemia becomes compromised, resulting in exaggerated secretion of TG and apoB48-

containing CM from the intestine and hepatic VLDL (Elam *et al.* 2001, Vine *et al.* 2008). Similarly, JCR:LA-*cp* rats have been shown to develop impaired post-prandial lipemia with an associated increase in CM production (measured as apoB48) (Vine *et al.* 2008). In this study, VA-fed obese JCR:LA-*cp* rats showed a substantial decrease in the post-prandial apoB48 AUC after an oral fat challenge compared to obese control rats. It was proposed that improvements in post-prandial dyslipidemia may result from either reduced intestinal nascent CM secretion or accelerated clearance of CM by the liver (which includes lipolysis and tissue uptake). VA decreased intestinal CM secretion as assessed by CM particle number, supporting the findings of a reduced apo B48 iAUC in VA-treated rats compared to controls. However, we did not measure lipolysis or tissue uptake directly to determine the effect of VA on clearance pathways. In addition to lymphatic CM, TG concentration in obese rats infused with triolein-VA relative to triolein-alone, without effects on the size of CM particles. These results collectively suggest that VA may mediate changes in enterocytic TG synthesis and/or CM particle assembly in obese JCR:LA-*cp* rats. Bases on these findings, it is therefore proposed that reduced CM secretion from the intestine may not only result in a lower burden for CM-r clearance by the liver and other peripheral tissure, but may also partially contribute to suppressed VLDL over-production as proposed earlier in the discussion, by delivering considerably less amount of TG to fuel VLDL assembly in the liver. Reduced remnant VLDL particles may in turn allow increased CM-r clearance via lipolytic and receptor mediated uptake pathways.

Major regulators of intestinal fatty acid synthesis were assessed to study the effect of VA on enterocytic *de novo* lipogenesis. Obese JCR:LA-*cp* rats had lower relative abundance of enterocyte mature SREBP-1, compared to their lean counterparts. It is proposed that the obese phenotype may have a compensatory intestinal adaptation to down-regulate lipidogenesis in response to hyperphagia and the associated greater total dietary lipid intake compared to lean controls. Following VA dietary supplementation the abundance of mature SREBP-1 was increased to a level comparable with lean controls, suggesting a VA-mediated normalization of lipogenic pathways involved in CM production. However, VA did not alter the relative protein abundance of FAS or ACC-1 in enterocytes. It is feasible that VA may play a specific role in the regulation of CM assembly pathways rather than fatty acid *de novo* synthesis *per se* in the intestine. It may also lend support to the notion that lipid absorption transport pathways are preferentially downregulated in

response to VA treatment, as lymph CM TG was significantly reduced following triolein-VA infusion. Lampen *et al* reported that VA enhances the expression of PPAR-alpha, beta and delta in a Caco-2 cell culture model (Lampen *et al.* 2005) which may translate to changes in fatty acid transporter expression and LCFA uptake into the enterocyte, however these aspects of VA mediated PPAR effects on lipid absorption require further investigation.

5.5 Conclusion

Long term dietary supplementation of VA (1.0% w/w) in obese, dyslipidemic JCR:LA-*cp* rats results in a substantial improvement in the associated atherogenic plasma lipid profile and post-prandial lipemia. Specifically, results from this chapter indicated novel effects of dietary VA on *de novo* lipogenesis pathways, including suppression of hepatic fatty acid synthesis enzymes and intestinal CM secretion under conditions of dyslipidemia in a rat model of MetS.

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Chapter 6 *Trans*-11 vaccenic acid activates PPAR-α and enhances intestinal expression of both PPAR-α and PPAR-γ in JCR:LA-*cp* rats

6.1 Introduction

Over the past few decades, increasing epidemiological and biochemical studies suggest that *trans* fatty acid (TFA) consumption (particularly from partially hydrogenated vegetable oils), is causally related to elevated cardiovascular events (Micha *et al.* 2009). Consequently, the American Heart Association currently recommends eliminating *trans* fat to less than 1% of daily energy intake in order to prevent a potential deleterious impact on cardiovascular health (Lichtenstein *et al.* 2006). However, emerging evidence (including data presented as part of my thesis in chapters 4 and 5) suggests that certain TFA from ruminant sources (rTFA), have neutral or even beneficial effects on cardiovascular health. Conjugated linoleic acid (CLA) was the first rTFA to be recognized as having health benefits (anti-carcinogenic and lipid-lowering) and excluded from most legislation surrounding TFA (Bhattacharya *et al.* 2006). Curiously, despite *trans*1-18:1 (vaccenic acid or VA) being the most prominent rTFA in the food chain and a precursor to CLA, it has not received significant scientific or public attention until recently.

As discussed earlier, VA accounts for 50%-80% of total *trans* fat in ruminant-derived lipids (Allison *et al.*1995). It is produced from linoleic acid and/or α-linolenic acid via bacterial hydrogenation in ruminant lumen and VA abundance can be raised up to 12% of total fat in dairy food and ruminant meat (e.g. beef, lamb) (Mendis *et al.* 2008). As part of the series of studies presented in my thesis thus far, I have demonstrated that pure VA supplementation may have substantial hypolipidemic properties during conditions of dyslipidemia and the metabolic syndrome (MetS) (Chapter 4). Additional follow-up studies have confirmed that VA can significantly improve the symptoms of hepatic steatosis, post-prandial lipaemia, intestinal chylomicron overproduction and inflammatory dysregulation in the JCR:LA-*cp* rodent model of MetS (Ruth *et al.* 2010, Chapter 5). The lipid-lowering properties of VA have been also been reported in other dyslipidemic rodent models (Tyburczy *et al.* 2010, Bassett *et al.* 2010), but to date no reported studies have addressed the metabolic pathways that may potentially be modulated as a result of VA dietary supplementation, particularly related to dyslipidemia and intestinal lipid metabolism.

Nuclear receptors peroxisome proliferator-activated receptors (PPAR) are a family of nuclear receptors existing as three subtypes (α, δ/β and γ), responsible for modulating lipid and carbohydrate metabolism, and maintaining energy homeostasis (Brown *et al.* 2007). Pharmaceutical compounds that activate PPAR-α and PPAR-γ (e.g. fibrates and thiazolidinedione, respectivey) have been widely recognized as potent lipid-lowering and insulin-sensitizing therapies for patients with hyperlipidemia and type-2 diabetes (Najib *et al.* 2002, Brown *et al.* 2007). Consequently, the PPAR pathways have become key targets in the effort to refine synthetic or naturally occurring PPAR ligands.

Analytical methods have been developed to help streamline the assessment of PPAR ligand activation; these include traditional gel filtration systems, differential protease sensitivity assays, electrophoretic mobility shift assays as well as newly developed scintillation proximity assays, competitive binding assays with radiolabelled or fluorescent reporting systems, are all common in-vitro techniques utilized to identify potential ligands, binding capacity and/or binding domains of various transcription factors (Mochizuki *et al.* 2006). Notably, a number of natural PPAR-α ligands have been identified among which, oleic acid (OA), arachadonic acid, eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA) are considered more potent activators than linoleic acid and most saturated fatty acids (Kliewer *et al.* 1997, Murakami *et al.* 1999). *In-vivo* studies have further confirmed that high DHA consumption is closely associated with plasma triglyceride (TG) reduction in various clinical, animal and cell culture studies in a PPAR-α dependent manner (Jump *et al.* 2005, Delarue *et al.* 2004, Clarke *et al.* 2001). Evidence from the Framingham cohort also reported similar inverse association between PUFA intake and plasma TG concentration, which is strongly correlated with PPAR-α polymorphism (Tai *et al.* 2005). As a result, fatty fish or fish oil high in EPA and DHA is recommended by the International Diabetes Federation as an effective first-line nutritional intervention for patients diagnosed with MetS.

