CONJUGATED LINOLEIC ACID (CLA) IN LIVER AND ADIPOSE METABOLISM:
Effects on insulin resistance, adiposity, lipidemia and hepatic steatosis in *fa/fa* and lean Zucker rats

By

Amy Dianne Noto

A thesis submitted to the Department of Human Nutritional Sciences
In partial fulfillment of the requirements for the degree of
Master of Science

Department of Human Nutritional Sciences
University of Manitoba
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THE UNIVERSITY OF MANITOBA

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ABSTRACT

CONJUGATED LINOLEIC ACID (CLA) IN LIVER AND ADIPOSE METABOLISM:

Effects on insulin resistance, adiposity, lipidemia and hepatic steatosis in *fa/fa* and lean Zucker rats

Amy Noto, MSc. Thesis, Department of Human Nutritional Sciences

Insulin resistance, obesity and non-alcoholic fatty liver disease are strongly interrelated conditions. In insulin-resistant rat models, dietary CLA improves insulin sensitivity and reduces lipid accumulation in adipose and liver tissue, however, contrasting data exists in other models.

It is hypothesized that CLA may act through peroxisome proliferator-activated receptor (PPAR)-, sterol regulatory binding element (SREBP)-, and/or adipocytokine-mediated mechanisms. The objective of the study presented in this thesis was to determine the effects of an eight week intervention with CLA diet (1.5% CLA + 7% soybean oil) versus control diet (8.5% soybean oil) in six-week old *fa/fa* and lean Zucker rats with regard to insulin sensitivity, adiposity, adipose fatty acid composition, circulating adipocytokines, lipidemia, hepatic steatosis, liver fatty acid composition, liver fatty acid oxidation (acyl coA oxidase; ACO mRNA) and synthesis (acetyl coA carboxylase; ACC mRNA), and liver function.

As expected, only the *fa/fa* genotype displayed obesity, hyperleptinemia, insulin resistance, hepatic steatosis and hyperlipidemia. The *fa/fa* genotype had different fatty acid profiles in liver and adipose triacylglycerols and phospholipids that suggested reduced fatty acid elongation compared to the lean genotype. Additionally, the *fa/fa*

genotype had greater fasting serum haptoglobin and alanine aminotransferase compared to the lean genotype.

Within the *fa/fa* genotype, CLA-fed rats displayed greater visceral adiposity, attenuated hyperleptinemia, improved insulin sensitivity and ameliorated hepatic steatosis compared to the control-fed rats. Concomitant with these observations CLA-fed *fa/fa* rats had altered fatty acid profiles in adipose and liver triacylglycerols and liver phospholipids but not in adipose phospholipids, suggesting altered expression and/or activity of desaturation, elongation and/or peroxisomal β-oxidation enzymes in these tissues. The CLA-fed *fa/fa* rats also had lower fasting serum haptoglobin and alanine aminotransferase suggesting reduced inflammation and improved liver function, respectively, compared to the control-fed *fa/fa* rats.

The CLA diet elevated fasting serum adiponectin in both *fa/fa* and lean rats. CLA-feeding did not elevate hepatic ACO mRNA (a PPAR-controlled gene) or attenuate liver ACC mRNA levels (a SREBP controlled gene).

Together, these results confirm characteristics previously described in the *fa/fa* Zucker rat and in particular, indicated acute phase inflammation and deranged liver function in these animals compared to the lean genotype. Metabolic derangements related to adipose and liver function were improved in *fa/fa* Zucker rats with CLA supplementation.

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Acyl-CoA Oxidase	ACO
Acetyl-CoA Carboxylase	ACC
Adipocyte specific protein 2	aP2
Alanine aminotransferase	ALT
Area under the curve	AUC
Area under the curve glucose	AUCg
Area under the curve insulin	AUCi
ATP citrate lyase	ATP-CL
Body Mass Index	BMI
Carnitine Palmitoyltransferase	CPT
CCAT/enhancer binding protein	C/EBP
Cis	c
Conjugated Linoleic Acid	CLA
Cyclooxygenase	COX
Cytochrome P450 4A	CYP4A1
Delta 9	Δ^9
Delta 6	Δ^6
Delta 5	Δ^5
Diabetes Mellitus - Type 2	DM-2
Fatty Acid Binding Protein	FABP
Fatty Acid Synthase	FAS
Fatty Acyl-CoA Synthetase	FACS
Glucose transporter	GLUT
Glycerol-3-Phosphate Acyltransferase	G3P-AT
Non-Alcoholic Fatty Liver Disease	NAFLD
Non-Alcoholic Steatohepatitis	NASH
Lipoprotein Lipase	LPL
Lipoxygenase	LPOX
Liver-X-Activated Receptor	LXR
Prostaglandin	PG
Peroxisome Proliferator-Activated Receptor	PPAR
Peroxisome Proliferator Response Element	PPRE

Polyunsaturated Fatty Acid	PUFA
Phospholipid	PL
Phosphoenolpyruvate	PEP
Radioimmunoassay	RIA
Retinoid X Receptor	RXR
Reverse transcriptase polymer chain reaction	RT-PCR
Saturated fatty acid	SFA
Stearoyl CoA Desaturase	SCD
Sterol Regulatory Element-Binding Protein	SREBP
Trans	t
Triacylglycerol	TAG
Uncoupling Protein	UCP
Very low lipodensity lipoprotein	VLDL
World Health Organization	WHO
Zucker Diabetic Fatty	ZDF

I. LITERATURE REVIEW

Metabolic syndrome and nonalcoholic fatty liver disease

The metabolic syndrome is defined by the World Health Organization (WHO) as insulin resistance, impaired fasting glucose (≥6.1 mmol/L), hypertension (≥ 160/90 mm Hg), hypertriglyceridemia (≥ 1.7 mmol/L), low HDL-cholesterol (< 0.9 and 1.0 mmol/L in men and women, respectively), central obesity and/or microalbuminuria (albumin excretion rate ≥20 μg/minute or albumin:creatinine ≥20mg·g). According to the WHO definition, people with insulin resistance but normal glucose tolerance and at least two of these characteristics have the metabolic syndrome. Individuals with impaired fasting glucose or impaired glucose tolerance have the metabolic syndrome if two characteristics are present. Insulin resistance is defined as glucose uptake below the lowest quartile for the background population under investigation under hyperinsulinemic, euglycemic conditions (Alberti & Zimmel, 1998).

Diabetes Mellitus Type 2 (DM-2), a condition that accounts for 90% of all diabetes cases, occurs when target cells in the body cannot sufficiently take up glucose and hyperglycemia occurs. Eventually there may be impaired β-cell function. Insulin resistance may be due to low numbers and/or low binding affinity of insulin receptors themselves or due to intracellular post receptor defects (reviewed in Le Roith & Zick, 2001). People with DM-2 can also be characterized as having the metabolic syndrome.

Obesity is defined as a body mass index (BMI) of greater than 30 kg/m^2 due to excessive accumulation of adipose tissue or a waist: hip >0.9 and >0.85 in men and women, respectively (WHO, 1998). Obesity, specifically abdominal obesity, is associated with insulin resistance and low insulin sensitivity is a strong predictor of

weight gain (Ravussin & Gautier, 1999). Obesity and metabolic inabilities related to insulin may possibly aggravate each other (Groop, 2000). Obesity is the greatest risk factor for DM-2, implicated in the development of over 75% of cases (Shils et al, 1999).

In the same regard, obesity and DM-2 are risk factors for Nonalcoholic Fatty Liver Disease (NAFLD). NAFLD is a disease spectrum that begins with hepatic steatosis and with oxidative stress may progress to nonalcoholic steatohepatitis (NASH), cirrhosis and end-stage liver disease (Youseff & McCullough, 2002). The risk of NASH progressing to cirrhosis is approximately 20% over 10-20 years (Younossi, Diehl & Ong, 2002). NAFLD is prevalent in an estimated 63% of people with DM-2 (Kemmer et al., 2001). In fact, NAFLD may be the most prevalent form of liver disease in our society. For example, it affects 15 to 39% of people in the general population in the United States (reviewed in Younossi, Diehl & Ong, 2002). It has been proposed that hepatic steatosis be a feature of the metabolic syndrome (Marceau et al., 1999). The exact etiology of NAFLD is not known, however, it is associated with insulin resistance, obesity and abnormal lipid metabolism. Hepatic steatosis, the initial stage of NAFLD, is defined as excess accumulation of lipid, specifically triacylglycerol (TAG) in the liver, which may lead to degenerative changes in liver cells (McCullough, 2002; Thomas & Venes, 1997).

The prevalence of obesity and DM-2 are increasing in many populations throughout the world and in populations that were previously not at high risk for the disease (e.g. young people). The WHO estimates that by 2025 approximately 300 million people will be obese and the number of people with diabetes will double, also reaching about 300 million people worldwide (WHO, 1998). It has been estimated that 65-75% of diabetes would not occur if BMIs were less than 25 kg/m^2 (Seidell, 1998). In Canada,

approximately 2 million people have diabetes and by 2010 it may be 3 million (CDA, 2001). Given that hepatic steatosis is strongly associated with insulin resistance and obesity, it is reasonable to hypothesize that NAFLD will also become an increasingly significant cause of morbidity.

The stunning rate of obesity and DM-2 can in part, be attributed to environmental factors. People are increasingly consuming more energy and expending less. There is also a genetic component. For example, twin studies suggest that genetics contributes to up to two-thirds of BMI variability (Ravussin & Bogardus, 2000). Currently there are a number of genes being investigated for their role in obesity, DM-2 and NAFLD. Gene-nutrient interactions may play an important part in the pathophysiology of these conditions. These interactions will be explored further in this thesis, by using conjugated linoleic acid (CLA) as an example of a group of nutrients that may directly affect gene transcription.

The purpose of the following literature review is to investigate current knowledge surrounding CLA, as a group of dietary factors that play a role in the regulation of characteristics of the metabolic syndrome (e.g. insulin resistance, obesity, dyslipidemia) and NAFLD.

Definition, structure and sources of CLA

CLA is the collective name for a group of positional (carbon 7 to 12) and geometric (cis-cis, cis-trans, trans-cis and trans-trans) isomers derived from linoleic acid (C18:2 n-6). These isomers contain a conjugated double bond system, while linoleic acid has a methylene group between its two double bonds (Pariza, 1997). The major isomers include cis-9,trans-11 (c9, t11; also called rumenic acid); t9,c11; t9,t11; t10,t12; and

t10,c12 octadecadienoic acids. The minor isomers include t7,c9; c9,c11; c10,c12; c10,t12; and c11,c13 octadecadienoic acids (Lin et al., 1995; Yurawecz et al., 1998).

Dietary sources of CLA include those from ruminant meats (cow, sheep, goat), ruminant milk, dairy products (yogurt, cheese, butter) and human breast milk (reviewed in McGuire et al, 1999). A survey of CLA in dairy products by Lin et al. (1995) revealed that the CLA content of cheeses ranged between 3.59 to 7.96 mg CLA/g lipids. Fermented dairy products contained 3.82 to 4.66 mg CLA/g lipid. Fluid milk contained 3.38-6.39 mg CLA/g lipid. Seasonal and geographical variations in the CLA content of milk have been reported (Banni, et al., 1996). For example, the CLA concentrations in cow's milk is higher in spring and summer when cows are more likely to be pasture-fed, thus receiving better dietary sources of polyunsaturated fatty acids (Jahreis & Kraft, 2002).

More than 80% of the CLA found in foods is the c9,t11 isomer (Lin, et al., 1995). Mean dietary intakes of CLA by humans worldwide have variably been estimated to be between 15 and 1000 mg per day (McGuire et al., 1999). In a study of young Canadians, intake of c9,t11-CLA ranged between 15 to 174 mg per day (Ens et al., 2001).

Ruminant animals produce CLA from dietary linoleic acid in the rumen via microbial isomerization and incomplete biohydrogenation or desaturation. The pathway of CLA production in the rumen is shown in Figure 1. One microorganism that is capable of isomerizing linoleic acid to CLA is the gram negative *Butyrivibrio fibrisolvens* (Kepler, et al, 1966). Some CLA that is formed does not undergo complete biohydrogenation and can be released in milk or stored (Kelly, et al, 1998). The CLA that is biohydrogenated is converted to vaccenic or trans-11 octadeconoic acid, which

after absorption can be converted back to CLA by delta-9 (Δ^9) desaturase (Griinari et al., 1998).

For humans, the main source of CLA is dietary. However an additional, although minor, source of CLA is from the endogenous synthesis of CLA from the trans-vaccenic acid (found in ruminant fats) by microsomal Δ^9 desaturase. Vaccenic acid is not absorbed readily and is oxidized easily. It has been estimated that the percent conversion of dietary and stored trans-vaccenic acid to c9, t11 CLA is 11.4% and 50.8%, respectively (Palmquist & Santora, 1999).

CLA can be chemically synthesized and many experimental studies of CLA use its chemically synthesized form. The final concentration of CLA in a synthesized product depends on the amount of linoleic acid in the starting oil. Starting oils are first crystallized at –75 °C to separate saturated fats and oleic acid from linoleic acid. CLA can then be produced from a high linoleic starting oil by alkali isomerization. This involves combining propylene glycol with a strong alkali base (e.g. KOH) at 130-180°C, followed by acidification with sulfuric or hydrochloric acid and purification by distillation (Reany, et al., 1999; Saebo, 2003). The addition of acid results in separation of fatty acids from the glycerol backbone of triacylglycerol (TAG) in the starting oil (Saebo, 2003). Therefore, many of the CLA oils used in research provide CLA as free fatty acids (FFA), whereas naturally occurring sources of CLA are part of TAGs. More recently procedures using very low levels of catalysts and lower reaction temperature have been developed that allow for the majority of the final CLA oil to remain in TAG form. As well, CLA as FFA can be esterified with glycerol in the presence of a non-specific lipase to produce TAG (Saebo, 2003). Synthetic CLA oils are usually a mixture

of primarily two isomers (approximately 40% of each c9,t11 and t10,c12 CLA) or four isomers (approximately 30% of each c9, t11 and t10, c12, plus 15% of each t8,c10 and c11,t13 CLA). Recently, researchers have been investigating the biological effects of the c9,t11 and t10,c12 isomers independently. In this literature review, studies using CLA will refer to the chemically synthesized mixture, unless it is indicated that one isomer was examined independently. Published studies generally do not provide sufficient compositional analysis data to distinguish between the 'two' or 'four' isomer mixtures.

Metabolism of CLA

In rats, c9,t11 and t10,c12 CLA, as part of TAGs, are absorbed equally as well and comparably to linoleic acid, but the c9,t11 isomer seems to be released into absorbable form more quickly than the t10,c12 isomer (Martin et al., 2000). Following absorption, in animals and people, CLA can either go through Δ6 desaturation and elongation, oxidation or be stored in its original or metabolized form (Sebedio et al., 2003).

Most stored CLA appears to accumulate in the TAG portion of cells but the storage of CLA is species and tissue specific (Banni et al., 1999). For example, CLA incorporation in rat liver was about 79% into TAGs and 10% into phospholipids (PL). The accumulation of the c9,t11 isomer was greater than the t10,c12 isomer in rat liver PL (Belury & Kempa-Steczko, 1997). In rats, trans-cis and cis-trans isomers occured equally in each of sn-1, sn-2 and sn-3 positions of TAGs. The trans-trans isomers occured more in the sn-1 and sn-3 positions (Sugano et al., 1997). Both c9,t11 and t10,c12 CLA may be oxidized in the body significantly more than linoleic acid (Sergiel et al., 2001).

The metabolic effects of CLA may partially be attributed to it being a ligand for transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and sterol regulatory binding proteins (SREBPs).

Nutritional regulation of transcription

a) Peroxisome proliferator-activated receptors

PPARs are ligand-activated transcription factors of the receptor family that includes the steroid, retinoid and thyroid hormone receptor family. They function as heterodimers with the retinoid X receptor (RXR) (Latruffe & Vamecq, 1997). Transcription factors are proteins that recognize specific nucleotide sequences present in the promoter region of genes and induce transcription. Transcription is one of the major points of gene expression amenable to regulation (Shils et al., 1999). There are three main types of PPARs in mammals – PPARα, PPARγ and PPARβ/δ. PPARα is expressed mainly in the liver. PPARγ, specifically the PPARγ2 form is found mainly in adipose, while PPARγ1 is found in many tissues. PPARβ/δ is ubiquitously distributed in the body (Khan & Vanden Heuvel, 2003).

As a transcription factor, the PPAR-RXR heterodimer binds to peroxisome proliferator response elements (PPREs) that are contained in the promoter regions of a number of genes related to lipid metabolism (Latruffe & Vamecq, 1997). Genes may have multiple response elements. Gene products related to fatty acid metabolism that contain PPREs include carnitine palmitoyltransferase (CPT), acyl CoA oxidase (ACO), fatty acyl coA synthetase (FACS), mitochondrial HMG-CoA synthetase, lipoprotein lipase (LPL), fatty acid binding protein (FABP), cytochrome P450 4A (CYP4A1) and uncoupling proteins (UCP-1 and UCP-3; reviewed in Clarke, 2000).

It has been demonstrated that CLA is a ligand and activator of PPARα through scintillation proximity assay and chimeric protein assay, respectively. The c9,t11 isomer has the greatest affinity for PPARα, followed by t10,c12 (Moya-Camarena et al., 1999; Clement et al., 2002). It has also been demonstrated that CLA is a ligand for PPARγ (Belury & Vanden-Heuvel, 1999), although Clement et al. (2002) have reported that PPARγ activation by CLA is not different between c9,t11- and t10,c12-CLA. CLA may have a high affinity for PPARs, probably due to its unique positional double bond system and large hydrophobic region that may favor conformational change (Moya-Camarena et al., 1999). Other fatty acids such as oleic, linoleic, linolenic and arachidonic, and eicosanoids (products of 20 carbon fatty acid metabolism) such as prostaglandins (PG; e.g. PGJ_2) are also ligands for PPARs. The degree of activation of PPARs may rely on a variety of ligands (Kliewer et al., 1997).

b) Sterol regulatory-element binding proteins

SREBPs are also transcription factors that control genes related to fatty acid synthesis. They are located on the endoplasmic reticulum and the nuclear envelope of cells and translocate to the nucleus to up-regulate the expression of several cholesterol and fatty acid synthesis enzymes including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl CoA desaturase (SCD, isoform of delta-9 desaturase), glycerol-3-phosphate acyltransferase (G3P-AT) and ATP citrate lyase (ATP-CL). Figure 2 shows enzymes involved in cytoplasmic fatty acid synthesis. Figure 3 shows enzymes involved in fatty acid elongation. SREBP-1c and SREBP-2 are also involved in the synthesis of NADPH, which is needed throughout lipogenesis (Horton et al., 2002).

There is also evidence that elevated SREBP-1c expression plays a key role in fatty liver, as well as, hyperinsulinemia and hyperglycemia. Both ob/ob mice (model of DM-2) and ap2-nSREBP-1c mice (mice with high SREBP-1c and aP2 expression in adipose that display lipodystrophy) have hyperglycemia, hyperinsulinemia and fatty liver. Liver X-activated receptor-α (LXRα) is a transcription factors regulating SREBP-1c (Roche et al., 2002; Horton et. al., 2002). LXR-α is a nuclear hormone receptor that requires exogenous ligands and influences hepatic SREBP-1c (DeBose-Boyd et al., 2001). PUFA in general inhibit the activation of LXR ligands (Ou et al., 2002).

In summary, activators of PPARα may act to improve lipid and carbohydrate metabolism in the liver and activators of PPARγ may act to improve insulin sensitivity, glucose usage and enhance TAG synthesis in peripheral tissues such as adipose (Khan & Vanden Heuvel, 2003). Suppressors of SREBPs, such as PUFA, decrease the transcription of fatty acid synthesis and desaturase enzymes. The relationship of CLA to PPARs and SREBPs will be discussed throughout the following sections.

Effects of CLA on insulin resistance, adiposity, lipidemia and liver metabolism

The two major peripheral target tissues of insulin action are adipose and muscle. Normally, insulin acts to promote glucose and free fatty acid uptake into cells via glucose transporter-4 (GLUT4) and LPL, respectively. Inside the cell, insulin also down regulates hormone sensitive lipase, inhibiting the release of FFA. Adipocyte insulin resistance leads to elevated fatty acid lipolysis and reduced fatty acid esterification resulting in a diversion of fat to other tissues, including the liver. Therefore, with insulin resistance, cells cannot effectively take up glucose, and in particular, adipocytes cannot

efficiently store TAG, resulting in disordered lipid metabolism (e.g. hyperlipidemia, hepatic steatosis; Lewis et al., 2002).

The fatty acid composition of adipocyte membrane PLs may also be an important factor in peripheral insulin resistance. Greater polyunsaturated fatty acid (PUFA) content and lesser saturated fatty acid (SFA) content in PL results in enhanced membrane fluidity and greater glucose uptake, possibly due to changes in insulin binding and/or signaling (Clarke, 2000; Storlein, 1996).

Peripheral insulin resistance has implications for liver physiology, specifically lipid accumulation. Case in point, the incidence NAFLD is higher in people with DM-2/insulin resistance (discussed above). With excess lipid in the liver, fat storage is promoted and very low density lipoprotein (VLDL) production is elevated, resulting in elevated serum TAG concentrations. However, there are limits to VLDL production (e.g. limits on apoB-100) and TAG can accumulate in the liver. Therefore, improving peripheral insulin resistance can decrease the progression of hepatic steatosis.

a) CLA and insulin resistance

The effects of CLA on circulating glucose, insulin and glucose or insulin tolerance are shown in Table 1. Low glucose tolerance reflects a state of poor insulin use by target cells. CLA feeding has led to lower circulating glucose and insulin, and improved glucose tolerance in male Zucker Diabetic Fatty (ZDF) rats (obese, insulin resistant and hyperglycemic model) compared to control-fed rats (Housekecht et al., 1998; Ryder et al., 2001; Nagao et al., 2003). This may be due to the t10,c12 isomer, as feeding enriched c9,t11 butter to male ZDF rats had no effect on circulating glucose, insulin or glucose tolerance, compared to control rats (Ryder et al., 2001). Additionally,

Henricksen et al. (2003), have shown that t10,c12 was effective in lowering circulating glucose and insulin, and the t10,c12 and CLA mixtures improved insulin sensitivity, compared to control-fed female *fa/fa* Zucker rats (obese, insulin resistance, but not hyperglycemic model). CLA has also reduced fasting serum insulin, compared to controls, in male OLETF rats (obese, insulin resistant model) with two percent enrichment, but not one percent enrichment (Wang et al., 2003). However, in normal-weight, Sprague-Dawley rats, CLA does not exhibit an effect on fasting serum insulin and the t10,c12 actually increases fasting serum glucose, compared to control-fed rats (Akahoshi et al., 2003).

CLA reduces insulin resistance in insulin-resistant rats, but the opposite is seen in various mouse models. In mice, but not rats, CLA causes lipodystrophy (discussed below) and insulin resistance. CLA feeding increased circulating glucose, insulin and insulin resistance in C57BL/6J (obese, insulin resistant model) and high-metabolic rate mice (Tsuboyama-Kasaoka et al., 2000; Hargrave et al., 2003). However, one study has shown that this effect is normalized with feeding a high-fat (34%) diet (Tsyboyama-Kasaoka et al., 2003). The greater insulin resistance appears to be due to the t10,c12 isomer in C57BL/6J mice and ob/ob (obese model) mice (Clement et al., 2002; Roche et al., 2002).

In human trials, CLA supplementation had no effect on circulating plasma glucose, but compared to placebo decreased fasting insulin and improved insulin sensitivity in sedentary healthy adults (Eyjolfson et al., 2004). However, in men with the metabolic syndrome, t10,c12-CLA increased insulin resistance, based on the euglycemic clamp technique (Riserus et al., 2002b).

Insulin resistance is inter-related with adiposity as shown in mouse models. Research about CLA and adiposity may further elucidate the relationship between CLA and insulin resistance.

b) CLA and adiposity

The effects of CLA on circulating leptin, circulating adiponectin and adiposity are detailed in Table 2. CLA feeding reduced adipose fat pad weights in various animal models including male and female Sprague-Dawley rats (Azain et al., 2000; Yamasaki et al., 2003; Akahoshi et al., 2004; Koba et al., 2002; Ealey et al., 2002), male lean Zucker rats (Sisk et al., 2001), male ZDF rats (Ryder et al., 2001), male OLETF rats (Wang et al., 2003; Rahman et al., 2001; Nagao et al., 2003b), female Wistar rats (Mir et al., 2003; Czauderna et al., 2003), female C57BL/J mice (Tsuboyama-Kasaoka et al., 2000 & 2003; Ealey et al., 2002), PPARα-null mice (Peters et al, 2001), male Balb-C mice (Terpstra et al., 2002), male ICR mice (Akahoshi et al., 2002), male SCD (+/+ and -/-) mice (Kang et al., 2004) and female large White x Landrace pigs (Ostrowska et al., 2003a & 2003b), compared to control-fed animals. This effect was specific to the t10,c12 isomer in Sprague-Dawley rats (Akahoshi et al., 2003; Hargrave et al., 2002), female *fa/fa* Zucker rats (Henricksen et al., 2003), C57BL/6J mice (Clement et al., 2002; Warren et al., 2003; Degrace et al., 2004), ob/ob mice (Roche et al., 2002), ICR mice (Chardigny et al, 2003) and hamsters (Navarro et al., 2003).

However, CLA mixtures had no effect on white adipose tissue weight in hamsters (Bouthegourd et al., 2002) and epididymal adipose weight and brown adipose weight in Sprague-Dawley rats (Akahoshi et al., 2004) and actually increased retroperitoneal adipose weight in *fa/fa* Zucker rats (Sisk et al., 2001).

In randomized, placebo controlled, human trials, CLA decreased body fat mass in healthy men and women after 12 weeks (Blankson et al., 2000) and overweight men and women after 13 weeks (Kamphuis et al., 2003) and one year of supplementation (Gaullier et al., 2004). Others have shown no effect of CLA, compared to placebo, on fat mass in healthy men and women (Zambell et al., 2000; Mougios et al., 2001; Smedman et al., 2001; Petriou et al., 2003) and men with the metabolic syndrome (Riserus et al., 2002a & 2002b). In men with the metabolic syndrome, those within a t10c12-CLA treatment group showed reduced weight, BMI, waist girth, body fat and lean body mass compared to baseline (Riserus et al., 2002b). Additionally, Belury et al. (2003) showed an inverse negative correlation between plasma t10,c12 CLA and body weight in people with DM-2.

The effects of CLA on adipose mass may be explained in part by its relationship to PPARγ. PPARγ induces adipogenesis and maintains genes needed for mature adipocytes. The PPARγ-RXR heterodimer is enhanced by CCAT/enhancer binding protein-α (C/EBPα), and is needed for the differentiation of preadipocytes to adipocytes by regulating the gene, adipocyte fatty acid binding protein 2 (aP2), which in turn increases fatty acid storage and improves insulin sensitization. PPARγ agonists act in adipose tissue to elevate phosphoenolpyruvate (PEP) carboxykinase and LPL for TAG synthesis and elevate GLUT 4 expression, improving glucose use (Dana et al., 2001). Tamori et al (2002), found that PPARγ regulated free fatty acid uptake and TAG accumulation in mature 3T3-L1 adipocytes by controlling the expression of genes for insulin receptor, insulin receptor substrate and GLUT4. PPARγ may also act peripherally to regulate leptin (Unger et al., 1999). The functions of leptin are discussed below.

In ZDF rats, CLA improved glucose tolerance and elevated adipose aP2 mRNA expression, similarily to troglitazone - a known PPARγ agonist (Houseknecht et al., 1998). There may be an important difference related to the storage capacity of adipocytes between normal weight and obese models. For example, obese models of mice have lower expression of genes related to differentiation (e.g. PPARγ2, C/EBPα) and fatty acid synthesis (e.g. FAS; reviewed in Nadler & Attie, 2001). These differences lead to a lack of functional adipocytes in obesity and may partially explain why CLA improves insulin sensitivity and lipid metabolism, through increasing adipocyte differentiation in ZDF rats. To further support this idea, CLA feeding in C57BL/6J mice decreased adipose PPARγ mRNA expression, concomitant with inducing insulin resistance (Tsuboyama-Kasaoka et al., 2000). Additionally, in human stromal vascular cells containing newly differentiated adipocytes, t10,c12 (but not c9,t11) treatment decreased TAG content, and insulin stimulated glucose and fatty acid fatty acid uptake, at the same time as reducing PPARγ and aP2 mRNA, and increasing leptin mRNA expression (Brown et al., 2004; Brown et al., 2003; Granlund et al., 2003). Thus, CLA has the ability to regulate adipocyte differentiation/functionality – seemingly to improve functionality in rats and interfere with functionality in mice and human stromal vascular cell culture. Adipose tissue mass and functionality also have important implications for the body's capacity to release adipocytokines, which have important functions in metabolism.

c) CLA and circulating adipocytokines

Adipose tissue can be classified as an endocrine organ. The term adipocytokines refers to proteins that are released by adipocytes (Hoist & Grimaldi, 2002). Examples of adipocytokines are leptin, adiponectin and haptoglobin.

