The Effect of Butter, Naturally Enriched with Trans-10, Cis-12 Conjugated Linoleic Acid, on Insulin Resistance and Inflammation in High Fat-Fed Rodents

by

Amanda Stefanson

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Human Health & Nutritional Sciences

Guelph, Ontario, Canada

ABSTRACT

THE EFFECT OF BUTTER, NATURALLY ENRICHED WITH TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID, ON INSULIN RESISTANCE AND INFLAMMATION IN HIGH FAT-FED RODENTS

Amanda Stefanson
University of Guelph, 2013

Advisor:
Professor D C Wright

This study was conducted to evaluate dietary $CLA_{t10,c12}$, given the studies showing that supplemental doses has a negative impact on insulin resistance. To that end, we produced a 60% high fat diet composed of butter produced from milk collected from dairy cows suffering from subacute ruminal acidosis (SARA) – a condition that can sometimes result from the dairy industry production practice of feeding a high grain, low forage diet to milk-producing cows. This butter represents the most $CLA_{t10,c12}$-enriched source of naturally occurring $CLA_{t10,c12}$ for humans. We compared the effects of the high $CLA_{t10,c12}$ butter against a commercially available butter in a high fat diet-induced rodent model of insulin resistance. After 8 weeks, whole body glucose homeostasis was evaluated along with markers of inflammation, indicated by mitogen-activated protein kinase (MAPK) activation.

Acknowledgments

I would like to thank Dr. David Wright for his support, guidance, and good humor throughout this project. His positive attitude and advising style ensured that even the difficult times were a pleasure. I would also like to extend my thanks Dr. David Dyck and Dr. Brian McBride for their important contributions to this project. Tara MacDonald deserves special thanks for all her patient help in the lab, but to all my wonderful lab mates – it has been a joy to work with you all!

Table of Contents

ABSTRACT II

ACKNOWLEDGMENTS III

TABLE OF CONTENTS IV

LIST OF TABLES VI

LIST OF FIGURES VI

LIST OF ABBREVIATIONS VIII

CHAPTER ONE: LITERATURE REVIEW 1

DIABETES, OBESITY AND INSULIN RESISTANCE 1

CELL SIGNALING PATHWAYS INVOLVED IN INSULIN RESISTANCE 1

Overview of insulin signaling 1

Overview of mitogen-activated protein kinase-mediated pathways 3

Interference with insulin signaling by mitogen-activated protein kinases 4

HIGH FAT DIET INDUCED INSULIN RESISTANCE AND INFLAMMATION 6

Early development of insulin resistance and over-nutrition 7

Endoplasmic reticulum stress 7

Intermediate development of insulin resistance and increasing adiposity 9

Free fatty acids, insulin resistance and toll-like receptors 10

Later development of insulin resistance and established adiposity 11

Inflammation, insulin resistance and tumor necrosis factor-α 12

CONJUGATED LINOLEIC ACIDS 13

Origins of conjugated linoleic acids 13

HEALTH EFFECTS OF CLA 14

Effects of CLA on body composition and insulin resistance in at the whole body level in rodent models 15

Molecular mechanisms of CLA action 16

Health effects of CLA in human clinical trials 18

Human dietary intake of CLA 19

Effect of $CLA_{t10,c12}$ in healthy adults 19

Effect of $CLA_{t10,c12}$ in obese adults 20

CHAPTER TWO: INTRODUCTION 22

CHAPTER THREE: METHODS 24

DIET PREPARATION 24

ANIMAL, HOUSING & EXPERIMENTAL GROUPS 26

WHOLE BODY INSULIN SENSITIVITY TESTS 27

Glucose tolerance test 27

Insulin tolerance test 28

Pyruvate tolerance test 29

TISSUE HARVESTING 29

WESTERN BLOT ANALYSIS 30

STATISTICAL AND OTHER ANALYSIS 31

CHAPTER 4: RESULTS ____ **33**
RODENT DIETS ____ 33
BODY WEIGHT AND FOOD CONSUMPTION ____ 37
WHOLE BODY GLUCOSE TOLERANCE ____ 42
Glucose tolerance test results ____ *42*
Insulin tolerance test results ____ *46*
Pyruvate tolerance test results ____ *50*
MOLECULAR MARKERS OF CELL STRESS AND INFLAMMATION ____ 54
CHAPTER FIVE: DISCUSSION ____ **60**
CHAPTER SIX: INTEGRATIVE DISCUSSION ____ **66**
REFERENCES ____ **70**
APPENDIX A ____ **82**
APPENDIX B ____ **85**