CLA has emerged as a novel bioactive dietary fatty acid and has been shown to effectively improve plasma lipid profile and delay atheromatous plaque formation (Mitchell *et al*. 2008). Accumulating evidence suggest that the regulatory impact of CLA on PPAR and related pathways underpin its bioactivity. *Cis*9,*trans*11-CLA has been shown to effectively bind to human PPAR-α (IC_{50}=140 nM) in a scintillation proximity assay and enhance the expression of fatty acid oxidation-related genes (e.g. acyl-CoA oxidase, cytochrome p450IVA1) in FaO hepatoma cells (Moya-Camarena *et al.* 1999). In

addition to a direct activation of PPAR's activity, *cis*9,*trans*11-CLA has been shown to indirectly modulate PPAR-related pathways via increasing the expression of these nuclear receptors (Lampen *et al.* 2005, Toomey *et al.* 2006, Zhou *et al.* 2008). Importantly, and in relation to the objectives of this thesis, there has not been a study that has assessed the potential of VA as a ligand for PPAR-α or determined whether it can modulate the expression of PPAR genes and/or corresponding protein abundance. Therefore, the objectives of this study were to explore the potential regulatory effect of VA on intestinal expression of PPAR and related key regulators involved in major lipid metabolic pathways in primary enterocytes and/or intestinal mucosa (collected from the feeding study described in Chapter 5), as well as to assess the *in-vitro* binding capacity of VA to PPAR-α ligand binding domain compared to other known fatty acids using a fluorescent-labelled competitive binding assay.

6.2 Methods

6.2.1 Animal Model and Experimental Protocol

Male rats of the JCR:LA-*cp* strain, both obese (*cp*/*cp*) (n=24) and lean (+/?) (n=8), were raised in the established breeding colony at the University of Alberta, as previously described in chapter 5. Rat care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Alberta Animal Ethics Committee. At 8 weeks of age, obese rats were randomized for 16 weeks to either a lipid balanced control diet (CD) or an isocaloric diet containing 1.0% wt/wt synthetic VA in purified form. Macronutrient and fatty acid composition for each of these diets has previously described in chapter 4. Lean littermates were fed the CD for the same period of time to represent normolipidemic control. At the end of the feeding period, jejunal mucosa samples were collected and snap-frozen in liquid N_2 and stored at -80 ^{o}C.

6.2.2 Diet Preparation

The CD was designed to resemble the Western diet, and composed (w/w) of 1% cholesterol, 43.1% carbohydrate, 28% protein, 8% fiber and 15% lipid (w/w) with a PUFA/SFA ratio of 0.6 and (n-6)/(n-3) PUFA ratio of 10 (30% calories from fat) (see chapter 5 and Table 5-1). The VA diet was prepared by adjusting the lipid composition of the CD to provide 1.0% (w/w) of VA while maintaining the PUFA/SFA and (n-6)/(n-3)

PUFA ratio (Chapter 5). The fatty acid compositions of all meals were verified by GC (see previous chapter 5 and Table 5-2). The diet mixture was extruded into pellets, dried at room temperature and stored at 4 ºC in air-tight containers.

6.2.3 Jejunal Enterocyte Collection, Protein Extraction and Relative Protein Abundance of Lipogenic Factors

The jejunum was excised and enterocyte fractions (1–10) were collected using the Weiser method (Weiser *et al.* 1973 and described previously in chapter 5). Samples (50 mL) from each fraction were pooled and homogenized in 200 mL lysis buffer [PBS (pH 7.4) with 1.5% Triton X-100 and 1% protease inhibitor cocktail (Sigma)]. The concentration of enterocytic TG was measured as previously described in chapter 5. The remainder of the homogenate was centrifuged at 700 g for 15 min, and the supernatant collected and stored at -80 °C. PPAR-α and PPAR-γ protein abundance was determined by western blot analysis as previously described (Qin *et al.* 2009 and chapter 5). In brief, western blot membranes were incubated with primary antibodies raised against PPAR-α (1:1000 dillution, catalog no. sc-9000, Santa Cruz Biotechnology) and PPAR-γ (1:1000 dilution, catalog no. sc-7196, Santa Cruz Biotechnology) and □ -actin (1:20000 dilution, internal control) (catalog no. A5441, Sigma-Aldrich). Adherent antibodies were visualized by chemiluminescence using ECL Advance Western Blotting Detection kit (catalog no. RPN2135, GE Health and previously described in chapter 5). Protein bands were quantified using Image J (version 1.41) software developed by NIH. The final value of PPAR-α and PPAR-γ relative protein abundance was normalized based on the respective □ -actin protein mass.

6.2.4 RNA extraction procedure and real time PCR analysis

Total RNA was extracted from jejuna mucosa using the RNeasy Mini kit (Cat No.74104, Qiagen, USA). The concentration of total RNA was determined by measuring the absorbance at 260 nm using NanoDrop. Total RNA was reverse transcribed to cDNA per manufacturer's instructions (High capacity cDNA reverse transcription kit, catalog no. 4368814, Applied Biosystems, USA). Real Time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA). Relative mRNA expression of genes relating to PPAR-α, PPAR-γ was assessed in order to investigate the impact of VA supplementation on enterocytic pathways. These included PPAR-α, PPAR-γ, sterol response element binding protein (SREBP)-1, SREBP-2, fatty acid synthase (FAS),

acetyl-CoA carboxylase (ACC)-1, microsomal triglyceride transfer protein (MTP), apolipoprotein (apo) B, fatty acid translocase (FAT/CD36), fatty acid transport protein-4 (FATP-4), Nieman Pick C 1-like protein 1 (NPC1L1), liver-type fatty acid binding protein (L-FABP), intestine-type fatty acid binding protein (I-FABP), ATP-binding cassette (ABC) A-1, ABCG5, ABCG8, scavenger receptor B-type I (SR-BI), low-density lipoprotein receptor (LDL-r) was measured and normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

6.2.5 PPAR-α competitive binding assay

PPAR-α competitive binding assay was performed *in vitro* using LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) assays according to manufacturer's instructions (catalog no. PV4892, Invitrogen). The general principle of this assay is as follows: a terbium-labeled anti-GST antibody binds to the receptor's GST taq. When a fluorescent ligand (tracer) binds to the receptor, energy transfer from the antibody to the tracer occurs and a high fluorescent signal (indicated by TR-FRET ratio) is observed. Competitive ligand binding to the receptor is detected by a test compound's ability to displace the tracer from the receptor, resulting in a loss of fluorescent signal between the antibody and the tracer. Measurement was performed on a Tecan Safire 2 monochromator-based microplate reader with instrument settings as recommended from LanthaScreen. Free fatty acids including VA, *cis9*,*trans*11-CLA (Lipid Nutrition), OA (catalog no. O1008, Sigma) and DHA (catalog no.U-84-A, NuChek Prep) were prepared in serial dilutions from 1 nM to 1 mM (1% final DMSO concentration). GW7647 (1nM) (catalog no. 1677, Tocris Bioscience) a known PPAR-α agonist was used as the positive control. Assays were performed in triplicates. The IC_{50} value was assessed using an equation for a sigmmoidal dose-response provided by GraphPad Prism 5.0. All assays were validated for the robustness by determining the respective Z'-factors (Zhang *et al.* 1999).