Leptin is produced by the ob gene. Leptin receptors are dense in the hypothalamus and hippocampus and leptin acts to suppress appetite, increase energy expenditure and decrease adipogenesis. Circulating leptin is increased in obesity, due to cellular resistance. Insulin may positively modulate leptin secretion but leptin does not appear to affect insulin secretion (reviewed in Hoist & Grimaldi, 2002). The effects of CLA on fatty liver may be due, in part, to interactions with leptin. Recently, hepatic steatosis has been linked with leptin deficiency in mice (Brix et al., 2002) and leptin resistance in people (Younossi, 2002), despite observations that fatty acid oxidation is not down-regulated in leptin-deficient mice (Brix et al., 1999).

CLA feeding, compared to controls, has reduced fasting circulating leptin in male ZDF rats fed high fat diet (Ryder et al., 2001), male OLETF rats (Wang et a., 2003; Rahman et al., 2001), male Sprague-Dawley rats (Yamasaki et al., 2003), female C57BL/6J mice (Tsyboyama-Kasaoka et al., 2000) and male ICR mice (Akahoshi et al., 2004). Again, this effect was specific to t10,c12 CLA in C57BL/6J mice (Clement et al., 2002) in association with reduced adiposity. CLA feeding had no effect, compared to controls, on circulating leptin in male ZDF rats fed 6% fat diet (Nagao et al., 2003) and male Sprague-Dawley rats fed 0.1, 0.5 and 1.0% CLA (Akahoshi et al., 2004).

In placebo-controlled trials in humans, CLA had no effect on circulating leptin in healthy people (Medina et al., 2000; Guallier et al., 2004), sedentary women (Petridou et

al., 2003) and men with the metabolic syndrome (Riserus et al., 2002b). Belury et al. (2003) have shown an inverse negative relationship between plasma t10,c12 CLA and serum leptin in people with DM-2.

Adiponectin (also known as acrp30, adipoQ, amp1 and GBP28) is a newly identified adipocytokine that is associated with improved insulin sensitivity and glucose tolerance. In hepatocyte culture, adiponectin inhibits glucose production by decreasing the expression of PEP carboxykinase and glucose-6-phosphate. Its expression is decreased in DM-2 and obesity and increased in states of weight loss. Its expression and release is also increased by PPARγ activators (e.g. TZDs) and decreased by TNF-α (another adipocytokine that decreases insulin signaling leading to insulin resistance), glucocorticoids, β-adrenergic agonists and cyclic AMP (reviewed in Hoist & Grimaldi, 2002). Adiponectin may also function to increase β-oxidation in skeletal muscle and decrease accumulation of TAG in liver and muscle (Berg et al., 2002). There are limited reports on the effect of CLA on circulating adiponectin. Compared to controls, CLA feeding led to greater fasting circulating adiponectin in male ZDF rats and had no effect on fasting circulating adiponectin in male Sprague-Dawley rats (Akahoshi et al., 2004).

Haptoglobin is an acute phase reactant that is secreted from white adipose tissue and liver. The circulating concentrations of haptoglobin are elevated in diabetes and obesity (Schmidt et al., 1999; Engstrom et al., 2003). The mRNA expression of haptoglobin is elevated in the white adipose tissue of ob/ob and db/db mice (Chiellini et al., 2002). There are no reports on the effect of CLA on circulating haptoglobin.

d) CLA and lipidemia

The effects of CLA on lipidemia, including circulating FFA, TAG and cholesterol are detailed in Table 3.

CLA feeding, compared to controls, lowered circulating FFA in male ZDF rats (Houseknecht et al., 1998), *fa/fa* Zucker rats (Ryder el al., 2001), male OLETF rats (Rahman et al., 2001), male Sprague-Dawley rats (Yamasaki et al., 1999), male SCD1 (+/+ and -/-) mice (Kang et al., 2004) and male ob/ob mice (Roche et al., 2002). This effect was specific to the t10,c12 isomer in female *fa/fa* Zucker rats (Henricksen et al., 2003), but was not isomer specific in male C57BL/6J mice (Degrace et al., 2004) and was actually specific to the c9,t11 isomer in male ob/ob mice. Noone et al. (2002) showed that CLA had no effect on fasting plasma FFA in healthy adults.

CLA supplementation lowered circulating TAG in male *fa/fa* Zucker rats (Ryder et al., 2001), male OLETF rats (Rahman et al., 2001; Wang et al., 2003), male SCD1 (+/+ and -/-) mice (Kang et al., 2004), male C57BL/6J mice (Degrace et al., 2004) and male PPARα mice (Peters et al., 2001), compared to control-fed animals. This reduction in TAG was specific to the c9,t11 isomer in ob/ob mice.

In randomized, placebo controlled trials in humans, fasting plasma TAG was lower in a 50:50 c9,11:t10,c12 group compared to placebo, but not in a 80:20 c9,11:t10,c12, suggesting that t10,c12 lowers plasma TAG in healthy adults (Noone et al., 2002). However, t10,c12 CLA increased VLDL-TAG in men with the metabolic syndrome (Riserus et al., 2002b).

CLA feeding lowered circulating total and HDL-cholesterol in male OLETF rats (Rahman et al., 2002) and total cholesterol in male PPARα-null mice (Peters et al., 2001),

compared to control-fed animals. The t10,c12 isomer had no effect on total cholesterol but increased HDL-cholesterol in male Sprague-Dawley rats (Akahoshi et al., 2003) and decreased both total and LDL-cholesterol in hamsters (Navarro et al., 2003), compared to control-fed and c9,t11-CLA-fed animals.

In humans, randomized, placebo-controlled studies have shown that CLA does not have an effect on HDL- or LDL-cholesterol in healthy adults, but an 80:20 c9,t11:t12,c10 mixture lowered VLDL-cholesterol, while a 50:50 mixutre did not (Noone et al., 2002). Unfavorably, the t10,c12 isomer decreased plasma HDL-cholesterol in men with the metabolic syndrome, compared to placebo (Riserus et al., 2002b).

e) CLA and hepatic steatosis

Studies showing the effects of CLA on hepatic steatosis and liver function are detailed in Table 4. CLA, compared to controls, decreased hepatic liver lipid content in male OLETF rats (Wang et al., 2003; Rahman et al., 2002; Nagao et al., 2003), male Sprague-Dawley rats (Yamasaki et al., 1999) and male Wistar rats (Sebedio et al., 2001) but increased liver lipid content in various mouse models (Tsuboyama-Kasaoka et al., 2000 & 2003; Takahashi et al., 2003), an effect that seems to be specific to the t10,c12 CLA isomer (Clement et al., 2002; Chardigny et al., 2003; Warren et al., 2003; Kelley et al., 2004; Degrace et al., 2003; Degrace et al., 2004; Nakanishi et al., 2004).

Data from human trials on the effect of CLA on liver metabolism is scarce. CLA given in TAG form did not alter liver function tests including plasma alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in sedentary women (Petridou et al., 2003) and healthy adults (Gaullier et al., 2004). However, CLA given in free fatty acid form did increase circulating AST in healthy people, compared to placebo,

although not to a point indicating abnormal liver function (Gaullier et al., 2004). In NAFLD, mild to moderate elevation of plasma AST or ALT is usually the only biochemical abnormality present (Zafrani, 2004).

The effects of CLA on hepatic steatosis may be related to PPARα. PPARα regulates the transcription of many genes involved in lipid metabolism in the liver, including, LPL, FACS, thioesterases, FABP, fatty acid transport protein, fatty acid translocase, peroxisomal (e.g. ACO) and mitochondrial β-oxidizing enzymes (e.g. carnitine palmitoyltransferase; CPT), microsomal ώ-oxidizing enzymes, apoliproteins AI, AII and CIII, long chain acyl coA dehydrogenase (reviewed in Desvergne & Wahli, 1999; van Bilsen et al., 2002), Δ6 desaturase and Δ5 desaturase (Matsuzaka et al., 2002).

Figure 4 shows some of the enzymes involved in peroxisomal fatty acid β-oxidation. ACO is the rate-limiting enzyme in peroxisomal β-oxidation and its activity increases during peroxisome proliferation (Belury et al, 1997). PPARα is hyper-activated and very long chain fatty acids cannot be oxidized in ACO-deficient mice (Fan et al., 1998). Liver FABP is also peroxisomal and is involved with shuttling fatty acids to and from the plasma membrane. ACO (oxidation) and L-FABP (transport) mRNA expressions have been shown to be elevated by CLA. Both are elevated with CLA treatment in FaO rat hepatoma cell culture, presumably due to the activation of PPARα (Moya-Camarena et al., 1999). Liver ACO mRNA and protein expression were dose-dependently elevated in female SENCAR mice fed 0.5%, 1.0% and 1.5% CLA for 6 weeks (Belury et al., 1997). CLA-induced PPARα activation may control hepatic steatosis by increasing fatty acid oxidation and transport, thereby, decreasing the amount of fatty acids available that may contribute to oxidative damage and/or are available for

TAG synthesis. On the other hand, one hypothesis for the progression of hepatic steatosis to NASH, is that when the mitochondrial β-oxidation pathway is saturated, enzymes of the peroxisomal fatty acid oxidation pathway take over, leading to the generation of H_2O_2 and oxidative stress (Younossi, Diehl & Ong, 2002). CLA may not only act to increase fatty acid oxidation, but suppress fatty acid synthesis through the suppression of SREBP-1c. The c9,t11-CLA isomer (but not the t10,c12-CLA isomer) reduced the hepatic mRNA expression of SREBP-1c and LXRα in ob/ob mice (Roche et al., 2002). Suppression of SREBP-1c by CLA indicates less fatty acid synthesis in the liver.

Summary and limitations of published research

To summarize, CLA has a wide range of metabolic effects that are not consistent among models. The studies discussed used varying levels and durations of CLA supplementation. Results may have been influenced by this fact and by the form of the CLA used (e.g. FFA or TAG), the type of control fat used, and species-specific, strain-specific and/or age-related (e.g. growing or non-growing) factors. It may also be possible that the major isomers interact physiologically and when tested separately, exert different effects (Choi et al., 2000). Because of these difference, generalizations about the effects of CLA are difficult to make.

The literature clearly adds to the evidence that adiposity, insulin sensitivity and hepatic steatosis are closely related, however the mechanisms for CLA action requires elucidation. Several explanations may revolve around the alteration of lipid metabolism, which is different in insulin-resistant and non-insulin-resistant models. Firstly, the research does not clearly show if CLA supplementation affects the distribution of fatty

acids in liver and adipose TAG (storage), differently from PL, in insulin resistant models. Related to this, the research does not consider that CLA may improve peripheral insulin sensitivity by altering adipose PL fatty acid distribution. Secondly, the research shows that CLA up-regulates hepatic ACO (a PPAR-responsive gene involved in fatty acid oxidation) in vitro and in mice, and down-regulates hepatic ACC (a SREBP-1c responsive gene involved in fatty acid synthesis) in obese mice, but these effects should be confirmed in an insulin resistant rat model given the often species-specific effects of CLA. Thirdly, the literature surrounding the effect of CLA on circulating adiponectin in insulin resistant states is scarce. Finally, the literature does not address what the implications of hepatic steatosis are when, either induced or ameliorated by CLA, such as the case in mice and rats, respectively. It would be useful to understand the effect of CLA on liver function tests (e.g. circulating ALT) and inflammation (e.g. circulating haptogloblin) in a model with hepatic steatosis.

Using a model such as the *fa/fa* Zucker rat compared to lean littermates, may help to clarify the role of CLA in the pathology of insulin resistance and associated conditions, including obesity, dyslipidemia and hepatic steatosis for reasons described next.

Zucker Rat Model

In 1961, Lois and Thiodor Zucker discovered a subset of rats in their colony that showed hyperphagia, reduced energy expenditure, obesity and insulin resistance (Zucker & Zucker, 1961). The *fa/fa* Zucker rat was subsequently proven to be a carrier of the autosomal recessive *fa* gene. The *fa/fa* genotype manifests in leptin resistance caused by a mutation in the leptin receptor and thus the *fa/fa* Zucker rat has high circulating leptin concentrations, and dysregulation of food intake and energy output (Liu, et al., 2002). In

fa/fa Zucker rats, elevated leptin concentrations correspond with a developmental switch of the genes controlling growth and development. This occurs at 22 days of age and leads to hyperphagia. Obesity becomes apparent at approximately four weeks of age (Truett et al., 2000). By this time, *fa/fa* rats have 4 to 5 times more adipose tissue mass and greater fat cell size than lean littermates (Guerre-Millo, 1996). The *fa/fa* Zucker rats have reduced sympathetic activity to brown adipose tissue, reduced heat production, increased de novo fatty acid synthesis, elevated serum TAG, and increased glucose utilization and LPL activity in white adipose tissue (White & Martin, 1997).

White and Martin (1997) hypothesize that obesity in the *fa/fa* Zucker rats is the result of the relationship between leptin, neuropeptide Y, corticosterone and insulin (Figure 4). Neuropeptide Y is regulated by leptin. With the elevation in circulating leptin due to the mutation in the leptin receptor, there is an elevation in neuropeptide Y activity, causing elevations in circulating corticosterone and subsequently insulin. These effects lead to hyperphagia, reduced energy expenditure and obesity. With elevated adipose mass, more leptin is produced – aggravating the cycle.

The cause of insulin resistance in the *fa/fa* Zucker rat is yet to be elucidated. Liu et al. (2002) compared plasma concentrations of leptin, FFA and TNF-α in *fa/fa* and lean Zucker rats. They found that the *fa/fa* compared to lean rats had significantly elevated concentrations of all three circulating proteins when compared at six and 15 weeks of age. At six weeks of age, FFA concentrations were much higher than TNF-α concentrations. They concluded that FFA play a greater role in insulin resistance up to six weeks of age whereas FFA along with TNF-α act together to elevate insulin resistance after six weeks of age. Hyperinsulinemia in *fa/fa* Zucker rats is associated with elevated

expression of GLUT4 (2x), FAS (6x) and ACC (15x) in white adipose tissue and glucokinase (3x) in the liver (Assimacopoulos-Jeannet et al., 1995). The *fa/fa* Zucker rats also have three to four times more ACC activity in the liver leading to high hepatic lipid synthesis (Kim & Tae, 1994). For all of these reasons, the *fa/fa* Zucker rat can be considered a model of the prediabetic state with hepatic steatosis.

Table 1. Effects of CLA on circulating glucose, insulin and glucose or insulin tolerance: summary of in vivo studies by species

Reference	Model	Amount & type of CLA[a]	Total lipid (control)[a]	Length of study	Significant effects of CLA compared to control animals		
					Glucose	Insulin	Glucose or Insulin Tolerance
Rat models:							
Houseknecht et al. (1998)	8 week old male ZDF	1.5 % mixture	6.5% (5% corn oil + 1.5% lard)	2 weeks	-↓ fasting and fed	-↓ fasting	Intra-peritoneal glucose tolerance test: - normalized impaired glucose tolerance shown by ↓ glucose area under the curve
Ryder et al. (2001)	7 week old male ZDF	1.5% mixture or 1.5% c9, t11 enriched butter	40% (low CLA butter)	2 weeks	- mixture ↓ fed - c9,t11 no effect	-mixture ↓ fasting - c9,t11 no effect	Intra-peritoneal glucose tolerance test: - CLA mixture improved glucose tolerance shown by ↓glucose area under the curve - c9,t11 had no effect
Nagao et al. (2003)	7 week old male ZDF	1% mixture	6% (5% corn oil + 1% high-linoleic safflower oil)	8 weeks	- ↓ fasting	↓ fasting	ND
Wang et al. (2003)	Male OLETF	1% or 2% mixture	6.5% (linoleic acid – rich safflower oil)	4 weeks	ND	- 1% mixture no effect -2% CLA ↓ fasting	ND

Table 1. (continued) Effects of CLA on circulating glucose, insulin and glucose or insulin tolerance: summary of in vivo studies by species

Reference	Model	Amount & Type of CLA [a]	Total lipid (control) [a]	Length of study	Significant effects of CLA compared to control animals		
					Glucose	Insulin	Glucose or Insulin Tolerance
Rat models continued:							
Henricksen et al., (2003)	7-8 week old female fa/fa Zucker	1.5g/kg body weight by gavage CLA mixture or c9,t11 or t10,c12	1.5g/kg bwt (corn oil) - all animals fed chow	3 weeks	- t10,c12 ↓ fasting - CLA mixture and c9,t11 had no effect	- CLA mixture and t10,c12 ↓ fasting - c9,t11 had no effect	- Oral glucose tolerance test: – CLA mix ↓plasma glucose (t=30 min) and area under the curve for glucose - t10,c12 ↓ plasma glucose (t=30 & 60 min) and area under the curve for glucose - CLA mix and t10,c12 ↓ plasma insulin (t=30, 60 & 90 minutes) and area under the curve for insulin - CLA mix and t10,c12 ↓ glucose-insulin index
Akahoshi et al. (2003)	5 week old male Sprague Dawley	0.8% CLA mixture or 0.4% c9,t11 or 0.4% t10,c12	7% (6% soybean oils + 1% high-linoleic safflower oil)	3.7 weeks	- t10,c12 and mixture ↑ fasting - c9,t11 had no effect	- no effect of any treatment	ND

Table 1. (continued) Effects of CLA on circulating glucose, insulin and glucose or insulin tolerance: summary of in vivo studies by species

Reference	Model	Amount & Type of CLA [a]	Total lipid (control) [a]	Length of study	Significant effects of CLA compared to control animals		
					Glucose	Insulin	Glucose or Insulin Tolerance
Mouse models:							
Tsuboyama-Kasaoka et al. (2000)	8 week old female C57BL/J	1 % mixture	10% (high-oleic safflower oil)	17 weeks	ND	- ↑ fasting	Insulin tolerance test: - ↑ insulin resistance Glucose tolerance test: - no effect
Tsuboyama-Kasaoka et al. (2003)	8 week old Female C57BL/6J	0.1 or 1.0% CLA mixture	4, 13 or 34% (high-oleic sunflower oil)	9 weeks	-1.0% CLA in 4% fat diet ↑ fasting - normalized with 34% fat	ND	Insulin tolerance test: - 1% CLA diet in 4% fat diet impaired insulin-mediated glucose lowering - no effect of 0.1% CLA at any fat level or 1.0% at 13 and 34% fat levels
Hargrave et al. (2003)	High (MH) and low (ML) metabolic rate	1% CLA mixture	7% (soybean oil) Note: also included PW group	9 weeks	ND	ND	Insulin tolerance test: - ↓ insulin sensitivity with greater effect high-metabolic rate mice
Roche et al. (2002)	7 week old male ob/ob	1.3% c9, t11 or 1.4% t10, c12	30% (not specified)	4 weeks	- t10,c12 ↑ fasting - c9,t11 had no effect on fasting	- t10,c12 ↑ fasting - c9,t11 had no effect on fasting	ND
Clement et al. (2002)	Female C57B1/6J	0.4% Linoleic acid or c9,t11 or t10,c12	2.4% (sunflower oil)	4 weeks	- no effect of any treatment on fasting	- t10,c12 ↑ fasting (10x)	ND

Table 1. (continued) Effects of CLA on circulating glucose, insulin and glucose or insulin tolerance: summary of in vivo studies by species

Reference	Model	Amount & Type of CLA [a]	Placebo	Length of study	Significant effects of CLA compared to control animals		
Human trials:					**Glucose**	**Insulin**	**Glucose or Insulin Tolerance**
Eyjolfson et al., 2004 (Randomized, placebo controlled, double blind)	Sedentary healthy adults (n=12 females and 4 males)	CLA mixture 4 g/d as FFA	Safflower oil placebo 4 g/d	8 weeks	- no effect on fasting	- ↓ fasting	Oral glucose tolerance testing: - improved Matsuda insulin sensitivity index
Belury et al., 2003 (Randomized, placebo controlled, double blind)	People with Type 2 diabetes not taking medications for glucose control	CLA mixture in ffa form 6.0 g/d	Safflower oil placebo	8 weeks	- 9 of 11 CLA-supplemented subjects had ↓ fasting (vs 2/10 safflower supplement subjects)	ND	ND
Riserus, et al., (2002a, 2002b) (Randomized, placebo controlled, double blind)	35-65 year old men with metabolic syndrome without pharma-ceutical intervention	t10c12 - CLA or CLA mixture as FFA	Not specified	3 months	- no effect on fasting	ND	Euglycemic clamp: - t10c12 ↑ insulin resistance

[a] amount of CLA and total fat is expressed as % by weight in animal studies, unless otherwise indicated
ND = not determined in that study, TAG=triacylglycerol, FFA=free fatty acids, PL=phospholipids, CLA=conjugated linoleic acid

Table 2. Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of Study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Rat models:							
Azain et al. (2000)	Growing female Sprague-Dawley	0.25% & 0.5% CLA mixture	7% (soybean oil)	5 weeks	ND	ND	- ↓ retroperitoneal fat pad weight with 0.5% CLA
Sisk et al. (2001)	7-8 week old, male fa/fa & lean Zucker	0.5% CLA mixture	7% (soybean oil)	5 weeks	ND	ND	-↓ retroperitoneal fat pad weight in lean -↑ retroperitoneal fat pad weight in *fa/fa*
Ryder et al. (2001)	7 week old, male ZDF	1.5% CLA mixture	40% (low CLA butter)	2 weeks	↓ fasting serum	ND	- ↓ epididymal fat pad weight (↓ feed intake and growth rate)
Nagao et al. (2003)	7 week old Male ZDF	1% CLA mixture	6% (5% corn oil + 1% high-linoleic safflower oil)	8 weeks	No effect	↑ plasma	ND
Henricksen et al., (20030	7-8 week old Female fa/fa Zucker rats	1.5g/kg bwt by gavage CLA mixture or c9,t11 or t10,c12	1.5g/kg bwt (corn oil) - all animals fed chow	3 weeks	ND	ND	- ↓ body weight gain by mixture < t10,c12 - ↓ total abdominal fat by t10,c12
Wang et al. (2003)	Male OLETF	1% or 2% CLA mixture	6.5% (linoleic acid –rich safflower oil)	4 weeks	↓ fasting serum at both levels	ND ND	-↓ weight gain -↓ visceral adipose weight

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Rat models continued:							
Yamasaki et al. (1999)	4 week old, male Sprague Dawley	1% or 2% mixture	7% (safflower oil)	3 weeks	ND	ND	-1% CLA ↓ white adipose free fatty acids -2% CLA ↓ white adipose TAG -no effect of CLA on adipose or body weight
Yamasaki et al., (2003)	5 week old Male Sprague-Dawley	1.5% mixture	4%, 7% and 10% (safflower oil)	3 weeks	↓ fasting serum (no difference between fat level)	ND	-no effect on body weight - body weight increased only with increasing fat level - ↓ perirenal adipose weight – greater effects seen with lower fat levels
Rahman et al., (2001)	Male OLETF	?% CLA as free fatty acid or CLA as triglyceride		4 weeks	↓ fasting serum (42%)	ND	↓ body weight ↓ peri-renal and epididymal fat pad weights
Nagao et al., (2003)	5 week old Male OLETF	1% CLA mixture	10% (safflower oil)	4 weeks	ND	ND	↓ visceral adipose weight by 23%

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Rat models continued:							
Akahoshi et al. (2004)	5 week old Male Sprague-Dawley	0.1, 0.5 or 1% CLA mixture Note: type of protein was either casein or soy	7% (6% soybean + 1% high linoleic safflower oil)	4 weeks	No effect fasting Serum	No effect fasting serum	- ↓ perirenal adipose weight – more pronounced with soy vs. casein diets - No effect on epidiymal or brown adipose weights
Mir et al. (2003)	Weaned Wistar rats	1.82% CLA mixture as FFA (2 isomer; 60% pure)	7% (soybean oil)	7 weeks	ND	ND	- ↓ retro-peritoneal fat weight - adipocyte numbers not different - ↑ SFA and ↓ MUFA in adipose
Mir et al. (2003)	Weaned Wistar rats	CLA enhanced beef (contained 4.63 mg c9t11 and 0.44 mg t10c12/g lipid compared to 2.51 mg c9t11/g lipid in CTL)	14% (7% soybean oil+ beef fat)	7 weeks	ND	ND	-↓ adipocyte number/unit weight and total adipocyte number -↓PUFA (18:2, 18:3, 20:5 and 22:5)
Czauderna et al. (2003)	9 week old Female Wistar rats	1% CLA mixture, 2% CLA mixture 1% c9,t11 or 1% t10,c12	Not specified	4 weeks	ND	ND	-↓ body weight gain and peri-gonadal adipose weight by mixtures and t10,c12

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Rat models continued:							
Alasnier et al. (2002)	Weaned Male Wistar rats	1.5% c9,t11 or t9,t11 or t10,c12 or t10,t12 as TAG	5% (sunflower oil, high oleic oil and linseed oil)		ND	ND	ND
Akahoshi et al. (2003)	5 week old Male Sprague Dawley	0.8% CLA mixture or 0.4% c9,t11 or 0.4% t10,c12	7% (6% soybean oils + 1% high-linoleic safflower oil)	3.7 weeks	ND	ND	- ↓ epididymal adiose weight by t10,c12 and mixture -non-significant trend for ↓ epididymal weight by c9,t11 - ↓ perirenal adipose weight by mixture, c9,t11 and t10,c12 - ↑ brown adipose tissue weight by t10,c12
Koba et al. (2002)	5 week old Male Sprague Dawley	1% linoleic acid (LA) or α-linolenic acid (LNA) or CLA mixture or CLNA (8%c9,t11; 15% t10,c12; 49% conjugated 18:3)	6% (soybean oil)	4 weeks	ND	ND	- ↓ perirenal and epididymal adipose weights by CLA and CLNA

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Mouse models:							
Tsuboyama-Kasaoka et al. (2000)	8 week old female C57BL/J mice	1 % mixture	10% (high-oleic safflower oil)	17 weeks	↓ fasting and fed plasma	ND	- ↓ white adipose weight
Peters et al. (2001)	11-13 week old, male PPARα null mice Wild type mice	0.5% mixture	5.5% (corn oil)	4 weeks	ND	ND	- ↓body weight - ↓ gonadal fat pad weight
Terpstra et al. (2002)	5 week old Male Balb-C mice - food restricted and non-restricted	1.5% CLA mixture	38% (1.5% sunflower oil + corn, coconut, olive and palm oils)	5.6 weeks	ND	ND	- ↓ % body fat
Hargrave et al. (2002)	High energy expenditure (MH), low energy expenditure (ML) or no selection (MC) mice (crossed from Harlan Sprague-Dawley and CF-1 and CRW mice)	1.0% CLA mixture or 0.50% c9,t11 or 0.50% t10,c12	7% (soybean oil)	2 weeks	ND	ND	- ↓ % body fat, retroperitoneal and epididymal fat pad weights by CLA mix and t10,c12
Clement et al. (2002)	Female C57B1/6J mice	0.4% Linoleic acid or c9,t11 or t10,c12	2.4% (sunflower oil)	4 weeks	↓ by t10,c12	ND	- ↓ peri-uteral white adipose weight by t10,c12

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Mouse models continued:							
Roche et al. (2002)	7 week old, male ob/ob mice	1.4% t10, c12	30% (not specified)	4 weeks	ND	ND	- ↓ epididymal fat pad weight
Ealey et a. (2002)	5 week old female C57BL/6 mice and Sprague-Dawley rats	1% CLA mixture	7% Soybean oil	3 weeks	ND	ND	- ↓ retroperitoneal, abdominal white adipose in mice and rats and brown adipose weight in mice
Xu et al., (2003)	9 week old Female ICR mice	0.5% CLA mixture	5% (corn oil)	4 days	ND	ND	- no effect on body weight, fat mass, adipocyte size - ↓ number large (>90μm) adipocytes
Tsuboyama-Kasaoka et al. (2003)	8 week old Female C57BL/6J mice	0.1 & 1.0% CLA mixture in low fat diet; 1% in moderate and high fat diets	4, 13 & 34% (high-oleic sunflower oil)	↓ with low fat diet only	ND	ND	- ↓ subcutaneous and retroperitoneal WAT and BAT weights with 1% CLA diet – ameliorated by 34% fat diet

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Mouse models continued:							
Chardigny et al. (2003)	7 week old Male and female ICR mice	1% t8,t10,c1218:2 or c9,t11 or t10,c12 or CLA mixture	1% (98 high-oleic sunflower: 2 linseed w/w)	6 weeks	ND	ND	- ↓ body fat by c9,t11< CLA mixture< t10,c12
Warren et al. (2003)	9 week old Female C57BL/6N mice	0.5% c9,t11 or t10,c12 as FFA or fenofibrate (2g/kg)	5% (corn oil)	8 weeks	ND	ND	- ↓ adipose tissue weight by t10,c12 and fenofibrate - NS c9,t11
Kang et al. (2004)	8 week old Male SCD1 (+/+) mice or SCD1 (-/-) mice	0.2% t10c12 CLA	20% (corn oil)	4 weeks	ND	ND	- ↓ epididymal and retro-peritoneal adipose weights in both genotypes
Degrace et al. (2004)	Male C57BL/6J mice	1% c9t11 or 1% t10c12 (as TAG)	1% cis9C18:1 (4.9% sunflower oil + 0.1% linseed oil)	4 weeks	ND	ND	- ↓ epididymal adipose tissue weight by t10c12 only

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Total lipid (control fat) [a]	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Hamsters:							
Bouthegourd et al. (2002)	7 week old Male Syrian hamsters	1.6% c9,t11 or 3.2% CLA mixture	33% (not specified)	6 weeks	- no effect	ND	- no effect on white adipose tissue - ↑ brown adipose tissue relative weight by both CLA treatments - ↓ whole body TAG in CLA mix-fed goup compared to control and c9,t11 fed group
Navarro et al. (2003)	10 week old Syrian Golden hamsters	0.5% c9,t11 or t10,c12 or linoleic acid + 10% (palm oil)	chow	6 weeks	ND	ND	- t10,c12 CLA ↓ epididyml, perirenal and subcutaneous fat weighs similarily to chow
Pigs:							
Smith et al. (2002)	23-25 day old Crossbred barrows	1.5% CLA (actual 62% CLA), 1.5% corn oil or 1.5% beef tallow Note: cholesterol matched	4.5% (not specified - corn-soybean meal diet)	5 weeks	ND	ND	- no effect on number of adipose cells
Ostrowska et al. (2003a, 2003b)	Growing Female Large White x Landrace pigs	0.07, 0.14, 0.275, 0.41 and 0.55% CLA (4 isomer) as FFAs	2% (soybean oil)	8 weeks	ND	ND	↓ rate of fat deposition (DXA) and feed intake (greatest in first four weeks; dose responsive - t10,c12 least abundant isomer -c9,t11 most efficiently stored isomer

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Placebo	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Human trials:							
Blankson et al. (2000) (Randomized, placebo controlled, double blind)	Healthy men and women BMI between 25-35	1.7, 3.4, 5.1 or 6.8 g/d CLA mixture	9.0 g olive oil	12 weeks	ND	ND	- no effect on body weight or BMI - ↓ body fat mass vs placebo - ↓ body fat mass within group for 3.4 and 6.8g/d doses
Zambell et al., (2000) (Randomized, placebo controlled, double blind)	20-41 year old healthy women	3 g/d CLA mixture	30% 3 g/d sunflower oil	64 days interventi on after 30 day baseline	ND	ND	- NS changes in body weight or composition
Medina et al., (2000) (Randomized, placebo controlled, double blind)	20-41 year old healthy women	3 g/d CLA mixture	30% 3 g/d sunflower oil	64 days interventi on after 30 day baseline	-no effect	ND	ND

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Placebo	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Human trials continued:							
Mougios et al., (2001) (Randomized, placebo controlled, double blind)	19-24 year old Healthy Men and Women	600-1200 mg/d CLA mixture	36.9-38.8% (soy bean oil)	8 weeks (week 1-4 600 mg/d; week 5-8 1200 mg/d)	ND	ND	- ↓ sum of 10 skinfolds % body fat and fat mass between weeks 4 and 8 but no effect over entire study period
Zambell et al., (2001) (Randomized, placebo controlled, double blind)	24-41 year old Healthy Women	3.9 g/d CLA	30% 3.9 g/d sunflower oil	94 days	ND	ND	ND
Smedman & Vessby et al., (2001) (Randomized, placebo controlled, double blind)	23-63 year old Healthy mean and women	4.2 g/d CLA mixture	4.2 g/d olive oil	12 weeks	ND	ND	- no effect on body weight, waist:hip, sagital adominal diameter
Riserus, et al., (2002a, 2002b) (Randomized, placebo controlled, double blind)	35-65 year old Men with metabolic syndrome without pharmaceutical intervention	t10c12 -CLA or CLA mixture as FFA	Not specified	12 weeks	- No effect	ND	- no effect on body weight, BMI, body fat lean body mass wasit girth between groups - ↓weight, BMI, wasit girth, SA, body fat and lean body mass within t10,c12 group

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Placebo	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Human trials continued:							
Noone et al., 2002 (Randomized, placebo controlled, double blind)	Mean age 31.6 years Healthy Men and Women	3.0 g/d (1.0 g before each meal) 50:50 mixture Or 80:20 mixture (c9t11:t10c12)	3.0 g/d linoleic acid	8 weeks	ND	ND	ND
Kamphuis et al., 2003 (Randomized, placebo controlled, double blind)	20-50 year old Men and Women Overweight subjected to 3 week very low calorie diet	CLA mixture 1.8 g/d (600mg before each meal) and 3.6 g/d (1200 mg before each meal)	Oleic acid placebo	13 weeks	ND	ND	-↓ % body fat independent of regain
Belury et al., 2003 (Randomized, placebo controlled, double blind)	People with Type 2 diabetes not taking medications for glucose control	CLA mixture 6.0 g/d	Safflower oil placebo	8 weeks	- inverse negative correl-ation between plasma t10c12-CLA and serum leptin	ND	- inverse negative correlation between plasma t10c12 CLA and body weight

Table 2. (continued) Effects of CLA on adiposity and circulating leptin and adiponectin: summary of in vivo studies by species

Reference	Model	Amount & type CLA [a]	Placebo	Length of study	Effects or CLA on adiposity compared to control animals		
					Leptin	Adipo-nectin	Adiposity
Human trials continued:							
Petridou et al. (2003) (Randomized, double blind, crossover)	19-24 y.o. Sedentary Women n=16	CLA mixture (2 isomer 50:50) 2.1g/d	2.1g/d Soybean oil	45d treatment & 45d placebo	no effect	ND	-no effect on body mass, BMI, sum of skinfold thickness, % body fat
Gaullier et al., (2004) (Randomized, placebo controlled, double blind)	Healthy Men and Women Overweight (BMI 25-30) Ad libidum lifestlye n=180	CLA mixture (2 isomer 50:50) 4.5g as FFA (3.6g CLA) or 4.5g as TAG (3.4g CLA)	4.5 g Olive oil	1 year	no effect	ND	- ↓ body weight, body fat mass in groups fed CLA as FFA and TAG - ↑ lean body mass in group fed CLA as FFA (measured by DEXA) -no differences energy intake

[a] amount of CLA and total fat is expressed as % by weight in animal studies, unless otherwise indicated
ND = not determined in that study, TAG=triacylglycerol, FFA=free fatty acids, PL=phospholipids, CLA=conjugated linoleic acid

Table 3. Effects of CLA on circulating free fatty acids, triacylglycerols and cholesterol: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on lipidemia compared to control animals		
					Free Fatty Acids	Triacyl-glycerol	Cholesterol
Rat models:							
Houseknecht et al. (1998)	8 week old, male ZDF & lean	1.6 % mixture	6.5% (5% corn oil + 1.5% lard)	2 weeks	- ↓ fasting serum	ND	ND
Ryder et al. (2001)	7 week old, male Zucker fa/fa & lean	1.5% mixture	40% (low CLA butter)	2 weeks	- ↓ fasting serum	- ↓ fasting serum	ND
Henricksen et al., (20030	7-8 week old Female fa/fa Zucker rats	1.5g/kg bwt by gavage CLA mixture or c9,t11 or t10,c12	1.5g/kg bwt (corn oil) - all animals fed chow	3 weeks	- ↓ plasma by mix < t10,c12	ND	ND
Rahman et al., (2001)	Male OLETF	?% CLA as free fatty acid or CLA as triglyceride		4 weeks	- ↓ fasting serum	- ↓ fasting serum	ND

Table 3. (continued) Effects of CLA on circulating free fatty acids, triacylglycerols and cholesterol: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on lipidemia compared to control animals		
					Free Fatty Acids	Triacyl-glycerol	Cholesterol
Rat models continued:							
Rahman et al. (2002)	12 week old Male OLETF rats	7.5% CLA mixture	15% (safflower oil)	48 hours (following 24 hour fast)	ND	NS	- ↓ total and HDL cholesterol
Wang et al. (2003)	Male OLETF	1% Mixture	6.5% (linoleic acid –rich safflower oil)	4 weeks	ND	- ↓ fasting serum	ND
Yamasaki et al. (1999)	4 week old, male Sprague Dawley	1% or 2% mixture	7% (safflower oil)	3 weeks	- no effect on fasting serum with 1% CLA - ↓ fasting serum with 2% CLA	- ↓ fasting serum with both 1% and 2% CLA	ND
Akahoshi et al. (2003)	5 week old Male Sprague Dawley	0.8% CLA mixture or 0.4% c9,t11 or 0.4% t10,c12	7% (6% soybean oils + 1% high-linoleic safflower oil)	3.7 weeks	- no effect – tended to ↓ with t10,c12 and mix ($p<0.07$)	ND	- no effect on total cholesterol -↑ HDL by t10,c12 and mix

Table 3. (continued) Effects of CLA on circulating free fatty acids, triacylglycerols and cholesterol: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid[a] (control)	Length of Study	Effects on lipidemia compared to control animals		
					Free Fatty Acids	Triacyl-glycerol	Cholesterol
Rat models continued:							
Koba et al. (2002)	5 week old Male Sprague Dawley	1% linoleic acid (LA) or α-linolenic acid (LNA) or CLA mixture or CLNA (8%c9,t11; 15% t10,c12; 49% conjugated 18:3)	6% (soybean oil)	4 weeks	- ↑ by CLNA (not CLA)	- ↑ by CLNA (not CLA)	- ↓ total and HDL cholesterol by CLNA
Mouse models:							
Kang et al. (2004)	8 week old Male SCD1 (+/+) mice or SCD1 (-/-) mice	0.2% t10c12 CLA	20% (corn oil)	4 weeks	- ↓ in both genotypes	- ↓ in both genotypes	- no effect in either genotype
Degrace et al. (2004)	Male C57BL/6J mice	1% c9t11 or 1% t10c12 (as TAG)	1% cis9C18:1 (4.9% sunflower oil + 0.1% linseed oil)	4 weeks	↓ serum - by both c9t11 and t10 c12	↓ serum - by both c9t11 and t10 c12	ND

Table 3. (continued) Effects of CLA on circulating free fatty acids, triacylglycerols and cholesterol: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of Study	Effects on lipidemia compared to control animals		
					Free Fatty Acids	Triacyl-glycerol	Cholesterol
Mouse models continued:							
Roche et al. (2002)	7 w. o. male ob/ob mice	1.3% c9, t11 or 1.4% t10, c12	30% (not specified)	4 weeks	- ↓ fed serum by c9,t11 - no effect of t10,c12	- ↓ fed serum by c9,t11 - no effect of t10,c12	- no effect of either isomer
Peters et al., (2001)	11-13 week old, male PPARα null mice Wild type mice	0.5% mixture	5.5% (corn oil)	4 weeks	ND	- ↓ fasting serum	- ↓ fasting serum
Hamsters:							
Navarro et al. (2003)	10 week old Syrian Golden hamsters	0.5% c9,t11 or t10,c12 or linoleic acid + 10% (palm oil)	chow	6 weeks	ND	- no effect (all treatments ↑ compared to chow)	- ↓ total and LDL-C by t10,c12 CLA compared to linoleic and c9t11 CLS

Table 3. (continued) Effects of CLA on circulating free fatty acids, triacylglycerols and cholesterol: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Placebo	Length of study	Effects on lipidemia compared to control animals		
					Free Fatty Acids	Triacyl-glycerol	Cholesterol
Human trials:							
Noone et al., 2002 (Randomized, placebo controlled, double blind)	Mean age 31.6 years Healthy Men and Women	3.0 g/d (1.0 g before each meal) 50:50 mixture or 80:20 mixture	3.0 g/d Linoleic acid	8 weeks	- no effect	- ↓ fasting plasma by 50:50 mixture, not 80:20 - no effect of 80:20 mixture	- ↓ VLDL-cholesterol by 80:20 not 50:50 - no effect on HDL, LDL
Mougios et al., 2001 (Randomized, placebo controlled, double blind)	19-24 year old Healthy	600-1200 mg/d CLA mixture	36.9-38.8% (soy bean oil)	8 weeks (week 1-4 600 mg/d; week 5-8 1200 mg/d)	ND	- ↓ fasting plasma between weeks 0 & 4	ND
Riserus, et al., (2002a, 2002b) (Randomized, placebo controlled, double blind)	35-65 year old Men with metabolic syndrome without pharmaceutical intervention	t10c12 -CLA or CLA mixture as FFA	Not specified	3 months	ND	- ↑ VLDL TAG with t10,c12	- ↓ HDL with t10,c12 and mixture

[a] amount of CLA and total fat is expressed as % by weight in animal studies, unless otherwise indicated
ND = not determined in that study, TAG=triacylglycerol, FFA=free fatty acids, PL=phospholipids, CLA=conjugated linoleic acid

Table 4. Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on fatty liver compared to control animals
Rat models:					
Sisk et al. (2001)	7-8 week old, male fa/fa & lean Zucker	0.5% mixture	7% (soybean oil)	5 weeks	↓ liver weight in fa/fa
Wang et al. (2003)	Male OLETF	1% or 2% mixture	6.5% (linoleic acid –rich safflower oil)	4 weeks	- ↓ liver TAG concentration with 2% CLA
Yamasaki et al., 2003	5 week old Male Sprague-Dawley	1.5% mixture	4%, 7% and 10% (safflower oil)	3 weeks	- no effect on liver weights or hepatic injury
Yamasaki et al. (1999)	4 week old, male Sprague Dawley	1% or 2% mixture	7% (safflower oil)	3 weeks	- ↓ liver TAG & free fatty acid - no effect on liver weight
Rahman et al. (2002)	12 week old Male OLETF rats	7.5% CLA mixture	15% (safflower oil)	48 hours (following 24 hour fast)	- ↓ liver TAG and cholesterol concentration, no effect on liver phospholipids
Nagao et al., 2003	5 week old Male OLETF	1% CLA mixture	10% (safflower oil)	4 weeks	- ↓ liver TAG concentration
	8 week old Male OLETF	1% t10c12 CLA	1% c9t11 CLA	10 days	- ↓ liver TAG concentration

Table 4. (continued) Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on fatty liver compared to control animals
Rat models continued:					
Sebedio et al. (2001)	Weaned Male Wistar rats	1% mixture			- ↓ liver TAG concentration, no difference in total liver lipid content
Alasnier et al. (2002)	Weaned Male Wistar rats	150 mg/d c9,t11 or t9,t11 or t10,c12 or t10,t12 as TAG	5% (sunflower oil, high oleic oil and linseed oil (79:13:8)	6 days	- no effect on liver weight - no effect on total liver lipid and lipid class distribution - no changes in liver fatty acid distribution
Akahoshi et al. (2003)	5 week old Male Sprague Dawley	0.8% CLA mixture or 0.4% c9,t11 or 0.4% t10,c12	7% (6% soybean oils + 1% high-linoleic safflower oil)	3.7 weeks	- no effect of any treatment on liver weights
Koba et al. (2002)	5 week old Male Sprague Dawley	1% linoleic acid (LA) or α-linolenic acid (LNA) or CLA mixture or CLNA (8%c9,t11; 15% t10,c12; 49% conjugated 18:3)	6% (soybean oil)	4 weeks	- ↑ relative liver weight by CLNA - ↑ liver cholesterol by LNA - ↑ liver TAG by CLNA (no effect of CLA)

Table 4. (continued) Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on fatty liver compared to control animals
Mouse models:					
Clement et al. (2002)	Female C57B1/6J mice	0.4% Linoleic acid or c9,t11 or t10,c12	2.4% (sunflower oil)	4 weeks	- ↑ relative liver weight and liver lipid content by t10,c12
Tsuboyama-Kasaoka et al. (2003)	8 week old Female C57BL/6J mice	0.1 & 1.0% CLA mixture in low fat diet; 1% in moderate and high fat diets	4, 13 & 34% (high-oleic sunflower oil)	9 weeks	- ↑ liver weights with 1% CLA diet – ameliorated by increasing dietary fat level (↑ 190, 100 and 45% by low, moderate and high fat diets, respectively)
Chardigny et al. (2003)	7 week old Male and female ICR mice	1% t8,t10,c1218:3 or c9,t11 or t10,c12 or CLA mixture	1% (98 high-oleic sunflower: 2 linseed w/w)	6 weeks	- ↑ liver weights by t10,c12 and CLA mixture in male mice, by t10,c12 only in female mice - liver PL and neutral lipids: ↑ MUFA (18:1), ↓PUFA (18:2n-6) – most prominent with t10,c12, also apparent with mix and c9,t11
Warren et al. (2003)	9 week old Female C57BL/6N mice	0.5% c9,t11 or t10,c12 as FFA or fenofibrate (2g/kg)	5% (corn oil)	8 weeks	- ↑ liver weight by t10,c12 and fenofibrate only; - no effect of c9,t11 - ↑ liver total lipids by t10,c12 only; no effect of fenofibrate or c9,t11
Kelley et al. (2004)	9 week old Female C57BL/6N mice	0.5% c9,t11 or t10,c12 as FFA	5% (corn oil)	8 weeks	- no effect of c9,t11 on total liver lipids % - ↑ total liver lipid %(4x), ↑ liver TAG %, ↓ liver PL%, CE% and FFA% by t10,c12 - c9,t11 ↓MUFA/↑PUFA % in liver TAG but not PL - t10,c12 ↑MUFA/↓PUFA % in liver TAG and PL

Table 4. (continued) Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on fatty liver compared to control animals
Mouse models continued:					
Kang et al. (2004)	8 week old Male SCD1 (+/+) mice or SCD1 (-/-) mice	0.2% t10,c12 CLA	20% (corn oil)	4 weeks	- SCD1 (-/-) ↓ liver lipid content compared to SCD1 (+/+) mice but no effect of t10c12 in either genotype
Degrace et al. (2004)	Male C57BL/6J mice	1% c9,t11 or 1% t10,c12 (as TAG)	1% cis9C18:1 (4.9% sunflower oil + 0.1% linseed oil)	4 weeks	- ↑ liver TAG in mice fed t10c12
Nakanishi et al. (2004)	Male Sea:ddY mice	300 ul/d CLA mixture or	300ul/d (high linoleic acid safflower oil)	4 weeks	- ↑ liver weight (g/kg body weight) - ↑ liver total lipid 3x (% liver weight) - ↑ liver TAG 11x (% liver weight)
		250 ul/d γ-linoleic acid (GLA) or 250 ul/d CLA mixture or 250 ul GLA + 250ul high linoleic safflower oil or 250 ul CLA + 250ul high linoleic safflower oil or 250 ul GLA + 250ul CLA	250ul/d (high linoleic acid safflower oil)	4 weeks	- ↑ liver weight (g/kg body weight) - ↑ liver total lipid (% liver weight) - ↑ liver TAG (% liver weight) - in mice fed CLA alone and CLA + high oleic sunflower oil - Mice fed GLA, GLA+high oleic sunflower oil or GLA+CLA did not have hepatic steatosis

Table 4. (continued) Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Total lipid (control)[a]	Length of study	Effects on fatty liver compared to control animals
Mouse models continued:					
Tsuboyama-Kasaoka et al. (2000)	8 week old Female C57BL/J mice	1 % mixture	10% (high-oleic safflower oil)	17 weeks	↑ panlobular macrovesicular steatosis
Degrace et al. (2003)	8 week old Male C57BL/J mice	1% c9t1 -1 CLA 1% t10c12-CLA	5% (oleic acid)	4 weeks	- no effect on liver weight or triacylglycerol concentration -↑ liver weights (~1.5x -↑ liver triacylglycerol concentration (~7.5 x)
Takahashi et al. (2003)	6 week old Male C57BL/6J mice 6 week old Male ICR mice 6 week old Male ICR mice	1.5% Mixture 1.5% Mixture 1.5% Mixture	15% (13.5% palm oil + 1.5% linoleic acid) 15% (13.5% palm oil + 1.5% linoleic acid) (13.5% palm oil + 1.5% linoleic acid or 1.5% palmitic acid)	3 weeks	-↑ liver weights (~doubled0 -↑ liver triacylglycerol (8-13 x) cholesterol (3-5x) and phospholipids (40-70%) concentrations
Hamsters					
Navarro et al. (2003)	10 week old Syrian Golden hamsters	0.5% c9,t11 or t10,c12 or linoleic acid + 10% (palm oil)	chow	6 weeks	- t10,c12 CLA ↓ liver total and free liver cholesterol and liver TAG but ↑ liver weight compared to c9,t11 and linoleic acid - both isomers ↓ liver PL compared to linoleic acid
Chicks					
Badinga et al. (2003)	1 day old Male Broiler chicks (Ross x Ross)	5% CLA mixture	7.86% Corn oil	3 weeks	- ↓ liver TAG concentration - ↑ saturated fatty acids, ↓monounsaturated fatty acids in liver

Table 4 (continued) Effects of CLA on liver lipids and metabolism: summary of in vivo studies by species

Reference	Model	Amount & type CLA[a]	Placebo	Length of study	Effects on fatty liver compared to control animals
Human trials:					
Petridou et al. (2003) (Randomized, double blind, crossover)	19-24 y.o. Sedentary Women n=16	CLA mixture (2 isomer 50:50) 2.1g/d	2.1g/d Soybean oil	45d treatment + 45d placebo	- no effect on serum alanine aminiotransferase
Gaullier et al., 2004 (Randomized, placebo controlled, double blind)	Healthy Men and Women Overweight (BMI 25-30) Ad libidum lifestlye n=180	CLA mixture (2 isomer 50:50) 4.5g as FFA (3.6g CLA) *or* 4.5g as TAG (3.4g CLA)	4.5 g olive oil	1 year	- no effect on alanine aminotransferase in people given CLA as FFA or TAG - no effect on aspartate aminotransferase in people given CLA as TAG - ↑ circulating aspartate aminotransferase in people given CLA as FFA both as difference from placebo and change within group (↑ 2.35 ± 7.00 U/L vs. ↓ 0.32 ± 5.06 U/L over 12 months in placebo group)

[a] amount of CLA and total fat is expressed as % by weight in animal studies, unless otherwise indicated
TAG=triacylglycerol, FFA=free fatty acids, PL=phospholipids, CLA=conjugated linoleic acid

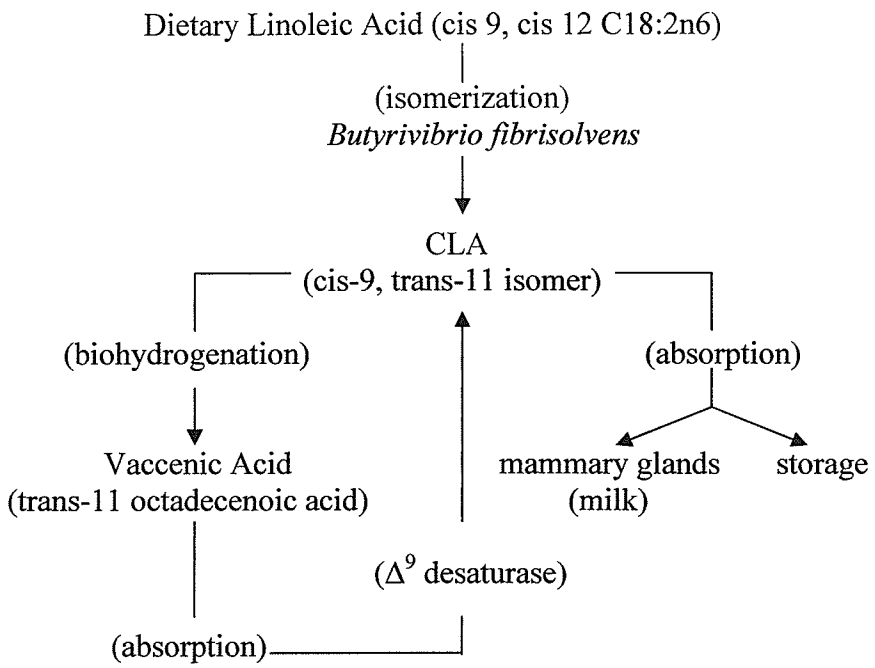


Figure 1. Production of CLA by rumen microorganisms and biohydrogenation (Adapted from Roche et al., 2001)

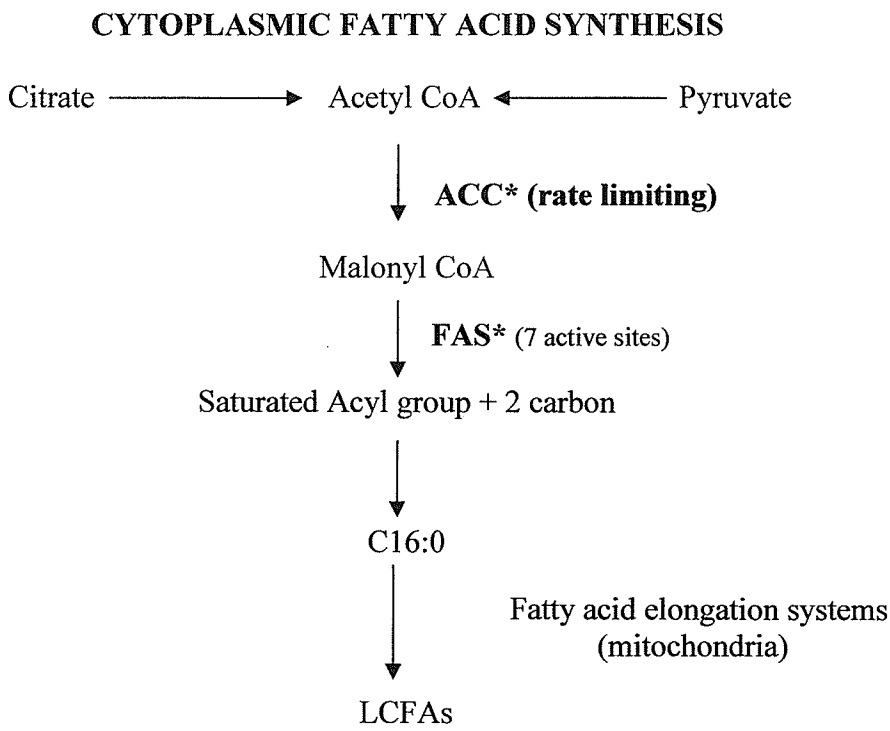


Figure 2. Regulatory genes in cytoplasmic fatty acid synthesis of long chain fatty acids (LCFAs). [*enzymes with genes up-regulated by SREBP-1c; ACC = acetyl CoA carboxylase, FAS = fatty acid synthase]

FATTY ACID DESATURATION AND ELONGATION

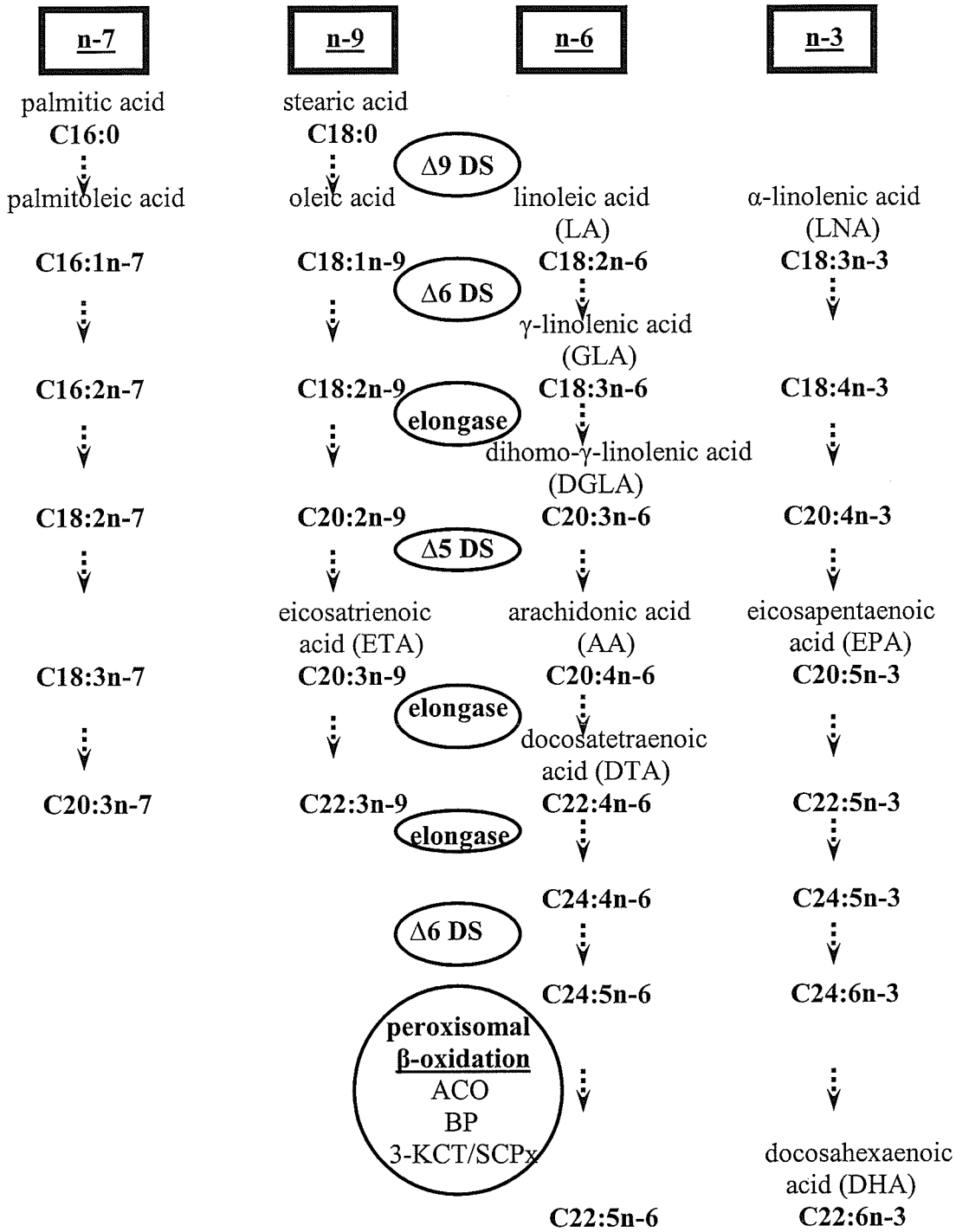


Figure 3. Fatty acid desaturation and elongation (adapted from Shils et al., 1999; Sprecher 2000; Ferdinandusse et al., 2001) ACO=acyl coA oxidase, BP=bifunctional protein, 3-KCT=3-ketoacyl CoA thiolase, SCPx=sterol carier protein X.