6.2.6 Statistical Analysis

All results are expressed as mean±SEM. Data were tested for normal distribution and differences among all groups for most variables were analyzed using 1-way ANOVA followed by Tukey's post hoc tests. The IC_{50} value of each fatty acid in PPAR-α competitive binding assay was calculated using an equation for a sigmmoidal dose-response. The level of significance was set at $p<0.05$ (Graph Pad Prism 5.0).

6.3 Results

6.3.1 Intestinal PPAR-α and-γ expression

In obese rats fed VA for 16 weeks, there was an 80% and 50% increase in PPAR-α and PPAR-γ relative mRNA abundance respectively in the jejunal mucosa compared to obese controls (Figure 6-1). Chronic VA supplementation resulted in a modest but significant increase in PPAR-γ relative protein abundance in the primary enterocytes isolated from VA-treated obese rats compared to obese controls ($p<0.05$, Figure 6-2). However, the ascending trend in the relative protein abundance of PPAR-α did not reach statistical significance in VA-treated rats (Figure 6-3).

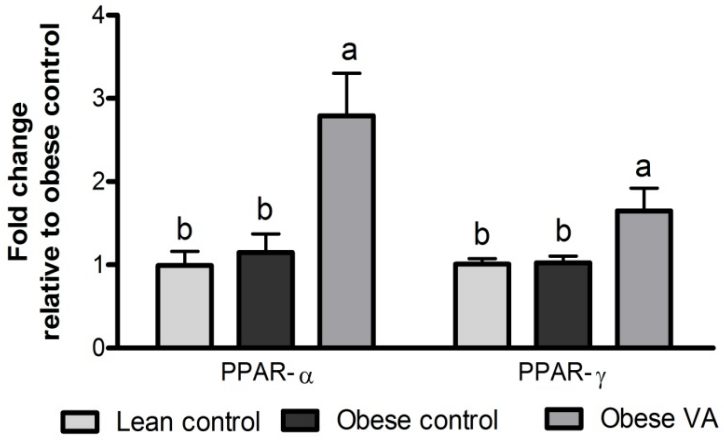

Figure 6-1. Fold change of mucosal mRNA expression of PPAR-α and PPAR-γ in lean and obese JCR:LA-*cp* rats fed the CD or VA diet for 16 weeks. Values are expressed as means±SEM and normalized to GAPDH, n=8. Means for each gene without a common letter differ; $p<0.05$.

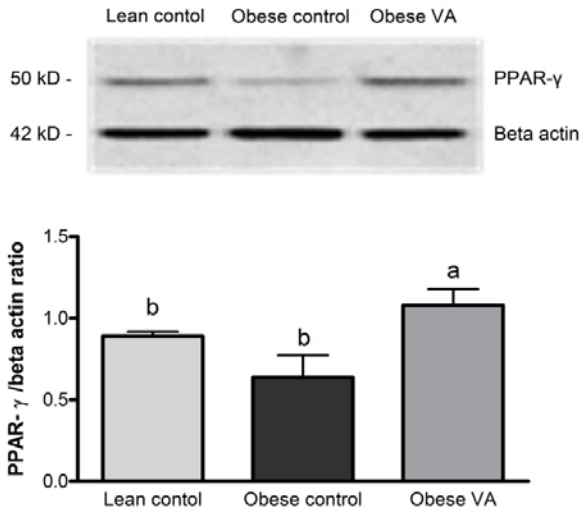

Figure 6-2. Relative intestinal protein abundance of PPAR-γ in lean and obese JCR:LA-*cp* rats fed with VA diet for 16 weeks. Values are expressed as means±SEM and normalized to β-actin, n=4. Means without a common letter differ; $p<0.05$.

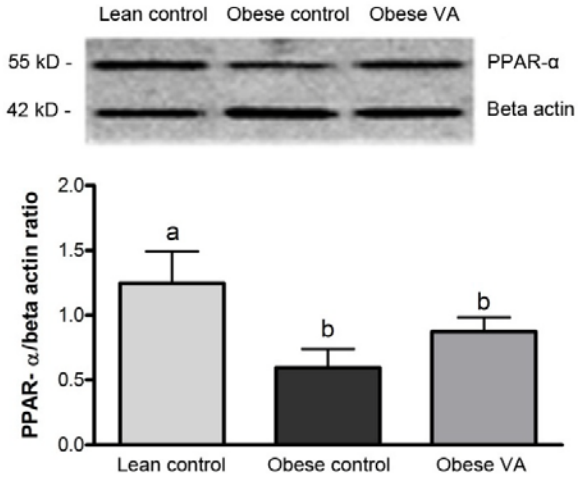

Figure 6-3. Relative intestinal protein abundance of PPAR-α in lean and obese JCR:LA-*cp* rats fed with VA diet for 16 weeks. Values are expressed as means±SEM and normalized to β-actin, n=4. Means without a common letter differ; $p<0.05$.

6.3.2 Mucosal expression of other genes involved in lipid metabolism

Relative gene expressions of FAT/CD36 and SREBP-1 were elevated in the jejunal mucosa of obese rats fed VA for 16 weeks compared to obese control (Figure 6-4 and 6-5). But no difference was detected in genes involved in several fatty acid transporters:L-FABP, I-FABP or FATP-4 (Figure 6-4), fatty acid/cholesterol synthesis:FAS, ACC-1, MTP, apoB48 or SREBP-2 (Figure 6-5) or cholesterol trafficking:NPC1L1, ABCG5/G8, ABCA-1, SR-BI or LDL-r (Figure 6-6) mRNA expression among groups.

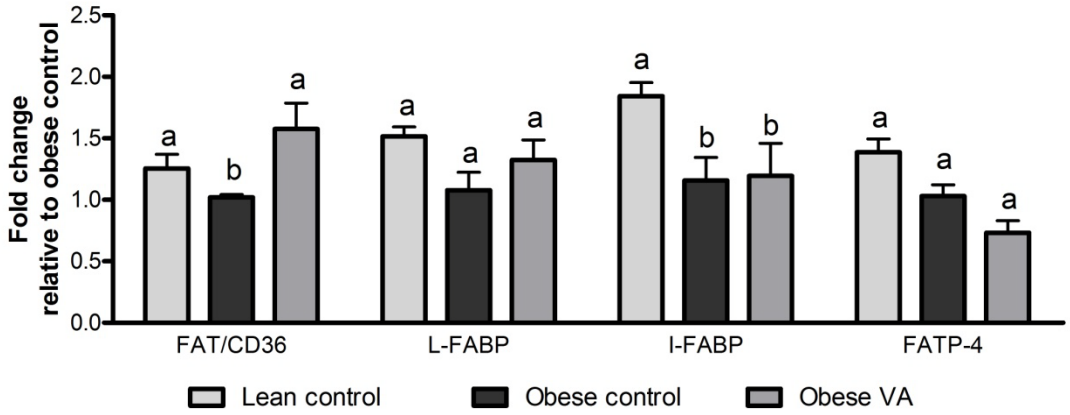

Figure 6-4. Fold change of mRNA expression of major fatty acid transporters in the mucosa of lean and obese JCR:LA-*cp* rats fed the CD or VA diet for 16 weeks. Values are expressed as means±SEM and normalized to GAPDH, n=8. Means for each gene without a common letter differ; $p<0.05$.