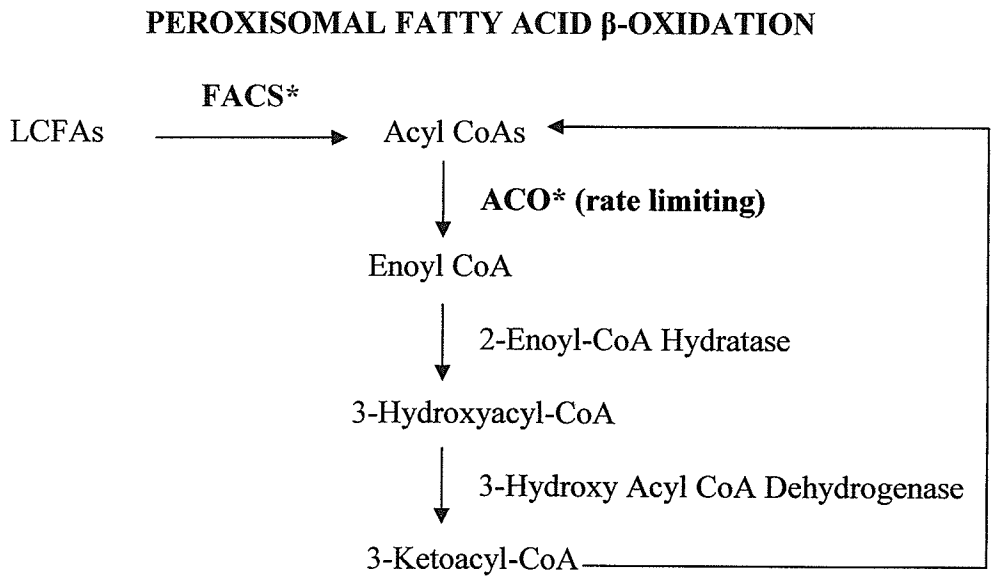


Figure 4. Regulatory enzymes in the peroxisomal β-oxidation of long chain fatty acids (LCFAs). [*genes that contain PPREs regulated by PPARα (Clarke, 2000); FACS = fatty acyl CoA synthase, ACO = acyl coA oxidase]

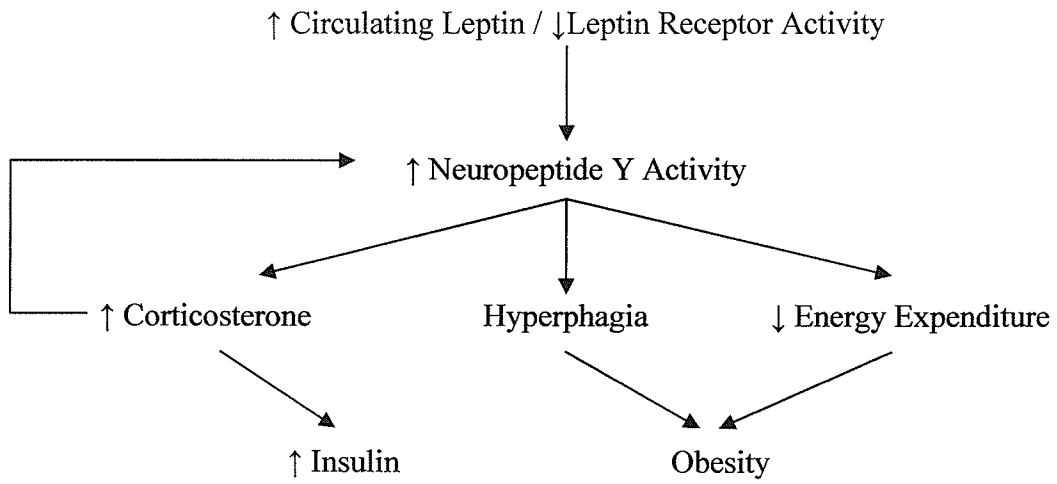


Figure 5. Relationship between leptin, neuropeptide Y, corticosterone and insulin in the *fa/fa* Zucker rat (Adapted from White & Martin, 1997)

II. STUDY RATIONALE

CLA may help control the manifestations of conditions such as the metabolic syndrome and NAFLD. At the time the study was designed, it was shown that CLA ameliorated insulin resistance in ZDF rats. Since that time this effect has been duplicated in other obese rat models and was concomitant with reducing hepatic lipid content and favourably modifying circulating lipid and adipocytokine status in some studies. However, other models, particularly mouse models, show that CLA worsens insulin resistance in association with lipodystrophy. Characteristics of the *fa/fa* Zucker rat including leptin resistance, insulin resistance and obesity make it an appropriate model to clarify the effects of CLA in liver and adipose metabolism. Furthermore, comparing *fa/fa* and lean genotypes allows the direct comparison of insulin-resistant to non-insulin-resistant animals.

The rationale for this study, in this model, is to: i) confirm that dietary CLA favourably modifies insulin sensitivity and adiposity; ii) demonstrate that dietary CLA reduces hepatic steatosis and demonstrate that this may have an effect on liver function and inflammation; iii) clarify the role for CLA in lipidemia and circulating adipocytokine status; iii) demonstrate that CLA modifies the fatty acid distribution of liver and adipose TAG, separately from phospholipids; and iv) demonstrate that CLA enhances liver fatty acid oxidation and reduces liver fatty acid synthesis, at the gene level, possibly through the regulation of PPARs and SREBPs, respectively.

III. HYPOTHESIS & OBJECTIVES

It is hypothesized that in the *fa/fa* Zucker rat, CLA feeding will improve peripheral insulin sensitivity in association with altered fatty acid profiles in liver and

adipose, and enhance adipocyte functionality, thus favourably modifying lipidemia and circulating adipocytokine status, and decreasing lipid storage in the liver. Furthermore, it is hypothesized that CLA will act as a PPAR-ligand and SREBP-suppressor in the liver to enhance fatty acid oxidation and inhibit fatty acid synthesis, respectively. These changes will improve liver function and reduce inflammation.

To investigate the action of CLA the following objectives were defined:

1. To determine the effects of an eight week dietary intervention with a CLA mixture in six-week old *fa/fa* and lean Zucker rats on:
    a) Glycemia and insulinemia (oral glucose tolerance testing - serum glucose and insulin and calculations for insulin sensitivity; fasting serum glucose, insulin, c-peptide, c-peptide:insulin ratio);
    b) adiposity and circulating adipocytokines (body weight, fat pad weights, fasting serum leptin, adiponectin and haptoglobin);
    c) lipidemia (fasting serum free fatty acids, triglycerides and cholesterol); and
    d) liver steatosis, function and inflammation (liver lipid content and fasting serum ALT and haptoglobin).
2. To determine the effects of dietary CLA on the fatty acid composition of TAG and PL in adipose and liver tissue of *fa/fa* and lean Zucker rats and to
    a) relate adipose phospholipid composition to calculations of insulin sensitivity; and
    b) calculate ratios of select fatty acids as indices for desaturase activity.

3. To determine if dietary CLA alters markers of

    a) hepatic fatty acid oxidation (ACO mRNA expression);

    b) hepatic fatty acid synthesis (ACC mRNA expression).

IV. METHODS

Experimental design

Weanling *fa/fa* (fa) and lean (ln) Zucker rats (n=20 per genotype) were fed either CLA or control (CTL) diets. The faCLA and lnCLA groups were fed the CLA diet, while the faCTL and lnCTL groups were fed control diet (n=10 per group).

Animals and diets

Following a five to seven day acclimatization period, 20 *fa/fa* and 20 lean six-week old Zucker rats (Charles River, St. Constant, PQ) were randomly assigned to receive either the CLA or CTL diet for 8 weeks. Pair-weighed groups (*fa/fa* and lean) were also included in the study design, but not analyzed because dietary treatment did not alter body weight.

The diet formulations were based on the AIN-93G diet (Reeves, 1993; Table 5). The exception was egg white which, was used instead of casein as the protein source. The dry ingredients for the diets were pre-mixed and stored at 4°C. The CLA oil was aliquoted and stored at -20°C. The CLA diet contained 1.5% CLA oil, in free fatty acid form (Nu-Check Prep. Inc., Eyslan, Minnesota) and 7% soy oil (w/w). The isomer distribution of the CLA mixture is shown in table 5. The CTL diet contained 8.5% soybean oil (w/w). The fatty acid composition of the soybean oil is shown in Table 5. Fresh, six kilogram batches of the diet, containing oil, were prepared weekly and stored at –20°C until used. Rats were given new feed cups with fresh feed three times per week. Feed consumption (corrected for spillage) and weekly body weights were recorded.

Oral glucose tolerance testing

At 7.5 weeks, oral glucose tolerance testing was done on rats after a five hour fast. Feed cups were removed at 8 a.m. and testing began at 1 p.m. Initial blood samples from the sephanous vein were collected (t=0), followed by administration of an oral glucose dose (1g glucose/kg body weight). Additional blood samples were collected at t=15, t=30 and t=60 minutes post glucose administration. Blood samples were stored on ice until centrifuged at 1500 rpm for 15 minutes at 4°C. The resulting serum layer was aliquoted and stored at -80°C until glucose and insulin concentrations were determined. Rats were pre-exposed to the oral glucose tolerance testing procedure, without blood collection, to reduce the stress response during the actual testing period.

Tissue collection

With cessation of the study period, rats were fasted overnight and euthanized by CO_2 asphyxiation and cervical dislocation according to the Canadian Council on Animal Care Guidelines (CCAC, 1993). Each rat was weighed, following asphyxiation. Trunk blood was collected following cervical dislocation and was immediately placed on ice until centrifuged to separate the serum fraction and stored at –80°C . Dissected organs, including epididymal adipose and livers were weighed, frozen in liquid nitrogen immediately and subsequently stored at –80°C .

Tissue analysis

a) Serum biochemistry

Fasting serum, obtained at the end of the 8 week study, was analyzed for glucose, insulin, c-peptide, leptin, adiponectin, FFA, TAG, cholesterol, ALT and haptoglobin, as detailed below. As well, OGTT serum was analyzed for glucose and insulin.

i) Enzymatic colorimetric endpoint spectrophotometric assays

Enzymatic colorimetric kits were used to quantitatively assess fasting and OGTT serum glucose (Sigma Chemicals, St. Louis, MO, #315-100), fasting serum FFA (Roche Diagnostics, Mannheim, Germany, #1383175), TAG (BioPacific, Vancouver, BC, #DCL 210-75), cholesterol (Biopacific, Vancouver, BC, #DCL225-26) and haptoglobin (Tri-Delta Diagnositcs, Ireland Morris Plains, NJ, #TP801).

Each assay involved the conversion of substrates to products that produce a color change which can be detected at a certain wavelength. The intensity of the color produced was proportional to the original concentration of the compound being measured. The linearity of all standard curves were ≥ 0.95. The percent coefficient of variation (%CV) for each set of samples was $\leq 10\%$.

The principles, reagents and procedures of each assay are described below. They all follow the calculation:

$$\text{Serum value} = \frac{(\text{Absorbance of Unknown} - \text{Absorbance of Blank})}{(\text{Absorbance of Standard} - \text{Absorbance of Blank})} \chi \text{ Concentration of Standard}$$

Glucose

Principle:

$$\text{Glucose} + H_20 + O_2 \xrightarrow{\text{glucose oxidase}} \text{Gluconic Acid} + H_20_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{peroxidase}} \text{Oxidized o-Dianisidine (brown colour)}$$

Reagents:

R1 Enzyme solution: 1 PGO enzyme capsule in 100 ml deionized H_20

R2 Color Reagent: 50 mg o-Dianisdine Dihydrochloride in 20 ml deionized H_20

Working Reagent: 100 mL R1 + 1.6 ml R2

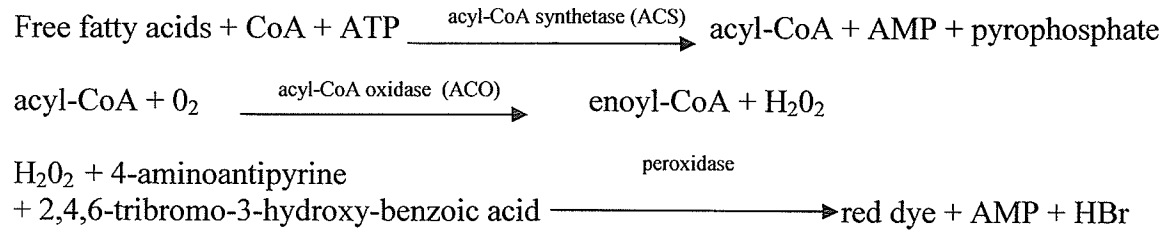
Glucose Standard (5.56 mmol/L)

Procedure:

A standard curve (0-0.83 mmol/L) was prepared from the glucose standard. Fasting and OGTT serum for both the *fa/fa* and lean genotypes were diluted 20-30χ in deinoized H_20. Twenty µl of blank, standards or samples were pipetted in triplicate, into a Costar EIA/RIA 96-well polystyrene plate. Two-hundred µl working reagent was added to each well. The plate was gently mixed, by tapping, and incubated at room temperature (18-26°C) for 45 minutes, then read at 450 nm in the microplate reader (Spectromax 340, Ramsey, MN).

Free fatty acids

Principle:



Reagents:

R1 Enzyme solution: 1 tablet bottle 2 (ATP, CoA, ACS, peroxidase, acorbate oxidase, 4 aminoantipyrine and stabilizers) into bottle 1 (11 ml potassium phosphate buffer, pH 7.8, tribromohydrooxybenzoic acid, magnesium chloride and stablilizers).

R2 Solution to remove excess CoA: Bottle 3 (3 ml aqueous N-ethyl-maleinimide solution with stablizers)

R3 Enzyme solution: 1 tablet bottle 5 (ACO and stabilizers) into bottle 4 (0.6 mL ACO dilution solution and stabilizers)

Procedure:

Two-hundred µl of R1 was pipetted into each well of a Costar EIA/RIA 96-well polystyrene plate. Ten µl blank, in-house control or undiluted samples were pipetted in triplicate into the plate. The plate was mixed for 30 seconds and incubated at room

temperature (22-23°C) for 10 minutes. Ten µl of R2 was added to each well. The plate was mixed for 30 seconds and the absorbance was read at 546 nm (A1) in the microplate reader (Spectramax 340, Ramsey, MN). The plate was removed and 10 µl of R3 was added to each well. The plate was then mixed for 30 seconds, incubated at room temperature (22-23 °C) for 20 minutes, mixed again for 30 seconds and read at 546 nm (A2). Calculation for serum free fatty acid concentration was:

$$C = \frac{V}{\varepsilon dv} \chi \Delta A \text{ (mmol/L sample solution)}$$

Where:
V = final volume (mL) = 0.230
ε = absorption coefficient of the dye at 546nm $(1 \cdot mmol^{-1} \cdot m^{-1})^3$ = 19.3
d = light path (cm) of microplate reader = 0.53326
v = sample volume (mL) = 0.010
$\Delta A = (A2\text{-}A1)_{sample} - (A2\text{-}1)_{blank}$

C (mmol/L) = 2.2347 χ ΔA

Triacylglycerols

Principle:

Triglycerides $\xrightarrow{\text{lipase}}$ Glycerol + Free Fatty Acids

Glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ Glycerol-1-Phosphate + ADP

Glycerol-1-Phosphate $\xrightarrow{\text{L-α-glycerol phosphate oxidase}}$ H_20_2 + Dihydrozyacetone Phosphate

4-Aminoantipyrine + H_20_2 + 3,5-dichloro-2-hydroxy-benzenesulfonic acid

$\xrightarrow{\text{peroxidase}}$ Quinoneimine dye (red colour) + HCl + $2H_20$

Reagents:

<u>R1</u> Enzyme color solution

Triacylglycerol standard (2 mmol/L)

Procedure:

A standard curve (0-2.0 mmol/L) was prepared from the triacylglycerol standard using 0.9% sodium chloride (Fisher Scientific, Nepean, ON, #BP358-212) in deionized water. Fasting serum from the *fa/fa* rats and lean rats were diluted 10χ and 4χ, respectively in 0.9% saline. One serum sample from a *fa/fa* rat required a 30χ dilution. Ten µl of blank, standards or samples were pipetted in triplicate, into a Costar EIA/RIA 96-well polystyrene plate. One-hundred µl of R1 and 140 µl deionized water were added to each well The plate was gently mixed and read at 515 nm in the microplate reader (Spectromax 340, Ramsey, MN).

Cholesterol

Principle:

Cholesterol Esters $\xrightarrow{\text{cholesterol esterase}}$ Cholesterol + Free Fatty Acids

Cholesterol + 0_2 $\xrightarrow{\text{cholesterol oxidase}}$ Cholesten-3-one + H_20_2

Reagents:

R1 Color reagent

R2 Phenol reagent

Working Reagent: equal parts R1 and R2

Cholesterol Standard (5.0 mmol/L)

Procedure:

A standard curve (0-5 mmol/L) was prepared from the cholesterol standard. Fasting serum from the *fa/fa* rats was diluted 5χ in 0.9% sodium chloride (Fisher Scientific, Nepean, ON, #BP358-212). Serum from lean rats was used undiluted. Ten µl of blank, standards or samples were pipetted in triplicate, into a Costar EIA/RIA 96-well

polystyrene plate. Two-hundred μl working reagent was added to each well. The plate was gently mixed for 30 seconds and incubated at 37°C for 5 minutes, then read at 505 nm in the microplate reader (Spectromax 340, Ramsey, MN).

Haptoglobin

Principle:

Haptoglobin preserves peroxidase activity of haemoglobin at low pH, therefore, the preservation of peroxidase activity is directly proportional to the amount of haptoglobin present in the sample.

Reagents:

R1 1 part Haemoglobin to 1 part Haemoglobin dilutent

R2 9 parts Chromagen to 5 parts Substrate

Phosphate Buffered Saline (PBS)

Haptoglobin Standard (2.0 mg/ml)

Procedure:

A standard curve (0-2 mg/ml) was prepared from the haptoglobin standard. Fasting serum from the *fa/fa* rats was diluted 2χ in PBS. Serum from the lean rats was used undiluted. Blank, standards or samples (7.5 μl) were pipetted in duplicate, into a Costar EIA/RIA 96-well polystyrene plate. One-hundred μl of R1 was added to each well and gently mixed, by tapping. One-hundred fourty μl of R2 was added to each well and the plate was gently mixed for 15 seconds and incubated at room temperature (20-25°C) for 5 minutes, and at 37°C for 8 minutes, then read at 630 nm in the microplate reader (Spectromax 340, Ramsey, MN).

ii) Enzymatic kinetic spectrophotometric assays

An enzymatic kinetic assay was performed to quantify fasting serum ALT (BioPacific Diagnostic, Vancouver, BC, #318-10). The assay measures the change in absorbance over time in the ultraviolet range of the electromagnetic spectrum.

Alanine aminotransferase

Principle:

$$\alpha\text{-ketoglutarate} + \text{L-alanine} \xrightarrow{\text{alanine aminotransferase}} \text{L-glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

Reagents:

R1 Enzyme Reagent: L-alanine, lactate dehydrogenase, buffer and preservative

R2 Substrate Reagent: 2-oxoglutarate, NADH and preservative

Working Reagent: 4 parts R1 + 1 part R2

DC-Trol Level 1 – serum quality control (BioPacific, Vancouver, BC, #SM-052)

Procedure:

Fifty µl of blank, standard or samples were pipetted into separate disposable methacrylate semi micro-cuvettes (Fisher Scientific, Nepean, ON, #14-385-938). Six cuvettes were read at a time (one blank, one standard, four samples). One mL of working reagent was added to each cuvette. The cuvettes were incubated for three minutes and absorbance was read at 30 second intervals for ten minutes using a Pharmacia Biotech Ultrospec 4000 (Piscataway, NJ) . ALT was calculated based on:

$$\text{ALT} = \frac{V}{\varepsilon dv} \chi \, \Delta A/\text{min}$$

Where: V = final volume (mL) = 1.05
ε = absorption coefficient of NADH at 340 nm $(1 \cdot mmol^{-1} \cdot m^{-1})^3$ = 6.22
d = light path (cm) of spectrophotometer = 1.0
v = sample volume (mL) = 0.05

ALT (U/L) = 3376 χ ΔA/min

iii) Radioimmunoassays (RIAs)

RIAs were completed for insulin, leptin, adiponectin and C-peptide (Linco Research Inc., St. Charles, MO; ^{125}I rat: insulin RIA kit, #SRI-13K; leptin RIA kit #RL-83K; adiponectin RIA kit, #MADP-60HK; c-peptide RIA kit #RCP-21K).

Principle:

A known concentration of labeled ^{125}I was incubated with antiserum so that the concentration of the antigen binding sites in the system was at a limited and fixed concentration. With the addition of the sample, unlabelled antigen competed with labeled antigen. Therefore, the amount of labeled ^{125}I that was bound to antibody decreased, as the amount of unlabeled antigen from insulin, leptin, adiponectin or C-peptide in the serum sample increased. The amount of ^{125}I actually bound was separated, counted and compared to a standard curve.

Dilution Factors:

	Insulin	Leptin	Adiponectin	C-Peptide
fa/fa rats:	125-200	10	500	40
lean rats:	10	1	1000	10

Reagents:

Assay buffer Phosphosaline with EDTA, sodium azide and BSA

Antiserum Guinea pig anti-rat insulin, leptin, adiponectin or C-peptide serum in assay buffer

^{125}I-labelled Insulin, Leptin, Adiponectin or C-peptide

Label hydrating buffer

Standards 0.2-1.0 ng/ml insulin, 0.5-50 ng/ml leptin, 1-100 ng/ml adiponectin or 25-1600 pM C-peptide

Quality controls

Precipitating reagent Goat anti-guinea pig IgG serum with PEG, TritonX-100, phosphosaline, EDTA and sodium azide

Procedure for insulin, leptin and c-peptide RIAs:

On day one, assay buffer was pipetted into 12 χ 75 mm borosilicate glass tubes (duplicate for each measure) in the following proportions: 0 µl into total counts (TC) tubes, 300 µl into the non-specific binding (NSB) tubes, 200 µl into the total binding reference tubes (B_o) and 100 µl into tubes for standards, quality controls and unknowns. Serum samples were diluted appropriately (see above) in assay buffer and pipetted in 100 µl volumes, in duplicate. One-hundred µl of the appropriate antibody was then added to the Bo, standard, quality control and unknown tubes. Tubes were vortexed, covered and incubated for 20-24 hours (4°C for insulin and c-peptide, room temperature for leptin).

On day two, 100 µl of the ^{125}I tracer was added to all tubes and tubes were vortexed, covered and incubated for another 20-24 hours.

On day three, 1 ml of precipitating reagent was added to all tubes, except the TC tubes, which were then vortexed, incubated for 20 minutes, centrifuged for 40 minutes at 2000 χ g and decanted by inverting tubes for 30 seconds and blotting excess supernate. The remaining pellet was counted for radioactivity in a Beckman Gamma 8000 (Scientific Instruments, Irvine, CA).

Procedure for adiponectin RIA:

On day one, assay buffer was pipetted into 12 χ 75 mm borosilicate glass tubes (duplicate for each measure) in the following proportions: 0 µl into total counts (TC) tubes, 300 µl into the non-specific binding (NSB) tubes, 200 µl into the total binding reference tubes (B_o) and 100 µl into tubes for standards, quality controls and unknowns. Serum samples were diluted appropriately (see above) in assay buffer and pipetted in 100 µl volumes, in duplicate. One-hundred µl of ^{125}I-Adiponectin was added to each tube. One-hundred µl of adiponectin antibody was then added to the Bo, standard, quality control and unknown tubes. Tubes were vortexed, covered and incubated for 20-24 hours at room temperature.

On day two, 10 µl of rabbit carrier was added to all tubes, except TC tubes. One ml of cold precipitating reagent was added to all tubes, except the TC tubes, which were then vortexed, incubated for 20 minutes, centrifuged for 40 minutes at 2000 χ g and decanted by inverting tubes for 30 seconds and blotting excess supernate. The remaining pellet was counted for radioactivity in a Beckman Gamma 8000 (Scientific Instruments, Irvine, CA).

Calculation for RIAs:

The RIAs follow the calculation:

$$\%\text{Bound/Total bound} = \frac{\text{Average counts} - \text{Non specific binding}}{\text{Total bound}} \chi 100$$

Serum values from %Bound/Total Bound were calculated from the standard curve using Graph Pad Prism 2.01 Software (Intuitive Software for Science, San Diego, CA) and multiplied by the appropriate dilution factor. Insulin values were converted from ng/ml to pmol/L using a molecular weight of 5800 for insulin.

Insulin Resistance Calculations

i) Extrapolating missing data points from OGTT

During OGTT, blood could not be collected from some animals at certain time points. For this reason, eleven values were estimated by regression, including:

Glucose: faCTL t=60 (n=1)
lnCTL t=0 (n=1)

Insulin: faCLA t=60 (n=1)
faCTL t=60 (n=2)
lnCLA t=0 (n=1), t=15 (n=1), t=30 (n=1), t=60 (n=1)
lnCTL t=15 (n=1), t=60 (n=1)

If there were ≥ two data points missing for any one animal, the data for that animal was not used in the insulin resistance calculations. This included one animal from each the lnCLA and lnCTL groups, therefore the following calculations are based on n=10 for each of the faCLA and faCTL groups and n=9 for each of the lnCLA and lnCTL groups.

First, parameter estimates (b_o, b_1, b_2 and b_3) for each treatment group were calculated, by Proc Reg in SAS 6.04 (SAS Institute, Cary, NC). Second, predicted values for each time point, within each treatment group, were calculated. Third, the intercept for the individual rat was calculated and based on this, the missing data point was extrapolated based on the assumption that all animals in a treatment group will have a commonly shaped curve parallel (above or below) the mean for that group. The equation used was: Insulin or Glucose (y) = $b_o + b_1T + b_2T^2 + b_3T^3$.

ii) Calculations for insulin sensitivity

Euglycemic insulin clamp is considered the most accurate measure of insulin sensitivity, however, several calculations have been developed that are an indirect

measures of insulin action on glucose disposal. These include, the homeostasis model assessment (HOMA; Matthews et al., 1985), quantitative insulin sensitivity check index (QUICKI; Katz et al., 2000), the Matsuda index (Matsuda & DeFronzo, 1999) and the glucose-insulin index (Myllynen et al., 1987; Cortez et al., 1991). The formulas for these calculations are shown in Tables 14, 15 and 16.

Area under the curve (AUC) was calculated for both glucose (AUCg) and insulin (AUCi) from OGTT by the Trapezoidal method (Purves, 1992), a method that has been validated for glucose tolerance testing (Allison et al., 1995):

$$AUC = [(\frac{t15 + t0}{2}) \chi 15(min)] + [(\frac{t30 + t15}{2}) \chi 15(min)] + [\frac{t60 + t30}{2}) \chi 30(min)]$$

where t0, t15, t30 and t60 are the values in mmol/L of glucose and pmol/L of insulin.

Total liver lipid

Total liver lipid was determined by the Folch method (1956). Liver samples were partially thawed. On day one, a 1.0 gram portion was weighed and homogenized in 22 mL 2:1 chloroform:methanol for 60 seconds using a Polytron homogenizer (PT 1020 3500, 115V, Brinkmann Instruments, Rexdale, ON). The homogenate was passed through a #1 Whatman filter into a 25 mL graduate cylinder with stopper. The volume of the eluate was recorded. Twenty percent of this volume was added as 0.73% NaCl and the mixture was shaken to form a milky suspension. The suspension was covered and allowed to separate overnight. Additionally, 25 mL glass vials were placed in a dessicator overnight for use the next day.

On day two, the volume of the lower, lipid-containing, chloroform layer was recorded and the upper, methanol layer was removed and discarded. The dried, 25 mL glass vials were weighed and a ten mL aliquot of the chloroform layer was transferred to

the vials. The chloroform was evaporated under nitrogen in a 30°C water bath (OA-SYS heating system, Organomation Associates, Berlin MA). The vials containing lipid were placed into the dessicator overnight.

On day three, the vials were weighed and lipid content was calculated:

$$\text{g lipid/g tissue} = \frac{(\text{dry weight vial+lipid}) - (\text{dry weight vial})}{\text{10 mL chloroform used}} \times \text{volume of chloroform layer}$$

Fatty acid analysis of epidydimal adipose and liver tissues

Both TAG and phospholipid (PL) fatty acid profiles were determined in adipose and liver tissue.

(i) Tissue lipid extraction

Lipids were extracted using a modified Bligh and Dyer extraction procedure (Bligh & Dyer, 1959). All solvents and the TLC plates were from Fisher Scientific (Nepean, ON). Butylated hydroxytoluene (BHT) was from Sigma-Aldrich (Oakville, ON).

A 0.25 gram sample of epididymal adipose or a 0.50 gram sample of liver was added to 10 ml of 2:1 chloroform (optima grade):methanol (pesticide grade) with 0.01% BHT. The mixture was homogenized four times by Polytron homogenizer (PT 1020 3500, 115V, Brinkmann Instruments, Rexdale, ON) for 15 seconds each and the rotor was cleaned in between to ensure complete homogenization. For adipose tissue, the solution was filtered through Whatman #4 filter paper (7 cm). The homogenate was washed with 2.3 ml of 0.73% sodium chloride prepared in double deionized water. The samples were vortexed for 30 seconds and centrifuged for 10 minutes at 1500 rpm using a GS-6 centrifuge (Beckman Instruments, Fullerton, CA). The top layer, consisting

primarily of methanol and water from each sample was removed and discarded. The remaining bottom layer was rinsed twice with 1-2 ml of theoretical upper layer composed of chloroform, methanol and water (3:48:47), which was subsequently removed and discarded. The remaining sample was transferred to a clean 15 ml tube and evaporated to dryness under nitrogen in a 30°C water bath (OA-SYS heating system, Organomation Associates, Berlin MA). Once dry, the samples were diluted in 2 ml of 2:1 chloroform:methanol with 0.01% BHT. The lipid classes of each sample were then separated using thin layer chromatography (TLC).

(ii) Thin layer chromatography (TLC)

Whatman K8 Silica Gel 80A plates were activated by heat (120°C oven) for 30 minutes. Once the plates had cooled in a dessicator for 30 minutes, they were spotted with the appropriate lipid sample.

For adipose tissue, TAG and PL samples were obtained on separate plates given the overabundance of TAG in each sample in relation to PL. For adipose TAGs, 100 μl of lipid extract, plus 50 μl internal standard (20 mg/ml C17:0 as triheptadecanoin; Nu-Check, Elsian, MN) was combined and 10 μl of this mixture was spotted onto the TLC plate. For adipose PLs, 1 ml of lipid extract, plus 50 μl internal standard (0.64 mg/ml C15:0 as 1,2-dipentadecanoyl-sn-glycero-3-phosphocoline; Avanti, Alabaster, AL) was combined, dried under nitrogen in a 30°C water bath, reconstituted in 100 μl of 2:1 chloroform:methanol and spotted onto the TLC plate in a 4 cm band.

For liver tissue, the TAG and PL samples were obtained on the same plate. For all animals, 100 μl of lipid extract was combined with 100 μl of PL internal standard (0.64 mg/ml C15:0 as 1,2-dipentadecanoyl-sn-glycero-3-phosphocoline; Avanti,

Alabaster, AL). For lean animals, the lipid extract was also combined with 10 μl of TAG internal standard (1.6 mg/ml C17:0 as triheptadecanoin; Nu-Check, Elsian, MN). For faCLA animals, the lipid extract was also combined with 25 μl of TAG internal standard (4 mg/ml C17:0 as triheptadecanoin; Nu-Check, Elsian, MN). For faCTL animals, the lipid extract was also combined with 60 μl of TAG internal standard (4 mg/ml C17:0 as triheptadecanoin; Nu-Check, Elsian, MN). All of the combined lipid extracts, plus internal standards were dried under nitrogen in a 30°C water bath, reconstituted in 50 μl 2:1 chloroform:methanol and spotted in 4 cm bands.

Once the plates were spotted, they were placed into a pre-prepared thin layer chamber. The chamber was lined with Whatman chromatography paper and soaked with a solution made up of optima grade petroleum ether, anhydrous certified A.C.S. ethyl ether and glacial acetic acid (80:20:1). The chamber was allowed to equilibrate for 30 minutes before the plates were added.

The plates were maintained in the chamber for 30-45 minutes, until the solvent had advanced to within ½ inch of the top of the plate. The plates were removed and sprayed with 0.1% 8-anilino-1-napthalen sulfonic acid (Sigma Chemical Co, St. Louis, MO). The bands representing the different lipid classes were illuminated under UV light. Using a razor blade, the PL and TAG bands were scraped from the plates into 8 ml screw-top vial containing 1.0 ml toluene with 0.01% BHT.

(iii) Methylation

The two lipid classes from each adipose and liver were methylated separately using a sodium methoxide ($NaOCH_3$) based procedure to ensure that isomerization of double bonds in the conjugated polyunsaturated fatty acids did not occur. Additionally,

the PL fraction contained sphingomyelin, and sphingomyelin is not methylated by $NaOCH_3$ (Christie, 2002). For this reason, the PL fraction from each adipose and liver sample was also methylated with methanolic hydrochloric acid (HCl). The results from the two methylation procedures were combined based on quantification by the internal standard.

$NaOCH_3$ procedure: Two mL 0.5 M $NaOCH_3$ was added to each tube. Each tube was capped, vortexed for 30 seconds and placed in a pre-heated 50°C oven for 15 minutes. Tubes were removed from the oven after 5 minutes, vortexed for 30 seconds and returned to the oven for the remaining 10 minutes. Tubes were allowed to cool for 10-15 minutes, before adding 0.1 mL acetic acid, 3.3 mL deionized H_2O and 2 mL hexane. Tubes were capped, vortexed for 30 seconds and centrifuged for ten minutes (1500-2000 rpm). The top layer from each tube was transferred to a clean 8 ml tube using a Pasteur pipette. One mL hexane was added and tubes were capped, vortexed for 15 seconds and centrifuged for ten minutes (1500-2000 rpm). Each top layer was combined with the previously removed top layer. To the combined layers, two mL of deionized H_2O was added and tubes were capped, vortexed for 15 seconds and centrifuged for 5 minutes (1500-2000 rpm). Each top layer was transferred to clean 8 ml tubes, anhydrous $NaSO_4$ was added to remove extra H_20 and each sample was filtered into a clean 8 ml tube. Samples were transferred to Target GC vials (Fisher scientific, Napean, ON, #03 377B). The samples were evaporated under nitrogen in 30 °C dry bath (Lab Line Instruments, Melrose Park, IL) and 50 µl hexane was added. Each sample was transferred to a 100 µl insert and the insert was returned to the vial. Vials were capped

with silicon lined screw-top caps (Fisher scientific, Napean, ON, #03 377 3B) and analyzed by gas chromatography (GC).

Methanolic HCl procedure: Methanolic HCl (1.2 mL of 0.5 M) was added to each tube. The tubes were capped, vortexed for 30 seconds and placed in a pre-heated 80°C oven for 2.5 hours. Tubes were allowed to cool for 10-15 minutes. One mL deionized H_2O was added to each tube and tubes were capped, vortexed for 30 seconds and centrifuged for ten minutes (1500-2000 rpm). The top layer from each tube was transferred to a clean 8 ml tube with Pasteur pipette. One mL petroleum ether was added and tubes were capped, vortexed for 15 seconds and centrifuged for ten minutes (1500-2000 rpm). The top layer was combined with the previously removed top layer. To the combined layers, two mL of deionized H_2O was added and tubes were capped, vortexed for 15 seconds and centrifuged for 5 minutes (1500-2000 rpm). The top layer was transferred to a conical GC vial with care not to include any of the bottom layer. The samples were evaporated under nitrogen in a 30 °C dry bath (Lab Line Instruments, Melrose Park, IL) and 50 µl hexane was added. Vials were capped with silicon lined lids and analyzed by gas chromatography (GC).

(iv) Gas chromatography

$NaOCH_3$-methylated samples were run on a Varian CP-3800 with FID detector (Mississauga, ON), and chrompack CP-select CB column for FAME, 100 m χ 0.25 mm diameter and 0.25 µm film thickness. The temperature program was: 45°C hold χ 4 minutes, 175°C at 13°C/minute χ 27 minutes, 190°C at 1°C/minute, 215°C at 4°C/minute χ 10 minutes, 240°C at 4°C/minute χ 5 minutes. Total run time was 83.5 minutes. All

samples were run with a 10:1 split ratio, except adipose PL samples, which were run splitless.

Methanolic HCl-methylated samples were run on a Varian 3400 with FID detector (Mississauga, ON), and J & W (Aligent) DB225MS column, 30 m χ 0.25 mm diameter and 0.25 μm film thickness. The temperature program was: 70°C hold χ 1 minute, 180°C at 20°C/minute, 220°C at 3°C/minute χ 15 minutes, 240°C at 20 °C/minute χ 10.5 minutes. Total run time was 35.0 minutes. All samples were run with 20:1 split ratio.

(v) Calculations estimating enzyme activity

Following analysis, where applicable, ratios indicating desaturase activity were calculated as follows:

Δ^9 desaturase:
total MUFA/(total MUFA+SFA)
C16:1n-7/C16:0
C18:1n-9/C18:0

Δ^6 desaturase:
C18:3n-6/C18:2-6

Δ^5 desaturase:
C20:4n-6/C20:3n-6

e) RT-PCR for analysis of mRNA

Liver tissue samples were homogenized in 1 mL TRI_{ZOL} reagent (Life Technologies, Burlington, ON, # 15596) per 50-100 mg of tissue. Samples were incubated for 5 minutes at 15 to 30°C to allow for the dissociation of nucleoprotein complexes. Chloroform (0.2 mL per 1 mL of TRI_{ZOL} reagent) was added to each sample. Each tube was capped, shaken by hand for 15 seconds and incubated at 15° to 30°C for 2-3 minutes. Samples were centrifuged at 12000 χ g for 15 minutes at 2-8°C. The RNA-

containing, colorless, aqueous phase was transferred to a new tube. The RNA was precipitated by adding 0.5 mL isopropyl alcohol per 1 mL TRI_{ZOL} reagent used in the initial homogenization. Samples were incubated at 15 to 30°C for 10 minutes and centrifuged at 12000 χ g for 10 minutes at 2-8°C. The supernate was removed. The RNA pellets were washed with 1 mL of 75% ethanol per mL of TRI_{ZOL} reagent used in the initial homogenization. Samples were vortexed and centrifuged at 7500 χ g for 5 minutes at 2-8°C. The RNA pellets were dried for 5 – 10 minute and redissolved in RNase-free water by passing the solution through a pipette tip a few times and incubating for 10 minutes at 55 to 60°C. RNA content was measured at 260 nm (1 A_{260} = 40 μg/mL).

PCR primers [Invitrogen Life Technologies, Burlington, ON, Rat ACO(S), ACO(AS), ACC(S), ACC(AS)] were based on published sequences or designed using Primer 3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi), a web-based program. Gene sequences were obtained from the Genbank database and transferred to Primer 3, which identified the best primer sequences within the parameters provided. The appropriateness of the sequences were tested with BLAST, a Genbank program that identifies sequence similarities. The sequences were: ACO(s) ttcgaggcttggaaaccact; ACO(as) ctgggcgtatttcatcagca; ACC(s) aggatcttaaggccaacgca; ACC(as) ttgggatctgcaatgtctgg.

For each gene, PCR amplification of 0.5 μg RNA was achieved using the 1-tube/2-step Promega Access RT-PCR system (Promega, Madison, WI, #A1250). Possible genomic DNA contamination was eliminated by incubating each RNAχ sample

with 1 μl DNase I, 1 μl 10χ reaction buffer and deionized water to 10 μl, for 15 min at room temperature prior to RT-PCR. To stop the reaction, 1 μl EDTA was added.

The RT-PCR mixture was be prepared by mixing 22 μl deionized water, 10 μl reaction buffer, 1 μl dNTP, 2 μl primer S, 2 μl primer AS, 2 μl $MgSO_4$, 1 μl RTranscriptase and 1 μl polymerase for each sample, and added to the RNA-DNase. The temperature program began with 45 minutes at 48 °C for reverse transcriptase and 2 minutes at 94 °C for AMV RT inactivation and RNA/cDNA/primer denaturation. Amplification was achieved by 25 cycles of 30 sec/cycle at 94°C, followed by 1 min at 62°C, and 2 min at 68°C. The final extension was completed at 68°C for 7 min. Each RNA sample was amplified in parallel with primers for a housekeeping gene (L32; Zahradka et al., 2004).

One μl of 1:500 Vistra green (Amersham, Baie d'Urfe, Quebec, #RPN5786) was added to 9 μl of RT-PCR product for each sample. Samples were incubated at room temperature for 15 min. Two μl of 6χ DNA loading buffer was added to each sample and Marker VI prior to electrophoresis on 2% agarose gels. Relative band intensity was quantified by scanning the gel (Storm Fluorimager, Amersham). Absolute values were normalized to the L32 band for comparison. Results are expressed as arbitrary units with the lnCTL group set as 1.

Statistical Analysis

Two-way ANOVA was used to detect differences due to genotype and dietary lipid and genotype χ lipid interaction (SAS 6.04, SAS Institute, Cary, NC). Duncan's multiple range test determined significant differences between means. Normality of each data set was based on the Shapiro-Wilk test. Pearson's correlation coefficient was used

for all correlation calculations. For RT-PCR data, gel effects were blocked, L32 was run as a covariate and contrasts were used to detect differences based on pre-planned comparisons. The significance level for all procedures was $p<0.05$.

Table 5. CLA and control (CTL) diet formulations per 100 grams

Ingredients (g)	CLA diet	CTL diet
Cornstarch	34.76	34.76
Maltodextrin	13.20	13.20
Sucrose	10.00	10.00
Egg white	21.25	21.25
Fiber (cellulose)	5.00	5.00
Mineral mix (zinc free)	3.50	3.50
Potassium phosphate	0.54	0.54
Vitamin mix	1.00	1.00
Choline	0.25	0.25
Biotin mix[a]	1.00	1.00
Zinc premix[b]	1.00	1.00
Tert-butylhydroquinone	0.0014	0.0014
Soybean Oil	7.00	8.50
CLA Oil	1.50	0.00
Fatty Acid Composition:		
C16:0	0.76	0.91
C18:0	0.32	0.38
C18:1n9	1.59	1.93
C18:1n7	0.10	0.12
C18:2n6	3.59	4.34
C18:3n6	0.05	0.05
C18:3n3	0.47	0.58
CLA c9,t11	0.37	0.00
CLA t8,c10	0.20	0.00
CLA c11,t13	0.23	0.00
CLA t10,c12	0.39	0.00
Totals (g):		
CLA (4 isomers identified)	1.30	0.00
all fatty acids	8.25	8.38
unidentified	0.25	0.12
cis/trans	1.20	0.00
cis/cis	3.63	4.30
trans/trans	0.03	0.00
saturated	1.13	1.37
monounsaturated	1.70	2.05
polyunsaturated	5.40	4.96
n9	1.59	1.93
n6	4.08	4.38
n3	0.47	0.58
Ratios:		
n6/n3	8.61	7.61
n9/n6	0.39	0.44

[a]Biotin premix = 200 mg/kg biotin in cornstarch
[b]Zinc premix = 5.775 g Zn carbonate + 994.2 g cornstarch

V. RESULTS

Total feed intake, body weight and adipose weight

Epididymal adipose to body weight ratio, peri-renal adipose weight, peri-renal to body weight ratio, total visceral adipose weight and total visceral to body weight ratio were approximately 1.2χ higher in *fa/fa* CLA-fed rats, compared to *fa/fa* CTL-fed rats (**Table 6**). Final body weight and total feed intake were 1.5χ higher in *fa/fa* rats compared to lean rats. As expected, epididymal adipose weight, peri-renal adipose weight and total visceral (epididymal + peri-renal) adipose weight and fat pad to body weight ratios were higher in the *fa/fa* compared to the lean genotype.

Liver steatosis and function

a) Liver weight and lipid content

Liver weight, liver to body weight ratio, total liver lipid and percent liver lipid was lower in *fa/fa* CLA-fed rats, compared to *fa/fa* CTL-fed rats. In fact, the liver to body weight ratio of the *fa/fa* CLA-fed group was not different from the lean groups (**Table 7; Figure 6**). Liver weight, liver to body weight ratio, total liver lipid and percent liver lipid, were higher in *fa/fa* compared to lean rats.

b) Hepatic fatty acid oxidation and synthesis

The faCLA rats had a trend ($p=0.0589$) towards greater ACO mRNA levels compared to faCTL rats. There was no difference in ACC mRNA levels within the *fa/fa* genotype. In lean rats, there were no differences in ACO and ACC mRNA levels (**Figure 7**).

c) Liver function: alanine aminotransferase

Fasting serum ALT was 54% lower in *fa/fa* rats fed CLA compared to *fa/fa* rats fed CTL diet. However, serum ALT was still elevated 3-fold in *fa/fa* rats fed CLA compared to the lean CTL-fed rats (**Figure 8**).

Fasting serum biochemistry

a) Glycemia and insulinemia

Fasting serum insulin and C-peptide were 65% and 54% lower, respectively in *fa/fa* CLA-fed rats compared to *fa/fa* CTL-fed rats (**Table 8**). Fasting serum glucose, insulin and C-peptide were higher in *fa/fa* rats, compared to lean rats. The insulin:C-peptide ratio was higher in the lean group compared to the *fa/fa* group.

b) Adipocytokines: leptin, adiponectin and haptoglobin

Fasting serum leptin and haptoglobin were 23% and 36% lower, respectively in *fa/fa* CLA-fed rats, compared to *fa/fa* CTL-fed rats (**Table 8**). Fasting serum leptin, adiponectin and haptoglobin were higher in *fa/fa* compared to lean rats. CLA-fed rats had higher fasting serum adiponectin compared to CTL-fed rats.

c) Lipidemia: free fatty acids, triacylglycerol and cholesterol

Fasting serum total cholesterol was 38% lower in *fa/fa* CLA-fed rats compared to *fa/fa* CTL-fed rats (**Table 8**). Fasting serum FFA, TAG and cholesterol were higher in *fa/fa* compared to lean rats.

Oral glucose tolerance testing

The *fa/fa* CLA-fed rats had lower serum glucose at t=30 minutes and lower serum insulin at all time points versus the *fa/fa* CTL-fed rats (**Figure 9; Appendix 1**). The *fa/fa* CLA-fed rats had improved insulin sensitivity compared to *fa/fa* CTL-fed rats based on HOMA and the glucose-insulin index, but not according to HOMA-IS, QUICKI and the

Matsuda index (**Table 9**). The *fa/fa* rats had higher serum glucose at t=15, 30 and 60 minutes and higher serum insulin at all time points, compared to lean rats. Measures of insulin resistance (HOMA, QUICKI, Matsuda index, glucose-insulin index) show that *fa/fa* rats have greater insulin resistance than lean rats.

Adipose and liver fatty acid composition

A summary of the fatty acid composition of adipose and liver TAG and PL is shown in **Table 10**. There were some major trends within the TAG portion of each tissue, however, the effects of CLA seemed to be lipid class, tissue and/or genotype specific.

In adipose TAG, CLA-fed rats, independent of genotype, had more SFA (e.g. C14:0, C16:0) and less MUFA (e.g. C18:1n-9, C18:1n-7; % composition) compared to control-fed rats, which was paralleled by lower Δ9 desaturase indices. Additionally, certain longer chain fatty acids (e.g. C18:2n-6, C18:3n-6, C20:4n-6) were present in lower amounts in CLA-fed compared to CTL-fed rats (**Table 11**). In liver TAG, the greatest changes were observed only in the *fa/fa* rats. For example, there were less MUFA (e.g. C16:1n-7, C18:1n-9, C18:1n-7), associating with lower Δ9 desaturase indices, and there was more of the longer chain PUFA including C20 fatty acids (e.g. C20:4n-6) and n-3 fatty acids (C18:3n-3, C22:5n-3 and C22:6n-3) in *fa/fa* rats fed CLA, compared to *fa/fa* rats fed CTL diet (**Table 12**). CLA did not alter the fatty acid composition of adipose PL (**Table 13**) or liver PL to a great extent in terms of total SFA, MUFA and PUFA, however CLA fed *fa/fa* rats had less C18:1n-9 and more C20:3n-6 in liver PL compared to CTL-fed *fa/fa* rats (**Table 14**). Overall, CLA lowered Δ9 desaturase indices in both adipose and liver. The results also suggest that in the liver of

fa/fa rats, more desaturation and elongation led to a greater relative proportion of longer chain PUFA in the TAG class.

In adipose and liver TAG and PL, the *fa/fa* rats had a higher percentage of SFA (e.g. C14:0, C16:0) compared to lean rats. In adipose and liver TAG and in liver PL, the *fa/fa* rats also had a greater percentage of MUFA (e.g. C16:1n-9, C16:1n-7, C18:n-9). The percentage of PUFA (e.g. C18:2n-6), the ratio of PUFA/SFA was lower in *fa/fa* rats compared to lean rats in TAG and PL of both tissues.

Adipose and liver PL fatty acid composition: Correlation to insulin resistance

There were positive correlations between the percent composition of SFA as part of PL and the insulin-glucose index (i.e. insulin resistance) in both adipose and liver. In liver, the MUFA and n-9 fatty acid composition of PL was also positively correlated with the glucose-insulin index. The percentage of PUFA and n-6 fatty acids found in liver PL was negatively correlated with the glucose-insulin index, while in adipose PL, only the-3 fatty acid composition was negatively associated with the glucose-insulin index (**Table 15**).

Table 6. Body weight, feed intake, adipose tissue weights and adipose tissue/body weight ratios of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

	faCLA	faCTL	lnCLA	lnCTL	Geno	Lipid	Geno x Lipid
Final Body weight (g)	552 ± 14	582 ± 12	355 ± 8	360 ± 9	<0.0001	0.1230	0.2635
Total feed intake (g)	1498 ± 40	1578 ± 35	1101 ± 34	1055 ± 28	<0.0001	0.6165	0.0740
Epididymal adipose (g)	18.9 ± 0.7	16.5 ± 0.5	6.1 ± 0.7	6.1 ±0.5	<0.0001	0.0537	0.0593
Epididymal adipose (g/100 g bwt)	3.4 ± 0.1 [a]	2.8 ± 0.1 [b]	1.7 ± 0.2 [c]	1.7 ± 0.1 [c]	<0.0001	0.0210	0.0489
Peri-renal adipose (g)	27.9 ± 1.5 [a]	24.4 ± 0.8 [b]	6.6 ± 0.7 [c]	7.4 ± 0.7 [c]	<0.0001	0.1817	0.0348
Peri-renal adipose (g/100 g bwt)	5.0 ± 0.2 [a]	4.2 ± 0.1 [b]	1.9 ± 0.2 [c]	2.1 ± 0.2 [c]	<0.0001	0.0496	0.0022
Total visceral adipose (g)	46.8 ± 2.1[a]	40.9 ± 1.2 [b]	12.8 ± 1.2[c]	13.5 ± 1.2[c]	<0.0001	0.0875	0.0282
Total visceral adipose (g/100g bwt)	8.5 ± 0.2 [a]	7.0 ± 0.2 [b]	3.6 ± 0.3 [a]	3.7 ± 0.3 [a]	<0.0001	0.0157	0.0036

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=10 rats/group; Means with different superscript letters are significantly different (P<0.05) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction.

Table 7 . Liver weight and lipid content of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks.[1]

	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
					Geno	Lipid	Geno x Lipid
Weight							
Liver (g)	17.9 ± 0.6 [b]	29.7 ± 0.9 [a]	11.1 ± 0.3 [c]	10.0 ± 0.9 [c]	<0.0001	<0.0001	<0.0001
Liver (g/100 g bwt)	3.2 ± 0.1 [b]	5.1 ± 0.1 [a]	3.1 ± 0.1 [b]	2.8 ± 0.2 [b]	<0.0001	<0.0001	<0.0001
Total Lipid[2]							
Total liver lipid (g)	1.65 ± 0.29 [b]	7.17 ± 0.49 [a]	0.52 ± 0.04 [c]	0.48 ± 0.04 [c]	<0.0001	<0.0001	<0.0001

[1]Means±SEM, bwt=body weight, TAG=triacylglycerol, PL=phospholipid, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=5 rats/group; means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction.

[2]Total liver lipid quantified by gravimetric method.