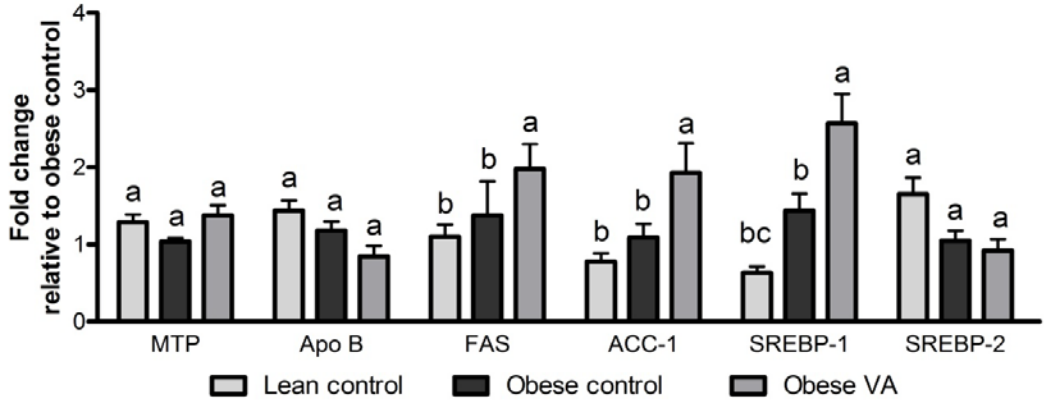

Figure 6-5. Fold change of mRNA expression of lipogenic genes in the mucosa of lean and obese JCR:LA-*cp* rats fed the CD or VA diet for 16 weeks. Values are expressed as means±SEM and normalized to GAPDH, n=8. Means for each gene without a common letter differ; $p<0.05$.

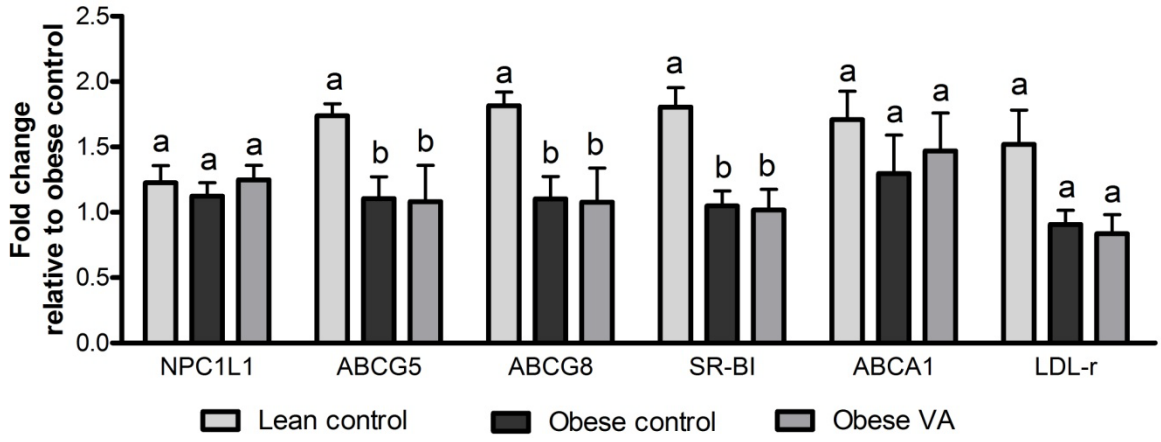

Figure 6-6. Fold change of mRNA expression of major cholesterol transporters in the mucosa of lean and obese JCR:LA-*cp* rats fed the CD or VA diet for 16 weeks. Values are expressed as means±SEM and normalized to GAPDH, n=8. Means for each gene without a common letter differ; $p<0.05$.

6.3.3 Affinity to PPAR-α ligand binding domain

The IC_{50} of several fatty acids to PPAR-α ligand binding domain were assessed using a TR-FRET competitive binding assay. The resultant inhibition curves (Figure 6-4) indicate that VA is a potent PPAR-α ligand with similar IC_{50} as *cis9*,*trans*11-CLA and VA has only one tenth of the binding compared to fenofibric acid (Table 6-1). Further, the 1:1 mixture of VA and *cis*9,*trans*11-CLA showed equivalent competence to activate this nuclear receptor to the individual fatty acids. VA, *cis*9,*trans*11-CLA and their 1:1 mixture had an IC_{50} value 50% lower compared to OA and 40% lower compared to DHA, suggesting an approximately one-fold higher affinity of these rTFA to PPAR-α than known fatty acid ligands.

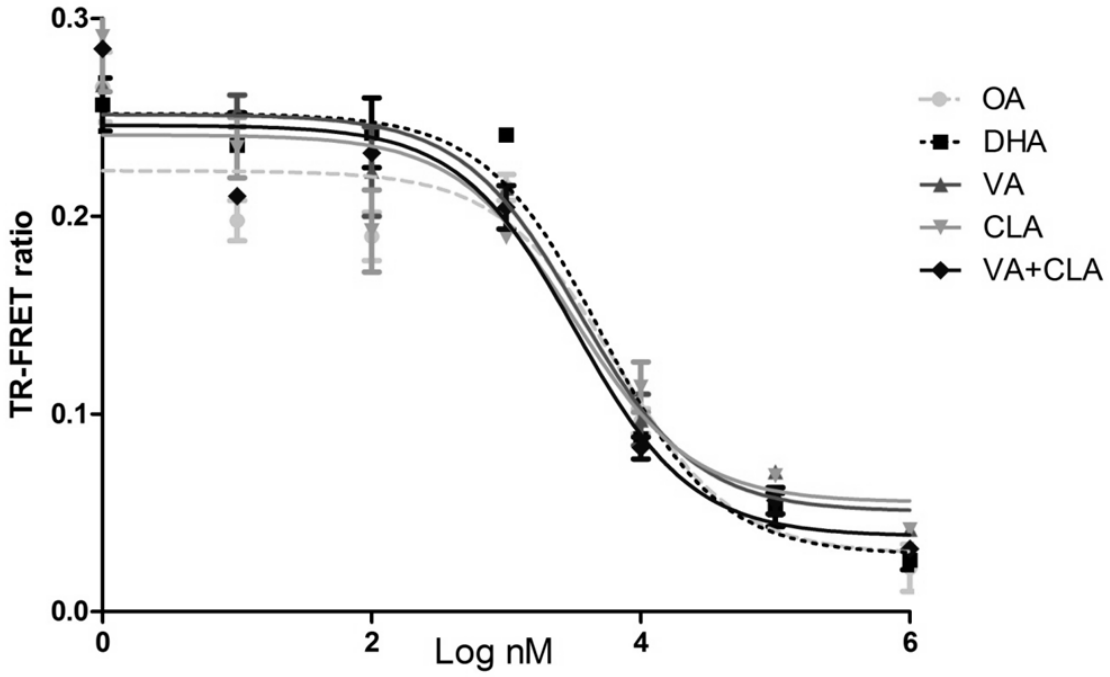

Figure 6-7. Competitive binding curve of OA, DHA, VA, *cis*9,*trans*11-CLA, a 1:1 mixture of VA and *cis*9,*trans*11-CLA to PPAR-α ligand binding domain. Value expressed as the 520 nm/495 nm emission ratio (TR-FRET ratio) at a serial concentration of fatty acids.

Table 6-1. The IC_{50} values of PPAR-α ligands in the TR-FRET competitive binding assay[1]

Ligand	IC_{50} (nM)
GW7647[2]	1.3
Fenofibric acid[2]	40000
VA	3499
Cis9,*trans*11-CLA	3188
VA+CLA mixture[3]	3284
OA	6505
DHA	5159

[1]IC_{50} is the concentration of the fatty acid that produces 50% displacement of the tracer
[2]The IC_{50} values of GW7647 and fenofibric acid are provided by the manufacturer of this assay
[3]VA+CLA mixture was composed of equal amounts of synthetic VA and *cis9*,*trans*11-CLA

6.4 Discussion

6.4.1 VA up-regulates the mucosal expression of PPAR-γ as well as certain PPAR-α target genes

Our study is the first to report that VA enhances the gene expression of both PPAR-α and PPAR-γ, as well as corresponding protein abundance of PPAR-γ after 16-week supplementation in JCR:LA-*cp* rats. Data from this series of studies also suggest that VA supplementation may result in an increased musocal expression of FAT/CD36 in obese JCR:LA-*cp* rats. FAT/CD36 is actively involved in long chain fatty acid absorption as well as intracellular transport in rats (Bonen *et al.* 2009). Very recent studies also suggest that mitochondrial FAT/CD36 maybe required to up-regulate mitochondrial fatty acid oxidation, which is one of the major metabolic pathways modulated by PPAR-α (Holloway *et al.* 2009). Although the cellular localization and/or protein abundance of FAT/CD36 was not assessed in this study, it is plausible to hypothesize that VA may function via activating PPAR-α activity in enterocytes, thus enhancing fatty acid oxidation.

6.4.2 VA is a natural PPAR-α ligand

To test the hypothesis that VA may activate PPAR-α activity, an in-vitro competitive binding assay was performed to assess the affinity of VA to the ligand binding domain (LBD) of PPAR-α. The result indicated that VA effectively displaced the fluorescent-labelled tracer from the LBD region with an IC IC_{50} of 3499 nM. More intriguingly, it was found that VA's affinity to PPAR-α was approximately 10-fold higher than fenofibric acid, a drug approved by US Food and Drug Administration (FDA) for the treatment of hypertriglyceridemia (RLD 21656/21695). It is thus feasible to speculate that a TG-lowering efficacy comparable to fenofibric acid administration may be alternatively achieved by a lower cellular abundance of VA when applied in neutraceutical intervention for hypertriglyceridemic patients. The affinity of VA, OA, DHA and a VA/CLA mixture has also been compared in the competitive binding assay. Notably, VA had an IC_{50} at least 1-fold lower than that of either OA or DHA. Indeed, the relative binding affinities from this chapter appear to be consistent with the *in-vivo* findings described in chapter 5. The substitution of OA in the control diet for VA in JCR:LA-*cp* rats (for 16 weeks) led to a substantial improvement in plasma TG during both the fasted and postprandial states in obese JCR:LA-*cp* rats, which is regarded as a charactistic pattern of clinical administration of PPAR-α agonists (Chapter 5).

The improvement in hepatic steatosis in JCR:LA-*cp* rats after VA supplementation also mimics typical clinical symptoms of non-alcoholic fatty liver disease using PPAR-α agonists (Kallwitz et al. 2008). In addition to the small intestine, the expression of PPAR-α has been identified in other tissues with high fatty acid catabolism including liver, heart, kidney, brown adipose tissue and muscle (Braissant *et al.* 1996, Pyper *et al.* 2010). Although the effect of VA on fatty acid oxidation was not directly measured in this study, recent findings have demonstrated an up-regulation of hepatic citrate synthase activity in obese JCR:LA-*cp* rats fed VA/CLA-supplemented diet, possibly leading to accelerated mitochondrial fatty acid oxidation (Jacome-Sosa *et al.* 2010) in response to VA and/or CLA supplementation. Consistent with this fingding, preliminary results also indicated increased energy expenditure in VA-fed obese rats compared to obese rats fed with a diet containing high saturated fatty acids (Jacome-Sosa *et al.* 2010), thus providing an indirect evidence of VA's regulatory effect on fatty acid and energy metabolism. It can therefore be speculated that an increase in energy expenture potentially contributed by hepatic fatty acid oxidation, combined with reduced fatty acid *de novo* synthesis (Chapter 5) may lead to a depletion of cellular fatty acid pool in the liver and subsequently insufficient TG synthesis to support very low density lipoprotein (VLDL) production in obese rats. Inhibition of VLDL secretion into the circulation may thus be partially responsible for reduced fasting TG observed in VA-fed obese rats. Future studies may continue to assess the interaction between VA and other PPAR subtypes (e.g. PPAR-γ and PPARδ) in order to understand the effect of VA on inflammation associated with MetS as reported earlier (Chapter 4 and Ruth *et al.* 2010).

6.4.3 VA did not affect *de novo* lipogenesis in the enterocytes

The expression of several key factors in lipogenesis was also examined in the jejunal mucosa. SREBP-1 and SREBP-2 are transcription factors regulating genes involved in fatty acid/TG and cholesterol biosynthesis respectively (Chen 2004). In this study, no change in SREBP-2 but an increase in SREBP-1 mRNA abundance was observed in the mucosa collected from VA-fed obese rats compared to controls. The increased SREBP-1 expression is consistent with the previously-reported increase in its protein abundance in primary enterocytes of VA-fed obese rats. It also supports the corresponding increase in the expression of target genes such as FAS and ACC-1 in the mucosa. Interestingly, the protein concentration of FAS and ACC-1 did not parallel the changes in respective

mRNA between groups, possibly due to increased degradation of these proteins (Chapter 5).

6.5 Conclusions

In conclusion, VA is a potent PPAR-α agonist and may possess greater TG-lowering capacity compared to some commonly used therapeutic agents to treat hypertriglyceridemia (e.g. DHA and fenofibrate). In addition, up-regulated expression of PPAR-α and PPAR-γ in the intestine may provide preliminary understanding regarding the metabolic pathways potentially modulated by VA supplementation.

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Chapter 7 General Final Discussion

7.1 Implication for dietary recommendation and *trans* fat policy

The consumption of *trans* fatty acid (TFA) being causally associated with the development of CVD has been strongly supported by epidemiological data, leading to a worldwide campaign of eliminating *trans* fat content in the food supply (Micha *et al.* 2009). In 2003, the World Health Organization recommended to eliminate *trans* fat intake to less than 1% of total energy intake. In December 2005, Canada became the first country to mandate total *trans* fat content per serving must be listed for all pre-packaged foods and was required to follow food labeling regulations. Food containing more than 0.2 g per serving cannot be claimed as "*trans* fat-free" in Canada. Other countries such the US, Brazil and New Zealand have also adopted similar mandatory labeling requirements in 2006, whereas in most European countries the '*trans* fat free' campaign remains on a voluntary basis (Colon-Ramos 2006, Hillyer 2007). However, the dietary guidelines or government regulations for *trans* fat in many countries have overlooked the types of *trans* fats and the complexity of this lipid class due to the nature of their origin. The increase in the proportion of ruminant TFA (rTFA) in dairy-derived products has further confounded the premise for minimizing total dietary TFA, but on the other hand has sparked a critical need to better understanding the bioactivity of specific rTFA isomers [e.g. *trans*11-vaccenic acid (VA) and *cis*9,*trans*11-conjugated linoleic acid (CLA)]. The benefits of CLA in cancer prevention and weight management has led to the exclusion of CLA from the legislatorial definition of TFA in Canada, the US and Denmark. Unfortunately, the appreciation of VA's bioactivity has remained rather limited, despite VA being the most prominent natural TFA in human food supply. Our group was the first to report that VA has substantial TG-lowering benefit specifically under conditions of dyslipidemia and the metabolic syndrome (MetS) (Chapters 4 and 5). Importantly, a number of other independent research groups have proposed similar lipid-lowing and anti-atherogenic effects of purified VA or VA-enriched butter in cholesterol-fed hamsters as well as LDL-r knockout mice (Tyburczy *et al.* 2009, Bassett *et al.* 2010). Although no human clinical study using a purified VA preparation has been published so far, the consistent hypolipidemic effects of dietary VA consumption reported in this thesis as well as other researchers supports the notion that VA does have putative health benefits and these are distinctive from the widely accepted detrimental health effects of industrial *trans* fatty acids (iTFA). As a result, it is proposed that it would be appropriate to now

recognize VA as pertaining to have at least equivalent biological properties compared to CLA.