Table 8. Serum biochemistry of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks [1]

	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
					Geno	Lipid	Geno x Lipid
Glucose, Insulin & C-Peptide							
Glucose (mmol/L)	14.98 ± 0.45	13.59 ± 0.54	11.82 ± 0.57	11.77 ± 0.40	<0.0001	0.1562	0.3089
Insulin (pmol/L)	2159.1 ± 153.2[b]	6323.7 ± 973.24[a]	213.4 ± 25.20[c]	213.6± 25.22[c]	<0.0001	0.0002	0.0002
C-Peptide (pmol/L)	5677.1± 652.1[b]	12388.5 ± 1393.7[a]	1277.0 ± 195.8[c]	1337.0 ± 150.9[c]	<0.0001	0.0001	0.0001
Insulin:C-peptide	2.59 ± 0.22	2.13 ± 0.18	5.96 ± 0.73	7.30 ± 1.52	<0.0001	0.8307	0.1741
Adipocytokines							
Leptin (ng/ml)	75.32 ± 5.27[b]	97.92 ± 4.91[a]	5.24 ± 1.06[c]	6.58 ± 1.11[c]	<0.0001	0.0025	0.0065
Adiponectin (ug/ml)	14.33 ± 0.65	10.58 ± 0.54	11.78 ± 0.60	9.61 ± 0.84	0.0127	<0.0001	0.2465
Haptoglobin (mg/ml)	1.62 ± 0.12[a]	2.46 ± 0.22[b]	0.50 ± 0.05[c]	0.57 ± 0.12[c]	<0.0001	0.0123	0.0328
Lipidemia							
Free Fatty Acids (mmol/L)	0.68 ± 0.05	0.56 ± 0.03	0.26 ± 0.02	0.28 ± 0.03	<0.0001	0.4597	0.1451
Triglycerides (mmol/L)	18.03 ± 1.58	15.07 ± 0.70	3.76 ± 0.48	4.05 ± 0.43	<0.0001	0.1584	0.0861
Cholesterol (mmo/L)	6.01 ± 0.47[b]	9.59 ± 0.63[a]	2.94 ± 0.15[c]	3.38 ± 0.16[c]	<0.0001	<0.0001	0.0007

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=10 rats/group except haptoglobin n=7 faCLA, n=6 lnCLA and n=8 lnCTL; Means with different superscript letters are significantly different (P<0.05) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid= genotype x lipid interaction.

Table 9. Insulin resistance calculations based on oral glucose tolerance testing (OGTT) serum measurements in *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA [1]

Index	Calculation	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
						Geno	Lipid	Geno x Lipid
HOMA[2]	$\frac{ins0'}{22.5^{-\ln glc0'}}$	98.41± 10.33[b]	199.35± 27.53[a]	13.18 ± 1.43[c]	15.22± 2.75[c]	<0.0001	0.0023	0.0032
HOMA IS[2]	$\frac{1}{\text{HOMA-IR}}$	0.0111 ± 0.0010	0.0058± 0.0006	0.0832 ± 0.0088	0.0795 ± 0.0101	<0.0001	0.4830	0.8994
QUICKI[3]	$\frac{1}{\log(ins0') + \log(glc0')}$	2.58 ± 0.02	2.58 ± 0.04	2.83 ± 0.01	2.79 ± 0.04	<0.0001	0.5989	0.4891
Matsuda[4]	$\frac{10\,000}{\sqrt{(glu0' \times ins0')}}$ (mean OGTTglc x mean OGTTins)	0.21 ± 0.01	0.11 ± 0.01	1.51 ± 0.24	1.31 ± 0.14	<0.0001	0.1345	0.6410
AUCg (mmol*min)	Trapezoidal	701 ± 44	830 ± 38	569 ± 27	595 ± 16	<0.0001	0.0236	0.1253
AUCi (pmol*min)	Trapezoidal	63477 ± 6363[b]	122443 ± 13508[a]	10923 ± 1462[c]	12411 ± 1781[c]	<0.0001	0.0006	0.0010
Glucose-Insulin Index (pmol*mmol*min^2)[5]	AUCg x AUCi	44 ± 4[b]	99 ± 9[a]	6 ± 1[c]	8 ± 1[c]	<0.0001	<0.0001	<0.0001

[1]Means±SEM, AUC=area under the curve, AUCg=area under the curve for glucose, AUCi=area under the curve for insulin, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=10 rats/group; Means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction.
[2]Matthews et al. (1985); units used for calculations were mmol/L glucose and uU/ml insulin
[3]Katz et al. (2000); units used for calculations are mg/dL glucose and uU/ml insulin
[4]Matsuda & DeFronzo (1999); units used for calculations are mg/dL glucose and uU/ml insulin
[5]Myllynen et al. (1987); result x 10^6

Table 10. Summary of fatty acid composition results in liver and adipose triacylglycerol and phospholipid[1]

	Adipose TAG			Liver TAG			Adipose PL			Liver PL		
	Geno	Lipid	G x L	Geno	Lipid	G x L	Geno	Lipid	G x L	Geno	Lipid	G x L
C14:0	↑	↑	↑b	↑	—	—	↑	—	—	n.d.	n.d.	n.d.
C16:0	↑	↑	↑fa	↑	—	↑ln	↑	—	—	—	—	—
C16:1n-9	↑	—	—	↑	—	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C16:1n-7	↑	↓	—	↑	↓	↓fa	↑	—	—	↑	↓	—
C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↓	—	—
C18:0	—	—	—	↑	↑	↑fa	—	—	—	↑	—	—
C18:1n-9	↑	↓	↓b	↑	↓	↓fa	—	—	—	↑	—	↓fa
C18:1n-7	↑	↓	↓b	↑	↓	↓fa	—	↓	—	—	↓	—
C18:2n-6	↓	↓	↓b	↓	—	—	↓	—	—	↓	↑	—
C18:3n-6	↓	↓	—	↓	—	—	n.d.	n.d.	—	n.d.	n.d.	—
C18:3n-3	↓	↓	↓b	↓	↑	↑fa	—	—	—	n.d.	n.d.	—
CLA C18:2 c9,t11/t8,c10	↓	↑	↑b	↓	↑	↑b	—	↑	—	—	↑	—
CLA C18:2 c11,t13	↓	↑	↑b	—	↑	—	—	↑	—	↓	↑	↑ln
CLA C18:2 t10,c12	↓	↑	↑b	—	↑	—	—	↑	—	—	↑	—
C20:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↓	—	—	n.d.	n.d.	n.d.
C20:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:2n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	↓	—	—
C20:3n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↑	—	—	↑	↑	↑fa
C20:4n-6	—	↓	↓b	↓	—	↑fa	—	—	—	—	—	—
C20:5n-3	n.d.	n.d.	n.d.	↓	—	↑fa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C22:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	—	—	—
C22:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	—	—	↑	—	—

Table 10. (continued) Summary of fatty acid composition results in liver and adipose TAG and phospholipids

	Adipose TAG			Liver TAG			Adipose PL			Liver PL		
	Geno	Lipid	G x L	Geno	Lipid	G x L	Geno	Lipid	G x L	Geno	Lipid	G x L
C22:5n-3	n.d.	n.d.	n.d.	—	↑	↑fa	—	—	—	↑	↑	—
C22:6n-3	n.d.	n.d.	n.d.	—	—	↑fa	—	—	—	—	—	—
C24:0	n.d.	n.d.	n.d.	—	—	n.d.	—	—	—	—	—	—
C24:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	—	↓	—	—	—	—
Totals												
SFA	↑	↑	↑b	↑	—	—	↑	—	—	↑	—	—
MUFA	↑	↓	↓b	↑	↓	↓fa	—	—	—	↑	↓	—
PUFA	↓	—	—	↓	↑	↑fa	↓	—	—	↓	—	—
n-9	↓	↓	—	↑	↓	↓fa	—	—	—	—	—	↓fa
n-6	↑	↓	↓b	↓	—	↑fa	↓	—	—	↓	—	—
n-3	↓	↓	↓b	↓	↑	↑fa	—	—	—	—	—	—
PUFA/SFA	↓	—	—	↓	—	↑fa	↓	—	—	↓	—	—
n-9/n-6	↑	↓	—	↑	↓	↓fa	↑	—	—	↑	—	—
n-6/n-3	↓	↑	↑ln	—	↓	↓fa	↓	—	—	↓	—	—
Δ9 DS index (MUFA/SFA+MUFA)	—	↓	—	—	↓	↓b	—	—	—	—	↓	—
Δ9 DS C16:1n-7/C16:0	↑	↓	—	↑	↓	↓fa	↑	—	—	↑	↓	—
Δ9 DS C18:1n-9/c18:0	↑	↓	—	—	↓	↓fa	—	—	—	—	—	↓fa
Δ6 DS C18:3n-6/c18:2n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↑	↓	↓fa
Δ5 DS C20:4n-6/C20:3n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	↓	—	—

[1]TAG=triacylglycerol; G=genotype; L=lipid; G x L=genotype x lipid interaction; fa=*fa/fa* rats; ln=lean rats; n.d.=no data reported; ↑=fa/fa>lean or CLA-fed >control-fed,↑fa= fa/fa CLA-fed>fa/fa control-fed, ↑ln= lean CLA-fed>lean control-fed, ↑b= fa/fa CLA-fed>fa/fa control-fed and lean CLA-fed>lean control-fed; ↓=fa/fa<lean or CLA-fed <control-fed, ↓fa= fa/fa CLA-fed<fa/fa control-fed, ↓ln= lean CLA-fed<lean control-fed, ↓b= fa/fa CLA-fed<fa/fa control-fed and lean CLA-fed<lean control-fed; — = not statistically different ($p>0.05$).

Table 11. Adipose triacylglycerol fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks [1]

FATTY ACID	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
% Composition					**Geno**	**Lipid**	**Geno x Lipid**
C14:0	2.33 ± 0.03[a]	1.61 ± 0.03[b]	1.28 ± 0.10[c]	0.97 ± 0.05[d]	<0.0001	<0.0001	0.0032
C16:0	33.44 ± 0.84[a]	27.36 ± 0.30[b]	21.86 ± 1.22[c]	19.47 ± 0.59[c]	<0.0001	<0.0001	0.0365
C16:1n-9	0.44 ± 0.03	0.40 ± 0.02	0.31 ± 0.03	0.35 ± 0.01	0.0013	0.9321	0.1386
C16:1n-7	6.81 ± 0.21	8.01 ± 0.24	1.92 ± 0.20	3.23 ± 0.20	<0.0001	<0.0001	0.7881
C18:0	2.78 ± 0.13	2.85 ± 0.11	2.87 ± 0.08	2.64 ± 0.03	0.5483	0.4010	0.1410
C18:1n-9	25.56 ± 0.34[b]	31.20 ± 0.30[a]	21.51 ± 0.21[c]	24.86 ± 0.56[b]	<0.0001	<0.0001	0.0077
C18:1n-7	2.15 ± 0.04[bc]	2.93 ± 0.09[a]	1.95 ± 0.06[c]	2.33 ± 0.08[b]	<0.0001	<0.0001	0.0158
C18:2n-6	17.70 ± 0.22	20 34 ± 0.28	34.43 ± 0.83	38.77 ± 0.50	<0.0001	<0.0001	0.1198
C18:3n-6	0.13 ± 0.03	0.19 ± 0.01	0.30 ± 0.01	0.36 ± 0.01	<0.0001	0.0056	0.8729
C18:3n-3	1.59 ± 0.03[d]	1.85 ± 0.05[c]	2.52 ± 0.07[b]	3.30 ± 0.13[a]	<0.0001	<0.0001	0.0051
CLA C18:2 c9,t11/t8,c10	1.70 ± 0.04[b]	0.00 ± 0.00[c]	3.73 ± 0.25[a]	0.00 ± 0.00[c]	<0.0001	<0.0001	<0.0001
CLA C18:2 c11,t13	0.86 ± 0.03[b]	0.00 ± 0.00[c]	1.88 ± 0.07[a]	0.00 ± 0.00[c]	<0.0001	<0.0001	<0.0001
CLA C18:2 t10,c12	0.97 ± 0.04[b]	0.00 ± 0.00[c]	1.77 ± 0.05[a]	0.00 ± 0.00[c]	<0.0001	<0.0001	<0.0001
C20:4n-6	0.17 ± 0.04	0.56 ± 0.02	0.17 ± 0.05	0.68 ± 0.08	0.2682	<0.0001	0.2682
Totals							
mg TG/g adipose	610.55 ± 25.70	648.47 ± 25.52	622.79 ± 35.03	639.15 ± 21.04	0.9580	0.3321	0.6973
SFA	38.73 ± 0.90[a]	31.87 ± 0.42[b]	26.24 ± 1.35[c]	23.25 ± 0.60[d]	<0.0001	<0.0001	0.0445
MUFA	35.37 ± 0.59[b]	43.07 ± 0.45[a]	25.87 ± 0.40[d]	30.99 ± 0.60[c]	<0.0001	<0.0001	0.0238
PUFA	24.35 ± 0.36	23.93 ± 0.38	46.25 ± 1.18	44.29 ± 0.67	<0.0001	0.1205	0.3073
SAT+ MUFA+PUFA	98.45 ± 0.15	98.87 ± 0.17	98.35 ± 0.25[a]	98.53 ± 0.23	0.3003	0.1708	0.5703
n-9	19.06 ± 0.29	21.65 ± 0.29	36.93 ± 0.90	40.09 ± 0.52	0.0001	<0.0001	0.6116

Table 11. (continued) Adipose triacylglycerol fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks [1]

Totals continued							
n-6	26.14 ± 0.37[b]	31.79 ± 0.31[a]	21.90 ± 0.20[c]	25.32 ± 0.57[b]	<0.0001	<0.0001	0.0096
n-3	1.94 ± 0.05[c]	2.45 ± 0.011[b]	2.84 ± 0.19[b]	4.28 ± 0.23[a]	<0.0001	<0.0001	0.0110
CLA	4.31 ± 0.17[b]	0.00 ± 0.00[c]	8.77 ± 0.09[a]	0.00 ± 0.00[c]	<0.0001	<0.0001	<0.0001
Ratios							
MUFA/SFA	0.90 ± 0.06	1.40 ± 0.07	1.02 ± 0.06	1.44 ± 0.04	0.4864	<0.0001	0.3058
PUFA/SFA	0.62 ± 0.03	0.76 ± 0.04	1.91 ± 0.16	2.02 ± 0.07	<0.0001	0.1266	0.9894
n-9/n-6	1.36 ± 0.01	1.51 ± 0.03	0.58 ± 0.01[c]	0.65 ± 0.01	<0.0001	0.0014	0.1166
n-6/n-3	9.55 ± 0.53[c]	9.51 ± 0.20[c]	13.13 ± 0.89[a]	10.10 ± 0.67[b]	0.0006	<0.0001	0.0086
Δ9 DS index (MUFA/SFA+MUFA)	0.48 ± 0.01	0.57 ± 0.01	0.50 ± 0.02	0.57 ± 0.01	0.4410	<0.0001	0.2842
Δ9 DS C16:1n-7/C16:0	0.20 ± 0.02	0.30 ± 0.02	0.08 ± 0.01	0.17 ± 0.01	<0.0001	<0.0001	0.4713
Δ9 DS C18:1n-9/c18:0	9.27 ± 0.50	11.04 ± 0.54	7.51 ± 0.27	9.40 ± 0.45	0.0006	0.0003	0.8711

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=5 rats/group; Means with different superscript letters are significantly different (P<0.05) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction; Only fatty acids >0.25% composition are reported.

Table 12. Liver triacylglycerol fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

FATTY ACID	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
% Composition					Geno	Lipid	Geno x Lipid
C14:0	2.09 ± 0.13	1.64 ± 0.03	1.05 ± 0.07	0.87 ± 0.13	<0.0001	0.0063	0.1828
C16:0	34.49 ± 0.70[a]	36.72 ± 0.47[a]	26.61 ± 0.82[b]	23.26 ± 1.31[c]	<0.0001	0.5469	0.0057
C16:1n-9	0.55 ± 0.04	0.60 ± 0.15	0.23 ± 0.10	0.46 ± 0.12	0.0495	0.2004	0.4305
C16:1n-7	6.89 ± 0.12[b]	9.75 ± 0.42[a]	2.69 ± 0.32[c]	2.59 ± 0.76[c]	<0.0001	0.0097	0.0060
C18:0	3.37 ± 0.33[a]	2.36 ± 0.17[b]	1.64 ± 0.06[c]	1.56 ± 0.20[c]	<0.0001	0.0208	0.0447
C18:1n-9	20.61 ± 0.88[b]	32.14 ± 0.40[a]	17.60 ± 0.83[c]	19.28 ± 1.32[bc]	<0.0001	<0.0001	<0.0001
C18:1n-7	2.07 ± 0.08[b]	3.58 ± 0.36[a]	1.77 ± 0.13[b]	2.12 ± 0.20	0.0011	0.0007	0.0185
C18:2n-6	14.38 ± 0.91	8.91 ± 0.56	33.27 ± 1.25	33.53 ± 2.57	<0.0001	0.1069	0.0789
C18:3n-6	0.23 ± 0.06	0.26 ± 0.02	0.64 ± 0.03	0.72 ± 0.19	0.0005	0.5950	0.8125
C18:3n-3	1.77 ± 0.14[b]	0.54 ± 0.06[c]	2.26 ± 0.07[a]	2.19 ± 0.18[a]	<0.0001	<0.0001	0.0002
CLA C18:2 c9,t11/t8,c10	1.60 ± 0.10[b]	0.00 ± 0.00[c]	2.54 ± 0.10[a]	0.00 ± 0.00[c]	<0.0001	<0.0001	<0.0001
CLA C18:2 c11,t13	0.49 ± 0.05	0.00 ± 0.00	0.72 ± 0.19	0.00 ± 0.00	0.2455	<0.0001	0.2455
CLA C18:2 t10,c12	0.34 ± 0.10	0.00 ± 0.00	0.47 ± 0.12	0.00 ± 0.00	0.4167	<0.0001	0.4167
C20:4n-6	2.16 ± 0.37[a]	0.49 ± 0.04[b]	2.37 ± 0.19[a]	3.58 ± 0.68[a]	0.0008	0.5703	0.0024
C20:5n-3	0.50 ± 0.05[a]	0.09 ± 0.01[b]	0.46 ± 0.13[a]	0.64 ± 0.19	0.0483	0.3309	0.0215
C22:5n-3	0.74 ± 0.09[a]	0.15 ± 0.01[b]	0.58 ± 0.08[a]	0.46 ± 0.14[a]	0.4058	0.0017	0.0218
C22:6n-3	2.35 ± 0.81	0.32 ± 0.09[b]	1.61 ± 0.19[ab]	1.86 ± 0.46[a]	0.4179	0.0806	0.0293
Totals							
SFA	40.47 ± 1.10	40.92 ± 0.61	29.47 ± 0.85	26.25 ± 1.35	<0.0001	0.1899	0.0898
MUFA	30.75 ± 0.86[b]	46.53 ± 1.06[a]	23.43 ± 1.29[c]	25.63 ± 2.13[c]	<0.0001	<0.0001	0.0002
PUFA	25.12 ± 1.70[b]	10.89 ± 0.74[c]	44.91 ± 1.99[a]	43.19 ± 3.95[a]	<0.0001	0.0043	0.0190
SAT+ MUFA+PUFA	96.34 ± 1.36	98.34 ± 0.55	97.81 ± 0.67	95.07 ± 2.15	0.5119	0.7848	0.0971

Table 12. (continued) Liver triacylglycerol fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

Totals continued	faCLA	faCTL	lnCLA	lnCTL			
n-9	21.21 ± 0.87 [b]	32.85 ± 0.49 [a]	17.84 ± 0.88 [c]	19.80 ± 1.29 [bc]	<0.0001	<0.0001	<0.0001
n-6	17.77 ± 1.05 [b]	9.79 ± 0.59 [c]	37.00 ± 1.51 [a]	38.03 ± 3.32 [a]	<0.0001	0.0891	0.0323
n-3	5.37 ± 0.91 [a]	1.10 ± 0.16 [b]	4.90 ± 0.43 [a]	5.15 ± 0.75 [a]	0.0125	0.0059	0.0026
CLA	2.44 ± 0.12 [b]	0.00 ± 0.00 [c]	3.74 ± 0.33 [a]	0.00 ± 0.00 [c]	0.0018	<0.0001	0.0018
Ratios							
MUFA/SFA	0.76 ± 0.03[c]	1.14 ± 0.04[a]	0.79 ± 0.03[c]	0.97 ± 0.04[b]	0.0653	<0.0001	0.0108
PUFA/SFA	0.63 ± 0.06	0.27 ± 0.02	1.54 ± 0.11	1.68 ± 0.21	<0.0001	0.4059	0.0589
n-9/n-6	1.21 ± 0.10[b]	3.41 ± 0.25[a]	0.49 ± 0.04[c]	0.55 ± 0.08[c]	<0.0001	<0.0001	<0.0001
n-6/n-3	3.65 ± 0.52[b]	9.43 ± 1.02[a]	7.70 ± 0.46[a]	7.73 ± 0.75 [a]	0.1274	0.0010	0.0011
Δ9 DS index (MUFA/SFA+MUFA)	0.43 ± 0.01 [c]	0.53 ± 0.01 [a]	0.44 ± 0.01 [c]	0.49 ± 0.01 [b]	0.1269	<0.0001	0.0166
Δ9 DS C16:1n-7/C16:0	0.20 ± 0.01[b]	0.27 ± 0.01[a]	0.10 ± 0.01[c]	0.11 ± 0.02[c]	<0.0001	0.0253	0.0533
Δ9 DS C18:1n-9/C18:0	6.38 ± 0.71[b]	13.94 ± 1.19[a]	10.76 ± 0.59[a]	12.92 ± 1.55[a]	0.1392	0.0004	0.0238

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=5 rats/group; Means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction; Only fatty acids >0.25% composition are reported.

Table 13. Adipose phospholipid fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks [1]

FATTY ACID	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
% Composition					Geno	Lipid	Geno x Lipid
C14:0	0.84 ± 0.23	0.51 ± 0.10	0.32 ± 0.08	0.23 ± 0.07	0.0112	0.1581	0.3875
C16:0	23.38 ± 1.48	21.87 ± 0.58	16.81 ± 0.42	17.80 ± 0.54	<0.0001	0.7674	0.1667
C16:1n-7	2.32 ± 0.51	2.03 ± 0.35	0.72 ± 0.20	0.91 ± 0.14	0.0009	0.8739	0.4909
C18:0	17.39 ± 2.78	19.80 ± 2.02	18.48 ± 2.13	18.96 ± 1.88	0.9563	0.5281	0.6713
C18:1n-9	11.94 ± 2.17	12.50 ± 1.84	9.29 ± 1.53	9.63 ± 1.41	0.1366	0.8019	0.9496
C18:1n-7	1.37 ± 0.11	1.90 ± 0.15	1.27 ± 0.17	1.58 ± 0.06	0.1269	0.0051	0.4051
C18:2n-6	15.03 ± 0.96	13.51 ± 1.03	22.10 ± 1.73	21.24 ± 2.06	0.0002	0.4466	0.8329
C18:3n-3	0.67 ± 0.22	0.44 ± 0.12	0.63 ± 0.28	0.69 ± 0.25	0.6528	0.7282	0.5300
CLA C18:2 c9,t11/t8,c10	1.12 ± 0.15	0.00 ± 0.00	1.04 ± 0.25	0.00 ± 0.00	0.7868	<0.0001	0.7868
CLA C18:2 c11,t13	0.59 ± 0.10	0.00 ± 0.00	0.90 ± 0.18	0.00 ± 0.00	0.1218	<0.0001	0.1218
CLA C18:2 t10,c12	0.53 ± 0.10	0.00 ± 0.00	0.67 ± 0.14	0.00 ± 0.00	0.4485	<0.0001	0.7868
C20:0	0.20 ± 0.02	0.23 ± 0.02	0.30 ± 0.04	0.37 ± 0.05	0.0047	0.1556	0.5772
C20:2n-6	0.45 ± 0.07	0.67 ± 0.04	0.63 ± 0.08	0.67 ± 0.09	0.2034	0.0819	0.2320
C20:3n-6	0.63 ± 0.13	0.70 ± 0.07	0.36 ± 0.03	0.38 ± 0.09	0.0041	0.5834	0.8135
C20:4n-6	8.83 ± 2.17	11.26 ± 1.14	10.66 ± 1.20	11.15 ± 0.99	0.5612	0.3285	0.5138
C22:0	1.01 ± 0.17	1.13 ± 0.16	1.10 ± 0.32	1.40 ± 0.20	0.4349	0.3535	0.6941
C22:4n-6	0.52 ± 0.11	0.85 ± 0.14	0.67 ± 0.09	0.68 ± 0.08	0.9054	0.1320	0.1643
C22:5n-3	0.54 ± 0.09	0.50 ± 0.04	0.57 ± 0.09	0.42 ± 0.05	0.7468	0.2459	0.4705
C22:6n-3	3.32 ± 0.46	3.81 ± 0.58	2.58 ± 0.41	2.91 ± 0.56	0.1253	0.4272	0.8738
C24:0	1.42 ± 0.21	1.62 ± 0.20	1.80 ± 0.23	1.85 ± 0.26	0.1944	0.5948	0.7581
C24:1n-9	0.26 ± 0.06	0.53 ± 0.05	0.29 ± 0.04	0.36 ± 0.05	0.1815	0.0030	0.0567

Table 13. (continued) Adipose phospholipid fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

Totals							
mg PL/g adipose	1.07 ± 0.17	1.07 ± 0.12	1.07 ± 0.10	1.46 ± 0.33	0.3504	0.3468	0.3425
SFA	44.66 ± 1.68	45.56 ± 1.94	39.34 ± 2.39	40.76 ± 2.74	0.0369	0.6101	0.9082
MUFA	16.23 ± 2.77	17.26 ± 2.23	11.93 ± 1.87	12.81 ± 1.65	0.0613	0.6653	0.9722
PUFA	32.37 ± 1.50	31.97 ± 0.76	40.98 ± 1.27	38.42 ± 1.93	<0.0001	0.3140	0.4615
SAT+ MUFA+PUFA	93.27 ± 0.28	94.79 ± 0.25	92.25 ± 0.77	91.99 ± 0.92	0.0081	0.3328	0.1753
n-9	12.53 ± 2.15	13.33 ± 1.82	9.94 ± 1.55	10.32 ± 1.45	0.1322	0.7430	0.9093
n-6	26.08 ± 1.84	27.10 ± 1.03[b]	35.13 ± 1.06	34.21 ± 2.07	<0.0001	0.9748	0.5449
n-3	4.58 ± 0.40	4.86 ± 0.48	3.92 ± 0.32	4.21 ± 0.36	0.1161	0.4769	0.9929
CLA	2.24 ± 0.27	0.00 ± 0.00	2.60 ± 0.54	0.00 ± 0.00	0.5573	<0.0001	0.5573
Ratios							
MUFA/SFA	0.37 ± 0.07	0.39 ± 0.06	0.32 ± 0.08	0.33 ± 0.07	0.4410	0.8574	0.9726
PUFA/SFA	0.73 ± 0.03	0.71 ± 0.04	1.07 ± 0.10	0.97 ± 0.11	0.0016	0.4889	0.6499
n-9/n-6	0.51 ± 0.10	0.49 ± 0.07	0.28 ± 0.04	0.30 ± 0.03	0.0066	0.9731	0.8381
n-6/n-3	6.01 ± 0.98	5.89 ± 0.83	9.22 ± 0.82	8.45 ± 1.05	0.0065	0.6337	0.7259
Δ9 DS index MUFA/SFA+MUFA	0.26 ± 0.04	0.27 ± 0.03	0.23 ± 0.04	0.24 ± 0.04	0.4295	0.8091	0.9697
Δ9 DS C16:1n-7/C16:0	0.10 ± 0.02	0.09 ± 0.02	0.04 ± 0.01	0.05 ± 0.01	0.0040	0.8441	0.6657
Δ9 DS C18:1n-9/c18:0	0.85 ± 0.26	0.69 ± 0.14	0.60 ± 0.20	0.56 ± 0.14	0.3410	0.6170	0.7397

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=5 rats/group; Means with different superscript letters are significantly different (P<0.05) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction; Only fatty acids >0.25% composition are reported.