7.2 Bioavailability of *trans*11-vaccenic acid

In the first objective of this thesis, it was confirmed that chemically-synthesized purified VA was bioavailable in both lean and obese phenotypes of the JCR:LA-*cp* strain (chapter 3). Obese rats appeared to have higher VA incorporation into lymphatic chylomicrons (CM) compared to their lean counterparts, perhaps indicative of overall higher lipid absorption efficiency in the obese phenotype. Indeed, our group has previously reported that obese JCR rats have longer jejunums as well as increased villus size and number, thus greater absorptive surface area (Hassanali *et al.* 2008). Consistent with these findings, an anatomical study of human intestine also concluded that the length of the small intestine was positively correlated with body weight (Hounnou *et al.* 2002). The resemblance between obese humans and rats in intestinal morphology suggests the intestinal bioavailability of dietary VA may be greater in obese individuals and thus VA may have a beneficial hypolipidemic potential in clinical interventions targeted at patients with MetS and dyslipidemia.

On the other hand, a lower VA bioavailability in lean JCR:LA-*cp* rats may provide some alternative explanation to the inconsistent outcomes reported between human clinical trials and animal feeding studies using VA-enriched sources (Desroches *et al.* 2005, Tricon *et al.* 2006, Tardy *et al.* 2009). Most of the feeding trials published to date have been based on studies using diseased animal models such as hyperlipidemic hamsters or rabbit models (Valeille *et al.* 2005, Lock *et al.* 2005, Bauchart *et al.* 2007). However, subjects participating in the above-mentioned clinical trials are healthy volunteers with no severe lipid abnormalities. Due to the lack of positive outcomes from human studies, the World Health Organization has concluded in their latest *trans* fat update, that ruminant TFA may be harmless, without acknowledging any cardio-protective potential (Uauy *et al.* 2009). With better appreciation of the differential VA bioavailability between healthy and diseased conditions, perhaps future clinical studies focusing on the obese and dyslipidemic population may prove successful in demonstrating the benefits of rTFA.

When comparing VA from different sources, we demonstrated that in obese JCR:LA-*cp* rats, VA from naturally enriched beef fat had greater bioavailability than the chemically-synthesized purified form. The interpretation of these findings is in line with earlier

findings by Burdge *et al.* regarding a higher bioavailability and plasma TG incorporation of dairy conjugated linoleic acid (CLA) in healthy humans compared to encapsulated *cis*9,*trans*11-CLA supplements. (Burdge *et al.* 2004). Chardigny *et al.* showed that dairy TG may present rTFA to the body in a structure more readily hydrolyzed by lipases, which are better absorbed into the enterocyte (Chardigny *et al.* 2003). Based on these exciting findings, it seems that dairy fat highly enriched with rTFA may be applied as a new dietary intervention strategy assisting the treatment of hypertriglyceridemia.

7.3 Developing rTFA enriched ruminant products

While the dairy industry is highly motivated to develop rTFA enriched products, they are still challenged by the current public perception of dairy fat which continues to be negative. For example, dietary recommendations have been encouraging low-fat dairy options and educating consumers that saturated fats from animal products are associated with a higher risk of developing CVD. However, dairy fat only contributes to a small part (less than 15%) of total dietary saturated fatty acid intake compared to red meat. The worldwide prevalence of CVD keeps growing in spite of a steady decline in milk fat consumption (Cash *et al.* 2004, Lloyd-Jones *et al.* 2010). Indeed, dairy consumption has been shown to be inversely associated with dyslipidemia and hypertension, particularly in the obese people (Pereira *et al.* 2002). As a result, high consumption of dairy fat may not necessarily be responsible for the prevalence of CVD *per se*. Secondly, the skimming process required for manufacturing low-fat or fat-free dairy products has concomitantly sacrificed other beneficial components associated with dairy fat (e.g. CLA and fat-soluble vitamins), not only in their absolute abundance, but also in their bioavailability.

Traditionally, rTFA has been considered to constitute a rather small part of the fat in dairy products (2% to 5% of total fatty acids), and beef and lamb (3%-9%) (Precht *et al.* 1996). However, it is now appreciated that fatty acid composition is largely dependent on bovine feeding practices, and can vary substantially due to geographical and/or seasonal change. For example, alpine pastures in Northern European countries yield greater concentrations of rTFA in milk compared to lowland pastures (Precht *et al.* 1995). Milk produced during the long daylight of summer often contains more total rTFA than milk produced during the winter months (Precht *et al.* 1995). Moreover, modification to agricultural practices have been developed to enhance the content of rTFA, such as

grass feeding (Daley *et al.* 2010, Mohammed *et al.* 2009), supplementing feed with high PUFA oil seeds (Rego *et al.* 2009), changing the microflora of the rumen (Boeckaert *et al.* 2008) and pre-selecting cows that are genetically high 'rTFA producers' (Taniguchi *et al.* 2004). Among these, modifying cow feed may be the most economic and efficient strategy to increase rTFA. The concept behind this is to supply sufficient substrate (predominantly linoleic acid and linolenic acid) for luminal bacterial fermentation (Figure 1-1). Sources such sunflower oil, fish oil and marine algae have been implemented in basic cow feed (Duckett *et al.* 2010), and as a consequence, rTFA content as high as 13% of total fat is now a regular occurrence in dairy products (with additional reductions in saturated fat) (Palmquist *et al.* 2005, Mendis *et al.* 2008, Abughazaleh *et al.* 2009). Another interestingly study published recently suggests that supplemental quebracho tannins may increase the specific microbial profile that converts LA to *cis*9,*trans*11-CLA and then to VA, whilst inhibiting the bacteria which convert VA to stearic acid (Vasta *et al.* 2010).

Another noteworthy point is the milk fat reduction that accompanies altered rumen biohydrogenation (Bauman *et al.* 2003). Reduced milk fat production may be related to certain by-products from rTFA enrichment, such as *trans*10-containing 18:1 and 18:2, which act as putative inhibitors of lipid synthesis (Griinari *et al.* 1998, Fuentes *et al.* 2009). Recent findings suggest that the noticeable decrease in stearic acid and oleic acid synthesis may ultimately limit the ability of the mammary gland to maintain a desirable milk fat content to allow the appropriate fluidity (Toral *et al.* 2010). Interestingly, supplementation of α-tocopherol to cow feed tends to increase VA while reducing trans10-18:1 production, which may lead to an overall improvement in milk fat content and rTFA profile (Pottier *et al.* 2006).

It may be necessary to point out that the ability to enrich rTFA in ruminant fat via supplementing cow feed is rather limited. In addition, dairy production in higher latitude regions remain limited by the lack of grass finishing periods relative to lower-latitude production sites. This in turn places great pressure on producers from higher-latitude regions to supplement their feed regimens in order to reach a rTFA-enriched fatty acid profile. Nevertheless, it seems promising that the other non-dietary strategies such as the application of genomic and bioengineering technology could potentially assist with with higher rTFA biosynthesis. For instance, bacteria strain screening and selective

breeding of ruminant animals may ultimately push the limit of natural VA or total rTFA enrichment to a comparable level used in animal studies.

7.4 Vaccenic acid reduces atherogenic lipid profile

Dysregulated lipid metabolism has been involved in the initiation and progression of atherosclerosis as well as advanced CVD complications (Genest *et al.* 2003, Ginsberg *et al.* 2005). The findings of this thesis support a substantial hypolipidemic benefit of VA on pre-existing atherogenic lipid profiles, including plasma triglyceride (TG), total cholesterol (TC) and low density lipoprotein-cholesterol (LDL) in the obese JCR:LA-*cp* rodent model (Chapters 4 and 5). In addition to these traditional lipid risk factors, there has been increasing appreciation during the past decade on the role of intestinal lipid metabolism in CVD development. More specifically, intestinal chylomicrons (CM) and related remnant lipoproteins (CM-r) significantly contribute to postprandial lipaemia, hypertriglyceridemia, hypercholesterolemia and accelerated atherogenesis (Proctor *et al.* 2004, Vine *et al.* 2008). JCR:LA-*cp* rats have been shown to develop impaired postprandial lipaemia associated with chylomicron overproduction, which has been improved after chronic VA dietary supplementation (chapter 5). Further, it has also been demonstrated that TG concentration, as well as CM particle numbers (indicated by apo B48) were lower in nascent lymph collected over the post-prandial phase proceeding a one-time oral dose of VA-triolein emulsion (Chapter 5). It is likely that TG synthesis and CM production may have been suppressed upon acute exposure to dietary VA. Although *de novo* lipogenesis in the intestine was not affected by VA (indicated by enterocytic ACC-1 and FAS relative protein abundance), there was a 150% increase in PPAR-α, a 60% increase in PPAR-γ and a 60% increase FAT/CD36mRNA abundance in the mucosa of VA-fed obese rats compared to obese controls, suggesting possible changes in intestinal fatty acid trafficking and/or metabolism in response to VA supplementation (Chapter 6).

Chronic low-grade inflammation plays an essential role in various stages of CVD development (Libby *et al.* 2006). The splenocytes of obese JCR:LA-*cp* rats have been shown to have impaired ability to respond to T-cell mitogen stimulation. Results from the chronic feeding study revealed a mild reduction in serum haptoglobin (a rodent acute phase protein equivalent to C-reactive protein in humans) concentration in VA-fed obese rats compared to the obese control (Chapter 5). Moreover, the impaired T-cell function

was normalized in splenocytes isolated from obese JCR:LA-*cp* rats fed VA either for 3 weeks or for 16 weeks, compared to obese control rats (Blewett *et al.* 2009, Ruth *et al.* 2010). The exact mechanism by which VA mediates immune-enhancing properties is unknown, but might possibly follow similar PPAR-γ agonizing pathways as reported for *cis*9,*trans*11-CLA (O'Shea *et al.* 2004). The enhanced mucosal mRNA and protein abundance of PPAR-γ in VA-fed obese rats (Chapter 6) may suggest immune-regulatory effect of VA on the intestine, but may require further confirmation by assessing the inflammatory markers associated with the intestine.

7.5 Proposed mechanism of action

As established the studies of this thesis, dietary VA supplementation can improve dyslipidemia by lowering fasting TG, LDL and free fatty acids (Chapter 5) as well as post-prandial TG and apoB48-containing lipoproteins, thus attenuating atherosclerotic progression. Reduced lymphatic TG and chylomicrons (CM) particle numbers (apo B48) in response to gastric infusion of VA may partially explain the improved post-prandial lipaemia reported in Chapter 5. It is further proposed that VA may potentially suppress *de novo* lipogenesis whilst up-regulating fatty acid oxidation, which may conjointly limit the cellular fatty acid pool that fuels the production of TG and TG-rich lipoprotein [i.e. CM and very low density lipoproteins (VLDL)] in the intestine and liver respectively, which is directly associated with improved fasting and post-prandial hypertriglyceridemia. The proposed retardation in fatty acid *de novo* synthesis due to VA supplementation was supported by the observation that the relative protein abundance of acetyl-CoA carboxylase (ACC)-1 and fatty acid synthase (FAS) was significantly lower in the liver harvested from VA-fed obese rats compared to those from obese controls (Chapter 5).

*Cis*9,*trans*-11 CLA, as an *in-vivo* metabolite of VA, has been implicated to up-regulate fatty acid oxidation and delay atherosclerosis in apo E knockout mice through PPAR-α dependent mechanisms (Toomey *et al.* 2003). Other PPAR-α agonists such as DHA and fibrates have been regularly used as nutraceutical or pharmaceutical agents in clinical settings to normalize hypertriglyceridemia and other cardiovascular risk markers (e.g. LDL and HDL) associated with the metabolic syndrome and type-2 diabetes (Staels *et al.* 2008). In Chapter 6 it was shown that VA is a potent agonist of peroxisome proliferator-activated receptor (PPAR)-α and has an affinity to the ligand binding domain similar to *cis*9,*trans*-11 CLA, approximately 100% higher than that of oleic acid (OA) and

decosahexaenoic acid (DHA). More strikingly, VA is a 10-fold more potent competitor for PPAR-α than the well known PPAR-α agonist—fenofibric acid, that is commonly prescribed to hypertriglyceridemic patients (Chapter 6). Therefore, it is plausible to suggest that the hypolipidemic effect of VA may be in part ascribed to the augmentation of PPAR-α associated pathways. Direct evidence that strongly support this proposed mechanism of action comes from a recently published study from our group, in which citrate synthase activity (a marker for fatty acid oxidation) was higher in the liver and inguinal adipose tissue of obese rats fed a combination of VA and CLA (the *cis*9,*trans*-11 isomer, 1% w/w for each fatty acid) (Jacome-Sosa *et al.* 2010).

In summary, the overall improvement in fasting and post-prandial dyslipidemia as well as inflammatory response associated with reduced CVD risk due to VA supplementation may be contributed by considerably down-regulated *de novo* lipogenesis as well as TG-rich lipoprotein production/secretion in the liver and intestine. It is possible that the enhancement of PPAR-α and PPAR-γ related pathways by VA plays a central role in mediating these metabolic changes; however, the specific cellular activities involved still requires further investigation. The proposed mechanism of action by VA in JCR:LA-*cp* rats is summarized in Figure 7-1.