Table 14. Liver phospholipid fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

FATTY ACID	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
% Composition					Geno	Lipid	Geno x Lipid
C16:0	15.45 ± 1.17	16.89 ± 0.54	17.28 ± 0.45	17.18 ± 0.44	0.1593	0.3594	0.2997
C16:1n-7	0.88 ± 0.11	1.11 ± 0.07	0.32 ± 0.03	0.44 ± 0.08	<0.0001	0.0410	0.5151
C17:0	0.34 ± 0.10	0.30 ± 0.03	0.43 ± 0.04	0.49 ± 0.06	0.0460	0.9134	0.4151
C18:0	28.13 ± 0.91	26.74 ± 0.60	23.08 ± 0.50	24.49 ± 0.67	<0.0001	0.9897	0.0575
C18:1n-9	2.72 ± 0.23[b]	3.36 ± 0.16[a]	2.80 ± 0.10[b]	2.59 ± 0.11[b]	0.0444	0.2082	0.0162
C18:1n-7	1.26 ± 0.07	2.24 ± 0.23	1.39 ± 0.06	2.21 ± 0.07	0.7284	<0.0001	0.5338
C18:2n-6	8.19 ± 0.36	6.97 ± 0.45	12.38 ± 0.17	10.57 ± 0.40	<0.0001	0.0007	0.4215
CLA C18:2 c9,t11/t8,c10	0.16 ± 0.03	0.00 ± 0.00	0.21 ± 0.01	0.00 ± 0.00	0.1811	<0.0001	0.1811
CLA C18:2 c11,t13	0.21 ± 0.04[b]	0.00 ± 0.00[b]	0.54 ± 0.06[a]	0.00 ± 0.00[b]	0.0002	<0.0001	0.0002
CLA C18:2 t10,c12	0.21 ± 0.04	0.00 ± 0.00	0.19 ± 0.02	0.00 ± 0.00	0.6348	<0.0001	0.6348
C20:2n-6	0.30 ± 0.03	0.26 ± 0.03	0.37 ± 0.03	0.43 ± 0.03	0.0008	0.7094	0.1104
C20:3n-6	1.66 ± 0.10[a]	1.11 ± 0.10[b]	0.52 ± 0.05[c]	0.54 ± 0.10[c]	<0.0001	0.0102	0.0062
C20:4n-6	25.68 ± 1.44	27.45 ± 0.87	26.89 ± 0.32	27.46 ± 0.72	0.5227	0.2258	0.5281
C22:0	0.26 ± 0.03	0.30 ± 0.06	0.28 ± 0.02	0.28 ± 0.05	1.0000	0.7275	0.6635
C22:4n-6	0.45 ± 0.06	0.37 ± 0.06	0.31 ± 0.02	0.27 ± 0.03	0.0130	0.1850	0.7046
C22:5n-3	1.32 ± 0.09	1.06 ± 0.08	0.95 ± 0.04	0.76 ± 0.03	0.0001	0.0034	0.5867
C22:6n-3	8.06 ± 0.69	7.52 ± 0.44	7.58 ± 0.34	7.20 ± 0.54	0.4546	0.3825	0.8819
C24:0	0.60 ± 0.04	0.62 ± 0.04	0.65 ± 0.04	0.64 ± 0.05	0.4516	0.8793	0.8452
C24:1n-9	0.21 ± 0.04	0.34 ± 0.06	0.18 ± 0.01	0.21 ± 0.03	0.0678	0.0557	0.2189

Table 14. (continued) Liver phospholipid fatty acid composition of *fa/fa* and lean Zucker rats fed 0% or 1.5% CLA for 8 weeks[1]

Totals	faCLA	faCTL	lnCLA	lnCTL			
SFA	45.07 ± 0.41	45.18 ± 0.77	41.99 ± 0.22	43.47 ± 0.6	0.0005	0.1635	0.2261
MUFA	5.19 ± 0.40	7.17 ± 0.42	4.90 ± 1.18	5.68 ± 0.23	0.0147	0.0006	0.0837
PUFA	46.66 ± 1.93	45.09 ± 1.88	50.37 ± 0.22	47.68 ± 1.18	0.0487	0.1684	0.7081
SAT+ MUFA+PUFA	96.92 ± 1.16	97.44 ± 1.20	97.25 ± 0.16	96.83 ± 0.79	0.8826	0.9585	0.6136
n-9	3.05 ± 0.28[b]	3.83 ± 0.16[a]	3.19 ± 0.10[b]	3.03 ± 0.10[b]	0.0846	0.1036	0.0178
n-6	36.26 ± 1.19	36.01 ± 1.39	40.39 ± 0.16	38.97 ± 0.67	0.0023	0.4016	0.5571
n-3	9.73 ± 0.69	8.82 ± 0.52	8.86 ± 0.37	8.28 ± 0.55	0.2131	0.1919	0.7705
CLA	0.58 ± 0.07[b]	0.00 ± 0.00[c]	0.94 ± 0.08[a]	0.00 ± 0.00[c]	0.0041	<0.0001	0.0041
Ratios							
MUFA/SFA	0.12 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.1038	0.0015	0.0662
PUFA/SFA	1.04 ± 0.05	1.00 ± 0.06	1.20 ± 0.01	1.10 ± 0.04	0.0080	0.1304	0.4519
n-9/n-6	0.09 ± 0.01	0.11 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.0213	0.1671	0.1257
n-6/n-3	3.78 ± 0.18	4.11 ± 0.13	4.60 ± 0.22	4.77 ± 0.25	0.0021	0.2303	0.7099
Δ9 DS index	0.10 ± 0.01	0.14 ± 0.01	0.11 ± 0.00	0.12 ± 0.01	0.1116	0.0013	0.0667
Δ9 DS C16:1n-7/C16:0	0.056 ± 0.003	0.066 ± 0.004	0.018 ± 0.002	0.026 ± 0.005	<0.0001	0.0312	0.7498
Δ9 DS C18:1n-9/c18:0	0.098 ± 0.012[b]	0.126 ± 0.008[a]	0.122 ± 0.006[a]	0.106 ± 0.007[a]	0.8238	0.4843	0.0233
Δ6 DS C18:3n-6/c18:2n-6	0.009 ± 0.001[c]	0.015 ± 0.001[a]	0.009 ± 0.000[c]	0.012 ± 0.001[b]	0.0490	<0.0001	0.0425
Δ5 DS C20:4n-6/C20:3n-6	15.64 ± 1.21	25.64 ± 2.18	53.59 ± 5.71	56.18 ± 7.50	<0.0001	0.2151	0.4580

[1]Means±SEM, faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL= lean rats fed 0% CLA, n=5 rats/group; Means with different superscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (*fa/fa* vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid=genotype x lipid interaction; Only fatty acids >0.25% composition are reported.

Table 15. Adipose and liver phospholipids: correlations to the glucose-insulin index[1]

	% SFA	% MUFA	% PUFA	% n-9	% n-6	% n-3
Adipose	**0.47** (0.0472)	-0.05 (0.8344)	0.41 (0.1040)	-0.10 (0.7182)	0.06 (0.8349)	**-0.52** (0.0324)
Liver	**0.58** (0.0015)	**0.74** (0.0005)	**-0.47** (0.0491)	**0.64** (0.0045)	**-0.57** (0.0146)	0.04 (0.8740)

[1]Glucose-Insulin index = area under the curve for glucose χ area under the curve for insulin; Pearson correlation coefficients with p-values in parentheses

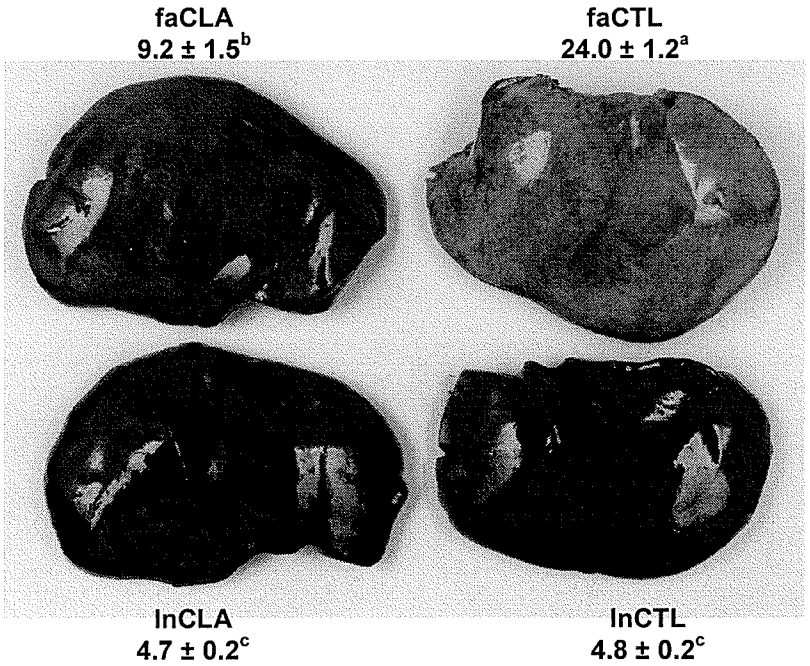


Figure 6. Hepatic steatosis and liver lipid concentration (%). The pale colour of faCTL liver portrays hepatic steatosis which was confirmed by analysis of liver lipid concentration; values are means ± SEM for n=10 rats/group; means with different subscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; faCLA=*fa/fa* rats fed 1.5% CLA, faCTL=*fa/fa* rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA and lnCTL=lean rats fed 0% CLA.

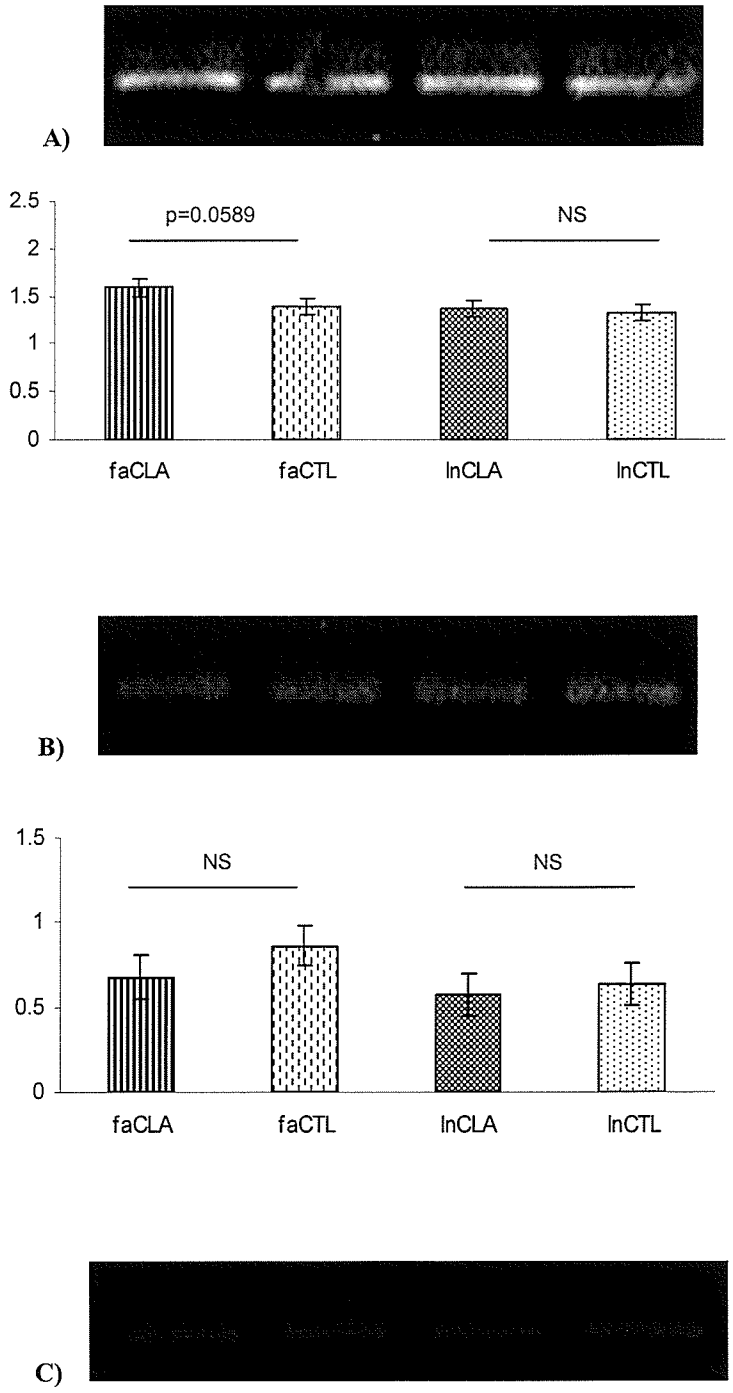


Figure 7. RT-PCR for mRNA levels of enzymes involved in A) peroxisomal fatty acid oxidation (ACO = Acyl CoA oxidase) and B) fatty acid synthesis (ACC = Acetyl CoA carboxylase). C) L32 Housekeeping gene. Results expressed as means for arbitrary units for n=10 rats/group except n=9 rats/group for L-FABP; p-values from pre-planned comparisons; faCLA=*fa/fa* rats fed 1.5% CLA, faCTL=*fa/fa* rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA and lnCTL=lean rats fed 0% CLA.

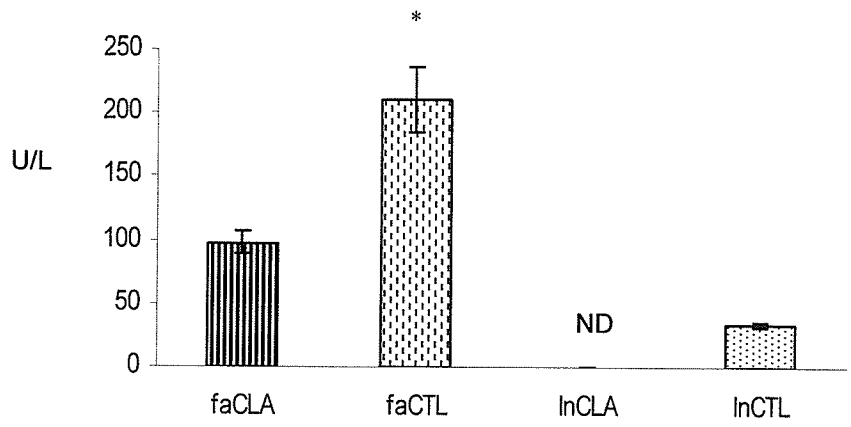


Figure 8. Fasting serum ALT (U/L). Results expressed as means ± SEM for n=10 rats/group; * denotes faCTL different from faCLA and lnCTL ($p<0.05$); faCLA=*fa/fa* rats fed 1.5% CLA, faCTL=*fa/fa* rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA and lnCTL=lean rats fed 0% CLA (pair weight group used as lnCTL); ND=not determined.

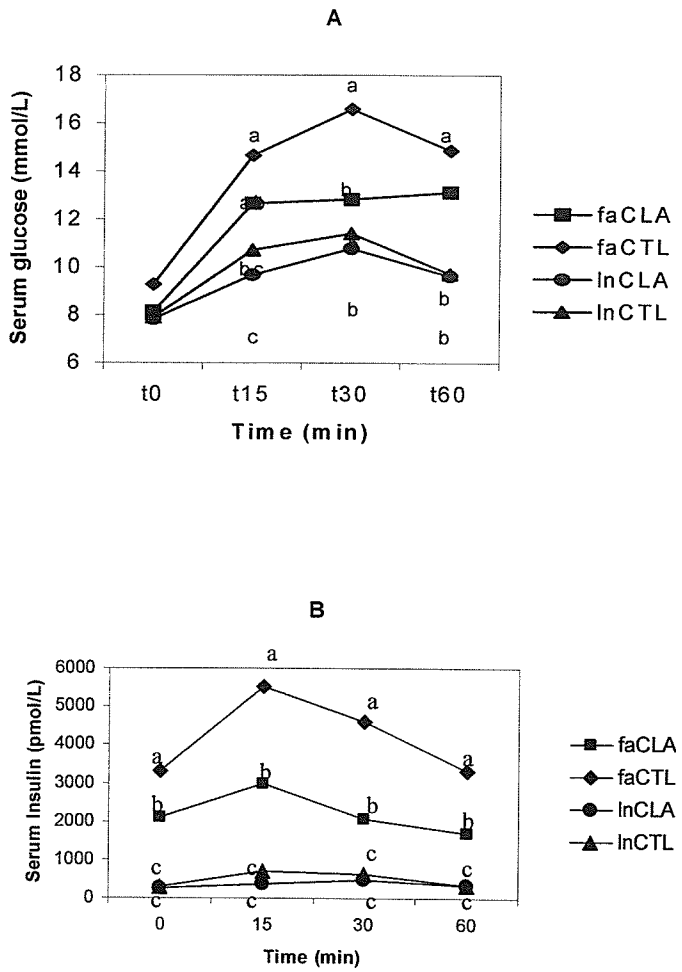


Figure 9. Oral glucose tolerance testing A) serum glucose B) serum insulin. (Results expressed as means ± SEM for n=10 rats/group; means with different subscript letters are significantly different ($p<0.05$) by Duncan's multiple range test; faCLA=*fa/fa* rats fed 1.5% CLA, faCTL=*fa/fa* rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA and lnCTL=lean rats fed 0% CLA)

VII. Discussion

A. Effect of *fa/fa* genotype

In the present study, *fa/fa* rats characteristically had greater total feed intake, final body weight, visceral adipose weight, fasting serum leptin, adiponectin, glucose, insulin, C-peptide, FFAs, TAG, and cholesterol, poorer glucose and insulin response to an oral glucose tolerance test, and greater insulin resistance compared to the lean genotype. These effects have previously been attributed to defects in the leptin receptor and unknown mechanisms, as discussed in the literature review. The *fa/fa* rats also had hepatic steatosis and higher fasting serum haptoglobin and ALT, compared to lean rats. This observation confirms that the *fa/fa* Zucker rat is an appropriate model to examine the relationship between insulin resistance, hepatic dysfunction and inflammation, although the mechanisms for these observations have not been well characterized.

The *fa/fa* rats also had different adipose and liver TAG and PL fatty acid profiles compared to lean rats. In general, the *fa/fa* rats had more SFA (%) and MUFA (%) and less PUFA (%) in adipose and liver TAG. The PUFA to SFA ratio was lower in *fa/fa* compared to lean rats in adipose and liver TAG and PL. The n-6 to n-3 ratio was lower in adipose TAG and PL and liver TAG while it was not different in liver PL in *fa/fa* rats compared to lean rats. These differences in the fatty acid composition of liver and adipose TAG and PL suggest different desaturase expression and/or activity in these tissues between genotypes. The highest tissue concentration of Δ^6 and Δ^5 desaturase in rats is in liver and Δ^5 desaturase is not found in rat adipose tissue (Brenner, 1971). Both Δ^6 and Δ^5 desaturase are deficient in animal models of type 1 diabetes (DM-1) and people with DM-1, due to insulin deficiency (Brenner, 2003; Boustani et al., 1989). In the liver

of eSS rats (DM-2 model), SCD mRNA expression and activity is higher and the expression of Δ^6 and Δ^5 desaturase is unchanged in the liver, compared to control rats (Montanaro et al., 2003). In OLETF rats (DM-2 model), Δ^5 desaturase activity is depressed compared to lean rats (Nishida et al, 2002). Desaturase activity affects the ratio of saturated to unsaturated fatty acids in the lipid bilayer in the membranes of cells. Increased desaturase activity means that more unsaturated fatty acids are produced. Incorporation of unsaturated fatty acids into membranes may increase the fluidity and/or alter the function of the cellular membranes (plasma and nuclear). This may be true in the case of insulin binding and signaling cascades (Storlein, 1996). Since adipose and liver are both insulin sensitive tissues and we saw differences in insulin resistance between *fa/fa* and lean Zucker rats during OGTT, we performed correlation statistics between the fatty acid profile of adipose and liver PL and the glucose insulin index as a marker of insulin resistance. Insulin resistance was reduced in correlation with more n-3 fatty acids and less SFA in adipose PL and with more PUFA and n-6 fatty acids and less SFA, MUFA and n-9 fatty acids in liver PL. This agrees with data from Field et al. (1988, 1990), where in rats, high PUFA to SFA ratio compared to low PUFA to SFA in adipocyte phospholipid membranes resulted in improved insulin binding. In cell culture, the membrane fatty acid composition of cells directly influences the insulin signaling system. Receptor autophosphorylation involves phosphoryation of the β-subunit of tyrosine residues. Subsequently, phosphorylated tyrosine can bind to cytoplasmic proteins (e.g. insulin receptor substrate-1 or IRS-1). In HepG2 cell culture, it was shown that increasing the amount of linoleic acid or eicosapentanoic acid in cell PL did not have an effect on insulin binding to the receptor, but it increased IRS-1 phosphorylation,

compared to control cells (Meuillet et al., 1999). Another general point is that insulin regulates the transcription of many genes and the fatty acid composition of the nuclear membrane may be important to this function (Storlein, 1996). In summary, the insulin defects present in the *fa/fa* rat may account for the effects on the fatty acid profile of insulin sensitive tissues including adipose and liver. The effects of other factors such as adipocytokine dysregulation on fatty acid metabolism have been less studied. The remaining discussion will focus on the effects of CLA supplementation or the interactions of genotype and CLA in this study.

B. Effect of CLA

a) Insulin sensitivity

This study showed that an eight week dietary intervention with a CLA mixture improved peripheral insulin sensitivity in *fa/fa* Zucker rats at 14 weeks of age based on improved glucose-insulin index and reduced fasting serum insulin and C-peptide concentrations compared to *fa/fa* rats fed control diet (Table 9; Figure 9). The glucose-insulin index was deemed relevant because there is a lack of data using QUICKI and Matsuda in the *fa/fa* Zucker rat. Improved insulin sensitivity is consistent with other reports where CLA mixtures were fed to male ZDF rats (Houseknecht et al., 1998; Ryder et al., 2001) and female *fa/fa* Zucker rats (Henricksen et al., 2003). In the study by Houseknecht, et al. (1998), CLA improved insulin sensitivity, similarly to troglitazone, a PPAR-agonist. However, this result is not consistent with various studies in mice, where CLA-feeding induced insulin resistance, likely due to the t10,c12 isomer (Hargrave et al., 2003; Tsuboyama-Kasaoka et al., 2003; Clement et al., 2002; Roche et al., 2002; Tsuboyama-Kasaoka et al., 2000). In mice, Roche et al. (2002) showed that the t10,c12

isomer down-regulated adipose GLUT4 expression along with inducing insulin resistance.

b) Adiposity

CLA-fed *fa/fa* rats had greater visceral adipose mass compared to *fa/fa* controls. This study did not demonstrate that CLA decreases adiposity in normal weight (i.e. lean) Zucker rats (Table 6). These results conflict with what others have shown in various other rodent models, including other obese/insulin resistant models such as ZDF rats, (Ryder et al., 2001) and OLETF rats (Wang et al., 2003; Rahman et al., 2003; Nagao et al., 2003). However, these results agree with data from *fa/fa* Zucker rats (Sisk et al., 2001). In fact, weight gain is a side effect of PPAR agonists such as thiazolidinediones (TZDs) that act to control insulin resistance and so the observations made in the current study are consistent with PPAR activation.

Since the completion of the current study, several reports have provided evidence that the t10,c12 CLA isomer is the anti-obesity factor in CLA diets that are fed to animal models (Degrace et al., 2004; Akahoshi et al., 2003; Chardigny et al, 2003; Czauderna et al., 2003; Henrickson et al., 2003; Warren et al., 2003; Clement et al., 2002; Hargrave et al., 2002). The actual level of t10,c12 CLA in our diet was 0.39%. It is possible, that the level of t10,c12 CLA used was not adequate to reduce peri-renal or epididymal adipose mass in our model. In another study using female *fa/fa* Zucker rats, the level of t10,c12-CLA administered (1.5 g/kg/d) was about three times higher than what was used in the present study, based on body weight and feed intake data (Henrickson et al., 2003).

The improved peripheral insulin sensitivity observed in *fa/fa* CLA-fed rats in the present study may involve other insulin sensitive tissues besides adipose. For example,

Ryder et al. (2001) showed that improved glucose tolerance in male ZDF rats fed CLA was paralleled by improved insulin-stimulated glucose uptake in isolated soleus muscles, increased muscle glucose transport, increased muscle glycogen synthase activity and normalization of muscle UCP mRNA levels. The authors note that all these effects are consistent with PPARγ activation.

Since mice are much more responsive to CLA-induced body fat reduction, the lack of functional adipose tissue can cause insulin resistance (Tsuboyama-Kasaoka et al., 2000). In fact, Hargrave et al. (2003) showed that in high- vs. low-metabolic rate mice, CLA caused greater insulin resistance in the high-metabolic rate mice – those with greater energy expenditure and greater reduction in adipose tissue mass. In mice with reduced adipose mass t10,c12 CLA lowers circulating leptin to the point of deficiency, which likely impairs its ability to aid in glucose disposal, further aggravating hyperglycemia and hyperinsulinema (Roche et al., 2002).

Direct mechanisms for the anti-obesity effect of CLA were not explored in the current study. Potential mechanism reported by others from in vivo studies include increasing the number of small adipocytes and decreasing the number of large adipocytes (Azain et al., 2000), increasing the rate of adipocyte apoptosis (Tsuboyama-Kasaoka et al., 2000; Hargrave et al., 2002), increasing energy expenditure (Azain et al., 2000; Nagao et al., 2003; Peters et al., 2001), increasing adipose UCP mRNA expression (Peters et al., 2001; Roche et al., 2002; Ealey et al., 2002; Kang et al., 2002) increasing adipose fatty acid oxidation via CPT (Rahman et al., 2001), decreasing fatty acid synthesis via FAS, ACC and/or aP2 (Tsuboyam-Kasaoka et al, 2000; Clement et al., 2002; Kang et al., 2002).

c) Circulating adipocytokines

This study showed that dietary CLA reduced fasting serum leptin in *fa/fa* Zucker rats (Table 8), despite the fact that visceral adipose tissue stores were higher, compared to *fa/fa* rats fed control diet. Leptin release may not completely dependent on the amount of adipose stores present and these results suggest reduced leptin release by adipocytes. Yamasaki et al. (2003) found no significant correlation between serum leptin and fat pad weights in male Sprague-Dawley rats. In other insulin resistant models, CLA-feeding has also decreased circulating leptin, compared to controls (Ryder et al., 2001; Wang et al., 2003; Rahman et al., 2001). Limited data in humans has failed to show an effect of CLA on plasma leptin (Medina et al., 2000; Riserus et al., 2002b; Petridou et al., 2003; Gaullier et al., 2004), although Belury et al. (2003) showed an inverse negative correlation between plasma t10,c12 CLA and plasma leptin in people with DM-2.

Given the wide range of physiological roles that leptin possesses, the leptin-lowering result by CLA in the present study has many implications. For example, leptin administration corrects insulin resistance and fatty liver in leptin deficient mice with truncated SREBP-1c (Shimomura et al., 1999). Interestingly, the SCD-1 gene is potently repressed by leptin and so leptin may also play a role in SCD-mediated effects in the liver (Kang et al., 2004), which are discussed further below.

In the present study, fasting serum adiponectin was higher in CLA-fed rats compared to control-fed rats (Table 8). Very few studies have reported the relationship between dietary CLA and circulating adiponectin. In agreement with the current study, Nagao et al. (2003) showed that CLA increased fasting plasma adiponectin in male ZDF rats compared to controls and that fasting plasma adiponectin was negatively correlated

to fasting plasma insulin. They also found that adiponectin mRNA in adipose tissue was up-regulated. Akahoshi et al. (2003) found that CLA did not have an effect on fasting serum adiponectin in Sprague-Dawley rats. Not only does decreased circulating adiponectin concentration correlate with insulin resistance in animal and human models (Yamauchi et al., 2001), adiponectin may in fact decrease lipogenesis and inflammation. Xu et al. (2003) showed that adiponectin administration to ob/ob C57BL-6 mice reduced hepatic steatosis and circulating ALT, TNF-α, TAG and FFA levels and improved glucose tolerance and insulin sensitivity, compared to untreated mice. In the same study, ethanol-induced fatty liver in FVB/n mice caused increased activity of hepatic lipid synthesis enzymes including ACC, however adiponectin administration decreased the activity of ACC without altering its mRNA expression. Xu et al. (2003) also reported that serum adiponectin and ALT were inversely correlated in obese Chinese adults, implicating an important role for adiponectin in NAFLD.

The present study showed that CLA lowered fasting serum haptoglobin in *fa/fa* CLA-fed, compared to *fa/fa* CTL-fed rats (Table 8). This indicates reduced inflammation, as haptoglobin is released by white adipose tissue and liver as an acute-phase reactant. No previous studies have reported the effect of CLA on fasting serum haptoglobin. This reduction in an inflammatory marker was in conjunction with improved insulin sensitivity in the *fa/fa* rats. Evidence for the role of oxidative stress and inflammation in insulin resistance is growing. In the only study to date looking at the effect of CLA on insulin resistance in people, Riserus et al. (2002a) showed that t10,c12 CLA increased insulin resistance in conjunction with increased F2 isoprostane excretion and increased plasma C-reactive protein, markers of oxidation and inflammation,

respectively. In a separate study, serum haptoglobin was positively associated with body fat in adults, as assessed by dual-energy x-ray absorptiometry (Chiellini et al., 2004).