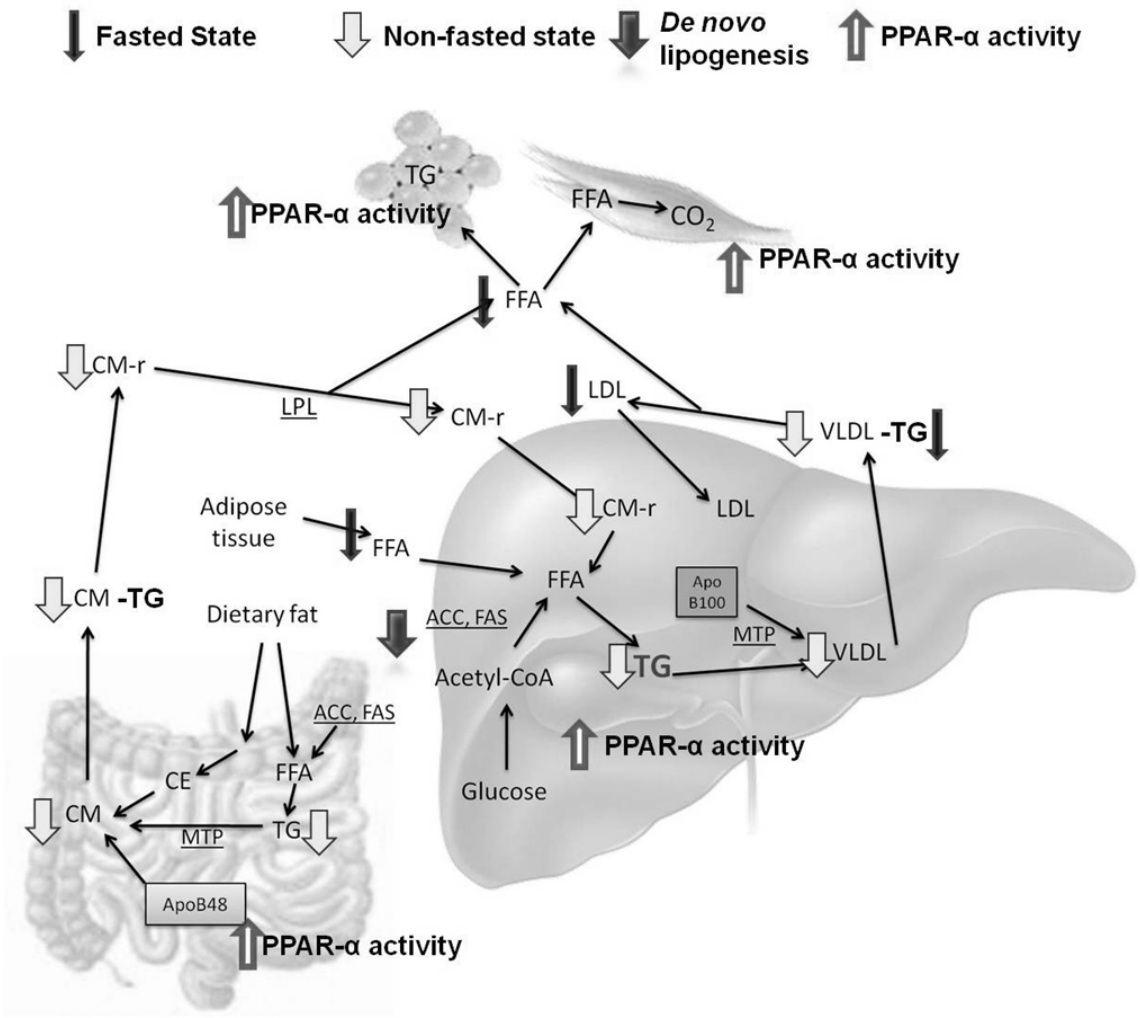

Figure 7-1. Schematic of the proposed mechanism by which VA could modulate lipid metabolism in JCR:LA-*cp* rats. Under conditions of MetS and dyslipidemia, VA reduces the secretion of CM from the intestine, accelerates CM-r clearance by the liver, reduces hepatic de novo lipogenesis and VLDL assembly, which collectively contributes to improvement in post-prandial lipaemia in obese JCR:LA-*cp* rats. The substantial hypolipidemic benefits of VA may partially be attributed to an upregulation of PPAR-α mediated fatty acid and energy metabolism.

7.6 Study limitations and future directions

It is appreciated that there are unresolved issues that may restrain the rapid translation of this theses findings to industrial or government action to change legislation around rTFA in the food supply. A time-dependent effect of VA has been demonstrated, acute verses chronic, however it still remains unclear what is the lowest or most effective dose of purified VA for a dietary intervention. So far the estimation of the average dietary VA consumption in human populations has only been reported in a limited number of case-control studies, which varies considerably from 280 mg/day to 800 mg/day (less than 0.5% energy based on a 2000 kcal/day diet) depending on the geographical location where the studies were conducted (Aro *et al.* 2000, Voorrips *et al.* 2002). Despite the accuracy of the methods used in estimating VA dietary intake, it certainly falls far below the pharmacological doses used in this studies (2.1% of energy), as well as in other animal or cell culture studies (3-5% of energy), even after accounting for differential bioavailability between synthetic and natural sources. Since it is unclear how long it may take for the food industry to commercialize rTFA enriched ruminant products, it would be efficient to first assess the dose response profile under conditions of MetS. After the differential bioavailability between natural and synthetic VA is evaluated in the MetS population, a well-controlled randomized clinical trial with VA supplementation (from purified synthetic source and rTFA-enriched dairy fat) at a consumption level achievable to humans could provide more valuable information to facilitate the implementation of this nutraceutical application.

In the animal feeding studies stated in chapters 4 and 5, synthetic VA was implemented into the diet by replacing a proportion of oleic acid in the control diet so as to maintain the total fat intake and PUFA:SFA ratio. Diet rich in cis-MUFA (predominantly oleic acid) has been shown to reduce plasma cholesterol and MetS risk in several clinical trials (Garg *et al.* 1998, Due *et al.* 2008, Jebb *et al.* 2010). It may be argued that oleic acid as the control fatty acid may pose a greater risk of a “false negative” outcome for VA’s bioactivity; nevertheless, the animal feeding trials (Chapters 4 and 5) and PPAR-α ligand binding assays (Chapter 6) accordingly suggest that VA may have greater lipid-lowering capacity compared to oleic acid. It is also noteworthy that the oleic acid content in both

control and VA diets was relatively low (17% and 9% w/w of total fat, respectively) compared to average human consumption (30% w/w of total fat) whereas the amount of stearic acid was considerably higher (approximately 50% greater than average human consumption). Caution needs to be applied when interpreting findings from this thesis in the context of human population. VA supplementation might lead to different outcomes when there are substantial changes in the composition of dietary lipids.

Another limitation of this thesis is inherited from the rodent model used in this study. The JCR:LA-*cp* rats is unique in that it spontaneously displays symptoms such as obesity, insulin resistance and hyperlipidemia that are characteristics for MetS and dyslipidemia in humans (Clark *et al.* 2000, Russell *et al.* 2006). However, the complete defect of leptin receptor signaling pathway in obese JCR rats makes it difficult to study the interaction between VA and leptin metabolism under the disease conditions.

The bioactivity and health implications of VA *per se* have just started to be appreciated during the past few years. However, an essential part of the metabolic pathways VA may participate in or modulate is still unknown. The results shown in this thesis have indicated the extensive involvement of VA in lipid metabolism and inflammatory regulations. Therefore, it will be worthwhile to assess the interaction between VA and other key nuclear receptors such as PPAR-γ, PPAR-δ and liver X receptor (LXR), as well as their respective target genes acting as key regulators. *In-vitro* studies using primary and/or secondary cultures of cells representing different tissues (e.g. hepatocytes, enterocytes, macrophages, smooth muscle cells, adipocytes, etc.) may be particularly useful to study the effect of VA on various metabolic pathways in different tissue. For instance, the rate of fatty acid synthesis/oxidation and lipoprotein synthesis/secretion could be traced using radiolabeled substrates. The cellular sub-localization of various transporters, rate-limiting enzymes and transcription factors could be visualized and quantified in these cell culture models. In terms of CVD end points, it has been shown that there was a substantial reduction in atherogenic lipid profile (e.g. fasting and post-prandial TG, CM/CM-r and LDL) after prolonged VA treatment, but the effect of VA on vasculopathy progression has not yet been assessed in this animal model. Further histological analysis on

atherosclerotic, myocardial and glomerular sclerotic lesions may offer direct support to the proposed anti-atherogenic effect of VA.

7.7 Conclusion

The research reported in this thesis has conclusively demonstrated a substantial hypolipidemic effect of VA on CVD risk factors in the JCR:LA-*cp* rodent model of the MetS. The findings also present the first evidence of the putative mechanisms in support of the hypolipidemic properties of dietary VA supplementation. It is essential to note that these studies have provided some insight into the actions of VA given the void of literature with regard to the bioactivity of this natural *trans* fat. Although future clinical studies are required, there is potential particularly during conditions of dyslipidemia and metabolic syndrome, that VA supplementation via dietary enriched foods or nutraceutical purified forms may benefit individuals at high risk of developing CVD and type-2 diabetes.

7.8 Literature Cited

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