Recent molecular work in genetic and diet-induced mouse models of insulin resistance have shown that chronic inflammation is part of the mechanism by which insulin resistance develops in obesity and that white adipose tissue contributes to the production of pro-inflammatory molecules. For example, Xu et al. (2003) have shown that six major inflammation genes known to function mainly in macrophages (immune cells that travel in the blood stream but are capable of entering tissues), are consistently up regulated in the white adipose tissue of ob/ob mice, db/db mice and diet-induced obese C57BL/6J mice. These observations were not made in any other tissue examined including brown adipose tissue, muscle, liver, stomach, hypothalamus, small intestine and pancreas. They also compared adipose tissue sections from wild type C57BL/6J mice and ob/ob mice, and discovered multi-nucleated cells (possibly fused macrophages) in the interstitial space of ob/ob, but not wild type mouse adipose tissue, that were not endothelial cells and that expressed the same inflammatory genes. They found that 2% of wild type and 33% of ob/ob mouse stromal vascular cells were macrophages. The trigger(s) for macrophage infiltration into adipose tissue and inflammation remain unknown. This increase in inflammatory gene expression occurred just prior to the onset of hyperinsulinemia. This could be due to the release of inflammatory cytokines, such as TNF-α, interleukin-1 and interleukin-6 by macrophages. Interestingly, in human stromal vascular cell culture, Brown et al. (2004) showed that t10,c12-CLA induced the production of interleukin-6 and interleukin-8, primarily from non-adipose cells. TNF-α has previously been implicated in the pathology of insulin resistance. It has been shown

that circulating concentrations are positively correlated with insulin resistance in people, apart from glucose tolerance (Zinman et al., 1999). Data from our laboratory, from the present study, showed that *fa/fa* CLA-fed rats had 2-3 times *less* adipose TNF-α mRNA expression compared to *fa/fa* CTL-fed rats (Yurkova et al., 2003). Furthermore, Xu et al. (2003) showed that rosiglitazone (a PPAR agonist) reduced the expression of inflammatory genes in ob/ob mice. In 3T3-L1 cell culture and db/db mouse adipose tissue, haptoglobin mRNA expression was increased by TNF-α. In 3T3-L1 cell culture haptoglobin was strongly inhibited by the PPARγ agonist, rosiglitazone (Oller do Nascimento et al., 2004; Chiellini et al., 2002). It is therefore possible, that in the current study, CLA acted as a PPAR ligand in adipose to reduce inflammation.

In summary, CLA supplementation in the present study positively modulated serum adipocytokine status compared to controls by increasing anti-inflammatory (adiponectin) and decreasing pro-inflammatory (haptoglobin) factors.

d) Lipidemia

Circulating lipids portray a balance between central (liver) lipid processing and peripheral (e.g. adipose) lipid storage and release. In the present study, CLA feeding did not have an effect on fasting serum TAG or FFAs (Table 8). The most common form of dyslipidemia in people with insulin resistance is high TAG and low HDL-cholesterol (Taskinen, 1995). In people, HDL-cholesterol carries much more TAG and is catabolized by endothelial lipase to a much greater extent in insulin resistant versus non-insulin resistant states (Rashid et al., 2002). The present study showed that CLA feeding decreased fasting serum total cholesterol if *fa/fa* rats and data from our laboratory shows that VLDL and LDL were lower, while HDL was higher in *fa/fa* CLA fed rats compared

to *fa/fa* control rats. Total cholesterol was also reduced in OLETF rats (Rahman et al., 2002) and PPARα-null mice fed CLA mixtures (Peters et al., 2001), and hamsters fed the t10,c12 isomer (Navarro et al., 2003). In OLETF rats, Wang et al. (2003) showed that CLA diets reduced hepatic microsomal TAG transfer protein activity, which is involved in lipoprotein packaging. In people, Noone et al. (2002) showed that 50:50 c9,t11:t10c12-CLA supplementation had no effect on fasting plasma HDL-, LDL- or VLDL-cholesterol in healthy adults. They also showed 80:20 c9,t11:t10c12-CLA supplementation did not affect fasting plasma HDL- and LDL-cholesterol but did lower VLDL-cholesterol in healthy adults. However, in men with the metabolic syndrome, CLA and t10,c12-CLA supplementation unfavorably decreased fasting plasma HDL-cholesterol, illustrating once again that the state of insulin resistance is an important determinant of CLA action.

e) Hepatic steatosis

The present study showed that dietary CLA was capable of ameliorating hepatic steatosis (liver TAG and total lipid concentration) in *fa/fa* Zucker rats (Table 7; Figure 6). This is in agreement with other data from CLA-fed insulin-resistant rats (Wang et al., 2003; Rahman et al., 2002; Nagao et al., 2003). Once again, this result conflicts with data from mice fed diets with a comparable total fat content (Clement et al., 2002; Tsuboyama-Kasaoka et al., 2003; Chardigny et al., 2003; Warren et al., 2003; Kelley et al., 2004; Degrace et al., 2004; Nakanishi et al., 2004; Tsuboyama-Kasaoka et al, 2000; Degrace et al., 2003; Takahashi et al., 2003). Despite this contradiction between species regarding the effect of CLA on liver lipid, it is clear that insulin resistance and hepatic steatosis are related. To illustrate the point of hormonal regulation and hepatic steatosis,

Tsuboyama-Kasaoka et al. (2003), showed that the amelioration of hepatic steatosis with diets containing 1% CLA plus increasing fat levels (4, 13 and 34%), was paralleled by reduced hyperinsulinema, hyperleptinemia and hypothyroidism.

Although we do not have any histological data on the progression of NAFLD in this study, we were able to demonstrate that fasting serum ALT was lower in *fa/fa* CLA-fed rats compared to *fa/fa* CTL-fed rats (Figure 8). The implication for reducing hepatic steatosis is to prevent the progression of the NAFLD disease spectrum. Lipid in the liver can become oxidized and result in progression to steatohepatitis and cirrhosis.

f) Hepatic gene expression

i) Fatty acid oxidation

There was a non-significant trend for elevated hepatic ACO mRNA expression in *fa/fa* CLA-fed rats compared to *fa/fa* control fed rats (Figure 7), possibly through PPAR-activation. Others have shown CLA-induced fatty acid oxidation in the liver. In mice, c9,t11-CLA and t10,c12-CLA similarly increased ACO mRNA expression (Warren et al., 2003; Degrace et al., 2004; Takahashi et al., 2003), but in one study, not to the extent of fenofibrate (PPARα agonist; Warren et al., 2003). In fact, PPARα mRNA expression was increased by both fenofibrate and c9,t11-CLA but was reduced by t10,c12 CLA. Others have shown that CLA feeding increases CPT expression and activity, a regulatory enzyme in mitochondrial fatty acid oxidation (Rahman et al., 2002; Akahoshi et al., 2003; Degrace et al., 2004; Takahashi et al., 2003). This effect may be particular to t10,c12-CLA in mice (Degrace et al., 2003). These data suggest that the mechanism by which t10,c12-CLA induces or protects against hepatic steatosis in mice and rats, respectively,

is independent of PPARα and fatty acid oxidation. Regulation of hepatic fatty acid synthesis may play a greater role.

ii) Fatty acid synthesis

In the present study, CLA feeding did not significantly reduce ACC mRNA expression in *fa/fa* rats (Figure 7), however there was a high variability among animals. To date, no other studies have reported liver ACC activity in rats fed CLA, however in OLETF rats, CLA diet decreased the activity of microsomal phosphatidate phosphohydrolase activity, a marker of TAG synthesis (Rahman et al., 2002).

ACC is the rate limiting enzyme in long chain fatty acid synthesis (Figure 3). ACC is regulated by SREBPs. The c9,t11 isomer reduced hepatic SREBP-1c and LXRα mRNA expression in ob/ob mice. The t10,c12 isomer had no effect on hepatic and adipose SREBP-1c or on LXRα (Roche et al., 2002). In normal weight, C57BL/6J mice, CLA had no effect on hepatic SREBP-1c, SREBP2 or LXR mRNA in one study (Clement et al., 2002), but increased hepatic SREBP-1, and its target genes (ACC and/or SCD) in others (Tsuboyama-Kasaoka et al., 2003; Degrace et al., 2004; Takahashi et al., 2003). To note, increased SREBP-1, ACC and SCD mRNA expression were not apparent in mice fed higher fat (34% vs. 4%) diet in one of these studies (Tsuboyama-Kasaoka et al., 2003). In vitro, CLA treatment of SKBR-3 cells (human cancer cells), inhibited FAS activity to a greater extent than a variety of other fatty acids, and CLA did not have an effect on ACC (Oku et al., 2003). Even though we did not observe an effect of CLA on ACC mRNA expression, CLA may have had an effect on ACC phosphorylation, protein expression and/or activity, or on other fatty acid synthesis enzymes (e.g. FAS).

g) Fatty acid profiles

i) Adipose TAG fatty acids

CLA was present as 4.31% and 8.77% of fatty acids in adipose TAG in the *fa/fa* and lean genotypes, respectively (Table 11). It has not previously been reported that CLA is incorporated less into tissues of insulin-resistant compared to non-insulin resistant animals. Notably, the current study provides indirect evidence that CLA-feeding reduces Δ^9 desaturase/SCD activity in adipose TAG, reflected by lower MUFA/SFA and Δ^9 desaturase indexes. SCD is the enzyme responsible for catalyzing Δ^9-cis desaturation of fatty acyl coA substrates such as palmitoyl CoA and stearoyl CoA (Ntambi et al., 1999). It has been shown in other models that CLA reduces SCD mRNA expression and/or activity, including in 3T3-L1 adipocytes (Lee et al., 1998; Choi et al., 2000) and pig adipose tissue (Smith et al., 2002), which reduces the level of monounsaturated fatty acids in the cell. Since SCD catalyzes the rate limiting step in MUFA synthesis and MUFA are required for normal rats of TAG, cholesterol ester and PL synthesis, it would be reasonable to think that inhibiting SCD would actually reduce adiposity. We did not however, observe a change in adiposity in the lean rats and in the *fa/fa* rats, CLA actually increased adiposity according to the ratios of fat stores to body weight (Table 6). This may suggest that de novo synthesis is less important than fatty acid import.

ii) Adipose PL fatty acids

In the present study, CLA represented 2.2% and 2.6% of fatty acids in adipose PL in *fa/fa* and lean rats, respectively (Table 13). The c9,t11 isomer seemed to be present in higher amounts than the t10,c12 isomer, although this can not be conclusively stated

because c9,t11 and t8,c10 overlap on the GC chromatogram. CLA-feeding did not alter lipid profile in terms of total SFA, MUFA, PUFA, n9, n6, n-3 and ratios calculating desaturase activity in adipose PL (Table 13), in CLA- versus control-fed rats within genotypes. The ability of CLA to improve peripheral insulin sensitivity was not mediated through changes in adipose phospholipid fatty acid composition, based on these results.

In 3T3-L1 adipocytes, the t10,c12 isomer decreased the amount of monounsaturated fatty acids in PL (Evans et al., 2001), but this was not evident in this study. Banni et al. (1999) found that rats fed 1% CLA had 50% less C20:4n-6 (arachidonic acid) in adipose tissue than control-fed rats. This agrees the present study where C20:4n-6 was present at about a third of the amount in the adipose TAG of CLA-fed rats compared to control-fed rats. However, there was no difference between CLA-fed and CTL-fed rats with regard to the amount of C20:4n-6 in adipose PL. This is perhaps more relevant to eicosanoid metabolism, which is discussed below.

iii) Liver TAG fatty acids

CLA represented 2.44% and 3.74% of fatty acids in the liver TAG of *fa/fa* and lean rats, respectively, fed CLA diet (Table 12). As in adipose TAG, the percent composition of CLA was lower in the *fa/fa* rats compared to the lean rats, a result that has not been previously reported. The c9,t11 isomer seemed to be preferentially incorporated into liver TAG, compared to the t10,c12 isomer.

CLA-feeding did change the fatty acid profile of liver TAG in *fa/fa* rats, by decreasing total MUFA and increasing total PUFA, but not altering total SFA. The same effect was seen in the liver TAG of mice fed c9,t11 CLA. However, the opposite effect was seen in the liver TAG of mice fed t10,c12 CLA (Kelley et al., 2004). This suggests

differential regulation of SCD by the two isomers. In our study, both isomers were fed in equal amounts.

As in adipose, the current study indirectly shows that CLA decreased liver Δ^9 desaturase/SCD activity (via calculation of Δ^9 desaturase indexes) in liver of *fa/fa* CLA-fed rats compared to *fa/fa* control-fed rats (Table 12). SCD inactivation may protect against hepatic steatosis as SCD (+/+) mice have greater liver lipid content than SCD (-/-) mice (Kang et al., 2004). It is known that SCD plays a key role in hepatic TAG and VLDL synthesis. This is because C18:1 (product of SCD activity) is essential for TAG and cholesterol ester synthesis – two lipids that are need for VLDL synthesis. SCD-1 deficient ob/ob mice have less adiposity and complete correction of hepatic steatosis, compared to ob/ob mice. SCD deficient mice display decreased lipid synthesis (e.g. decreased ACC mRNA) and increased lipid oxidation (e.g. increased ACO mRNA), independent of PPARα (Dobrzyn & Ntambi, 2004; Miyazaki et al., 2004). CLA decreases liver SCD mRNA expression in mice (Lee et al., 1998). Decreased ratios of C16:1 to C16:0 and C18:1 to C18:0 have been observed in the liver TAG of male ICR mice (Lee et al., 1998) and rats (Banni et al, 2001, Moya-Camerena et al, 1998; Sisk et al, 2001) fed a CLA mixture, compared to controls.

As well, in the current study, CLA feeding elevated the percentages of C18:3n-3, C20:4n-6, C20:5n-3, C22:5n-3 and C22:6n-3 be 3.5-5.5-fold in liver TAG of *fa/fa* rats compared to lean rats (Table 12). This suggests increased expression and/or activity of Δ^6 desaturase, Δ^5 desaturase, and/or enzymes of Sprecher's pathway for DHA biosynthesis (Figure 3). Matsuzaka et al. (2002) have shown that hepatic Δ^6 and Δ^5 desaturase gene expression in C57BL/6J mice are regulated differentially by SREBP-1

and PPARα. C57BL/6J mice are highly susceptible to diet-induced obesity and DM-2 (Jackson Laboratory, 2004). They studied the activation of Δ^6 and Δ^5 desaturase in SREBP-1a, -1c and -2 transgenic and wild-type mice. They showed that hepatic Δ^6 and Δ^5 desaturase mRNA expression were increased in all types of SREBP transgenic mice compared to wild-type mice, most prominently in the mice over-expressing SREBP-1a and -1c. Furthermore, they confirmed that PUFA (C18:2n-6, C22:5n-3, C22:6n-3) feeding suppressed Δ^6 and Δ^5 desaturase mRNA in wild-type mice, but showed that in SREBP-1c transgenic mice PUFA were not able to suppress Δ^6 and Δ^5 desaturase mRNA. This same study also showed that fibrate (PPARα agonist)-treated mice had increased expression of both Δ^6 and Δ^5 desaturase mRNA. In conclusion, SREBP-1 isoforms and PPARα activators up-regulate the expression of Δ^6 and Δ^5 desaturase mRNA during lipogenesis. In theory, since PUFA normally inhibit lipogenesis, SREBP-1 induction of desaturases would actually aid in the regulation of lipogenesis (i.e. suppress lipogenesis when adequate PUFA are available; Nakamura & Nara, 2003). It has been shown that human Δ^6 desaturase contains a sterol regulatory element (Nara et al., 2002) and a functional direct-repeat-1 element that is responsive to PPARα (Tang et al., 2003). It is possible that in the current study, CLA activated SREBP-1 and/or PPARα in the liver, which led to increased mRNA expression of Δ^6 and Δ^5 desaturase and this would explain why there were higher amounts of twenty-carbon fatty acids (C20:4n-6 and C20:5n-3) and long chain n-3 fatty acids (C22:5n-3 and C22:6n-3) in liver tissue of *fa/fa* rats fed CLA, whose liver TAG concentrations of these fatty acids are normalized based on lean animals.

iv) Liver PL fatty acids

CLA represented 0.58% and 0.94% of fatty acids in the liver PL of *fa/fa* and lean rats, respectively, fed the CLA diet (Table 14). There did not appear to be a difference between the incorporation of c9,t11- and t10,c12-CLA into liver PL. Others have suggested that dietary CLA may function to alter phospholipid membrane structure, C20:4n-6 metabolism and eicosanoid metabolism (Atkinson, 1999). Since CLA can be a substrate for Δ^6 desaturase it competes with linoleic acid for this enzyme. CLA is desaturated and elongated by the same pathway as linoleic acid. For example, metabolites including conjugated C18:3, conjugated C20:3 (eicosatrienoic acid) and conjugated C20:4 (eicosatetranoic acid) were identified in the liver of rats (Banni et al., 1995; Banni et al., 2001) and in the adipose and serum of humans (Banni et al, 1999). In rat liver, it has been shown that c9, t11 CLA is converted to conjugated c6,c9,t11-18:3, c8,c11,t13-20:3 and c5,c8,c11,t13 20:4 while, t10, c12 CLA is converted to c6,t10,c12-18:3, c8,t12,c14-20:3 and c5,c8,c11,t13-20:4 (Banni et al., 2001; Sebedio et al., 2001). It appears that CLA is metabolized to the same extent as linoleic acid as analyzed by ^{14}C labeling (Belury & Kempa-Steczko, 1997). These products may compete with other substrates for phospholipases, cyclooxygenases (COX) and lipoxygenases (LPOX) and thus influence eicosanoid production (Belury & Kempa-Steczko, 1997; Banni et al., 1999). Our results suggest, but do not show, that CLA was not acting in the liver to affect eicosanoid metabolism through C20:4n-6. In the present study, the amount of C20:4n-6 in the liver TAG of *fa/fa* rats was higher in those fed CLA versus controls, however CLA did not affect C20:4n-6 in liver PL. C20:4n-6 is a substrate for COX and LPOX and the precursor for the 2-series prostaglandins and thromboxanes and 4-series

leukotrienes (Shils et al., 1999), which has many implications for metabolism. CLA did reduce C20:4n-6 in total liver lipids in 42 day old female SENCAR mice fed 1% and 1.5% CLA for 6 weeks, compared to control-fed mice (Belury et al. 1997). In vitro studies have also shown that CLA decreases C20:4n-6 content by half. However, Banni et al. (1999) showed that CLA-feeding had no effect on C20:4n-6 in total liver lipid of rats. In mice t10,c12-CLA, but not c9,t11-CLA, decreased C20:4n-6 in liver TAG but neither CLA-isomer affected the composition of C20:4n-6 in liver PL (Kelley et al., 2004), which again is likely more relevant to eicosanoid metabolism and agrees with our results.

The concentration of Δ^6 desaturase in rat liver microsomes, similarily to humans, is high, compared to other tissues (Brenner, 1971; Cho et al., 1999). It has been reported that CLA decreases the Δ^6 desaturation of linoleic acid in vitro in liver microsomes (Bretillon et al., 1999) and t10,c12-CLA reduces Δ^{9-}, Δ^{6-} and Δ^{5-} desaturation in HepG2 cells (Eder et al., 2002), however our results suggest that the opposite is occurring in the Zucker *fa/fa* rat. This may be important because Nakanishi et al. (2004) showed that C18:3n-6 (the product of linoleic acid Δ^6 desaturation) plus CLA supplementation can prevent the steatosis inflicted by CLA supplementation alone, in mice, associated with increased prostaglandin E_2 content in the liver. Prostaglandin E_2 may suppress hepatic lipogenesis by inhibiting the transcription of genes important in this pathway (Michelle et al., 1999). In this study, there were no differences in the relative percent of C18:3n-6 (data not shown) or 20:4n-6 in liver PL (Table 14), but CLA did elevate the relative percent of 20:3n-6 in liver PL of *fa/fa* rats compared to those fed control diet, further suggesting increased activity of elongase and/or Δ^6 desaturase. C20:3n-6 is also a

substrate for COX and LPOX a precursor for the 1-series prostaglandins and 3-series leukotrienes. In HepG2 cells, t10,c12-CLA treatment resulted in greater release of prostaglandins ($F_{1\alpha}$ and $F_{2\alpha}$) compared to linoleic acid-treated control cells, despite lowering the composition of C20:4n-6. The authors hypothesized that t10,c12-CLA could cause deacylation of fatty acids from PLs, thus lowering their content in PL and making the FFAs available for eicosanoid production (Eder et al., 2002). We do not have data on PL subfractions or FFA composition to further comment.

SUMMARY

In summary, dietary CLA improved peripheral insulin sensitivity and favourably modified circulating adipocytokine status in the *fa/fa* Zucker rat, in a mechanism independent of reducing adiposity or changing the fatty acid profile in adipose PL. CLA reduced hepatic steatosis in the *fa/fa* Zucker rat, concomitant with a trend for increased PPAR-responsive ACO mRNA expression and altered liver fatty acid profiles that suggested a differential effect on liver desaturase activity. Additionally, dietary CLA reduced circulating ALT and haptoglobin in the *fa/fa* Zucker rat, suggesting improved liver function and reduced overall inflammation. Circulating FFA and TAG status was not modified by CLA feeding, but total serum cholesterol was lowered, in the *fa/fa* Zucker rat.

VIII. CONCLUSIONS

- The *fa/fa* genotype exhibits characteristics of obesity, insulin resistance and NAFLD. The *fa/fa*, compared to the lean genotype, exhibited:
    - Greater body weight, feed intake, visceral adipose weights
    - Greater calculated insulin resistance, fasting serum glucose, insulin and C-peptide and reduced insulin:C-peptide
    - Greater fasting serum leptin, adiponectin, FFAs, TAG and cholesterol
    - Greater SFA and MUFA, and reduced PUFA in TAG and PL of adipose and liver tissue
    - Greater n-9/n-6 and reduced n-6/n-3 fatty acid ratios in adipose TAG and PL and liver PL
    - Greater liver weight, TAG content and total lipid content
    - Greater fasting serum ALT and haptoglobin
    - Greater ACO and ACC mRNA expression in liver tissue
- Dietary CLA modifies characteristics of obesity, insulin resistance and NAFLD in the *fa/fa* genotype. The *fa/fa* CLA fed-rats, compared to the *fa/fa* CTL-fed rats exhibited:
    - Greater visceral adipose/body weight ratios and peri-renal fat weight
    - Greater fasting serum adiponectin and lesser fasting serum leptin
    - Lower fasting serum cholesterol
    - Improved insulin sensitivity and lower fasting serum insulin and C-peptide
    - Lower percent composition of C20:4 n-6 in adipose TAG
    - Greater SFA and lesser MUFA in adipose TAG, concomitant with indirect evidence for reduced Δ^9 desaturase/SCD activity

- Lesser MUFA and greater PUFA in liver TAG, concomitant with indirect evidence for reduced Δ^9 desaturase activity and greater Δ^6 and Δ^5 desaturase activity and/or enzymes of Sprecher's pathway
- Lower MUFA in liver PL, concomitant with indirect evidence for reduced Δ^9 desaturase activity
- Lower liver weight, liver TAG content and total liver lipid
- Lower fasting serum ALT and haptoglobin
- Trend for greater ACO mRNA expression

- Many of the effects of CLA described within the *fa/fa* genotype, were not apparent within the lean genotype. The exceptions were that the lean CLA-fed, compared to the lean CTL-fed rats exhibited:
  - Reduced C20:4n-6 in adipose TAG, increased SFA and reduced MUFA in adipose TAG, and increased SFA in liver TAG, and indirect evidence for reduced Δ9 desaturase (SCD) activity
  - Altered Δ6 desaturase activity in liver, based on liver PL fatty acid composition
  - Increased fasting serum adiponectin

This suggests that the effects of CLA on the desaturases and adiponectin may be independent of the metabolic derangements of the *fa/fa* Zucker rats (described in the first point).

IX. LIMITATIONS

- The animals in this study were growing, and therefore not in energy balance – this must be considered before the relevancy to adult human models can be speculated.
- The isomers of CLA were not tested independently, therefore, it is not known if one was more active than another.
- The amount of CLA fed to animals on a per kilogram basis was much higher than the amount of CLA a person would consume through dietary sources or compared to human CLA supplementation studies.
- Transcription factors such as PPARs and SREBPs were not directly measured
- Data suggesting a change in desaturase activity is not backed up by gene and protein expression data.
- Data on gene expression is not backed up with data for protein expression and enzyme activity.
- Histological data that would indicate any progression of NAFLD within the *fa/fa* rat was not collected, to support the data for fasting serum ALT.
- An eight-week study is relatively short-term, although adequate to observe effects.

X. FUTURE RESEARCH

The effect of CLA mixture vs. c9,t11 CLA vs. t10,c12 CLA on:

- liver Δ^9, Δ^6 and Δ^5 desaturase mRNA and protein expression
- eicosanoid production (e.g. PGE_2 given evidence that it has been shown to reduce the extent of hepatic steatosis)
- mRNA, protein expression and activity of proteins involved in the insulin signaling cascade and membrane glucose transport in insulin sensitive tissues, such as adipose and muscle
- expression of inflammatory genes and presence of macrophages in white adipose tissue
- oxidation products in circulation and in the liver
- expression of PPARγ in adipose tissue and PPARα in liver tissue
- ACO protein expression in liver
- ACC protein expression and phosphorylation in liver
- mRNA and protein expression of other hepatic fatty acid synthesis enzymes (e.g. FAS)
- Liver and adipose PPAR mRNA expression
- Liver and adipose SREBP mRNA and protein expression
- Lipoprotein subfractions of cholesterol in fasting serum

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X. Appendices

Appendix 1. Mean serum glucose and insulin in fa/fa and lean Zucker rats fed 0% or 1.5% CLA during Oral Glucose Tolerance Testing (OGTT)[1]

	faCLA	faCTL	lnCLA	lnCTL	Pr>F		
					Geno	Lipid	Geno x Lipid
Glucose (mmol/L)							
t=0	8.13 ± 0.29[a]	9.28 ± 0.87[a]	7.84 ± 0.41[a]	7.90 ± 0.49[a]	0.1598	0.3091	0.3619
t=15	12.66 ± 0.90[ab]	14.66 ± 0.79[a]	9.68 ± 0.70[c]	10.72 ± 0.35[bc]	<0.0001	0.0400	0.5070
t=30	12.84 ± 1.21[b]	16.59 ± 0.67[a]	10.78 ± 0.56[b]	11.42 ± 1.49[b]	<0.0001	0.0085	0.0556
t=60	13.13 ± 1.01[a]	14.86 ± 1.87[a]	9.64 ± 0.59[b]	9.73 ± 0.59[b]	0.0004	0.4096	0.4577
Insulin (pmol/L)							
t=0	2096.11 ± 248.20[b]	3331.23 ± 294.33[a]	245.14 ± 23.09[c]	301.17 ± 39.66[c]	<0.0001	0.0042	0.0078
t=15	2966.33 ± 529.84[b]	5528.26 ± 580.24[a]	360.13 ± 44.98[c]	690.64 ± 90.23[c]	0.0127	0.0022	0.0124
t=30	2055.68 ± 149.48[b]	4599.16 ± 720.93[a]	475.70 ± 54.74[c]	615.88 ± 126.24[c]	<0.0001	0.0042	0.0087
t=60	1680.81 ± 113.94[b]	3319.00 ± 267.82[a]	335.85 ± 33.83[c]	341.15 ± 42.11[c]	<0.0001	<0.0001	<0.0001

[1]Means±SEM; faCLA=fa/fa rats fed 1.5% CLA, faCTL=fa/fa rats fed 0% CLA, lnCLA=lean rats fed 1.5% CLA, lnCTL=lean rats fed 0% CLA, serum glucose n=10 rats/ diet group except: t=0 n=9 lnCTL, t=60 n=9 faCTL; serum insulin: n=10 rats/diet group except t=0 and t=15 lnCLA and lnCTL n=9, t=30 faCTL n=8, t=60 n=9 faCLA, n=8 faCTL, lnCLA and lnCTL; Means with difference superscript letters are significantly different (P<0.05) by Duncan's multiple range test; Main effects from ANOVA: Geno=genotype (fa/fa vs. lean rats), Lipid (0% vs. 1.5% CLA), and Geno x Lipid = Genotype x Lipid interaction