List of Tables

Table 1: Diet compositions 25

Table 2: Fatty acid profile comparison of butters (g/100 g) 34

Table 3: Blood glucose values (mmol/L) at each time point of a glucose tolerance test . 45

Table 4: Blood glucose values (mmol/L) of each time point of an insulin tolerance test 49

Table 5: Blood glucose values (mmol/L) of each time point of a pyruvate tolerance test 53

Table 6: Results of unpaired Student's t-test comparison of blood glucose values between commercial butter and NonSARA butter-fed rodents 53

List of Figures

Figure 1: Simplified insulin signaling cascade 3

Figure 2: MAPK signaling cascades 6

Figure 3: Experimental design of the current study 27

Figure 4: Growth curve of rats fed either low or high fat diets for 8 weeks 38

Figure 5: Final body weight of rats fed either low or high fat diets for 8 weeks 39

Figure 6: Mean daily food intake of rodents fed either a low or high fat diet for 8 weeks 40

Figure 7: Mean daily food intake in kcal of rats fed either low or high fat diets for 8 weeks 41

Figure 8: Glucose tolerance test results for rats fed either a low or high fat diet for 8 weeks 43

Figure 9: Total area under the curve for glucose tolerance test 44

Figure 10: Insulin tolerance test results for rats fed either a low or high fat diet for 8 weeks 47

Figure 11: Total area under the curve for insulin tolerance test 48

Figure 12: Pyruvate tolerance test for rats fed either a low or high fat diet for 8 weeks.. 51

Figure 13: Total area under the curve for pyruvate tolerance test 52

Figure 14: Markers of inflammation in gonadal adipose tissue of rodents fed either a low or high fat diet for 8 weeks 55

Figure 15: Markers of inflammation in the subcutaneous adipose tissue of rodents fed either a low or high fat diet for 8 weeks 56

Figure 16: Markers of inflammation in the tricep muscle tissue of rodents fed either a low or high fat diet for 8 weeks 57

Figure 17: Markers of inflammation in the soleus muscle tissue of rodents fed either a low or high fat diet for 8 weeks 58

Figure 18: Markers of inflammation in the liver tissue of rodents fed either a low or high fat diet for 8 weeks..... 59

Figure xix: Growth curve of female Sprague-Dawley rats fed either a low or high fat diet for 22 weeks..... 84

Figure xx: Individual AUC for the pyruvate tolerance test of twenty five female Sprague-Dawley rats 85

List of Abbreviations

AP-1	activator protein 1
AS160	Akt substrate of 160 kDa
BAT	brown adipose tissue
BiP	binding immunoglobulin protein
CLA	conjugated linoleic acid
$CLA_{c9,t11}$	*cis*-9, *trans*-11 conjugated linoleic acid
CLA_{mix}	commercially available ~50:50 blend of $CLA_{t10,c12}$ and $CLA_{c9,t11}$
$CLA_{t10,c12}$	*trans*-10, *cis*-12 conjugated linoleic acid
DAG	diacylglyceride
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FFA	free fatty acid
GLUT4	glucose transporter type 4
IKK	inhibitor of nuclear factor kappa B kinase
IL-6	interleukin 6
IL-8	interleukin 8
IRE-1	inositol-requiring enzyme 1
IRS	insulin receptor substrate
IκBα	inhibitor of nuclear factor kappa B
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MFD	milk fat depression
MUFA	mono-unsaturated fatty acid
Nfr2	nuclear factor erythroid 2-related factor 2
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
p38	p38 mitogen-activated protein kinase
PDK1	phosphoinositide-dependent kinase
PERK	PKR-like eukaryotic initiation factor 2 α kinase
PI3K	phosphatidylinositide 3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKB	protein kinase B
PUFA	polyunsaturated fatty acid
SARA	subacute rumenal acidosis
SAT	subcutaneous adipose tissue
SCD	stearoyl CoA desaturase
SFA	saturated fatty acid

T2D	type 2 diabetes
TLR4	toll-like receptor 4
TNFR	tumor necrosis factor-α receptor
TNFα	tumor necrosis factor-α
UCP	uncoupling protein
UPR	unfolded protein response
VAT	visceral adipose tissue
WAT	white adipose tissue
WT	wild type

Chapter One: Literature Review

DIABETES, OBESITY AND INSULIN RESISTANCE

Obesity rates have nearly doubled in the last 3 decades; according to the World Health Organization, more than 10% of the world's adult population was obese as of 2008[1]. Obesity is the greatest risk factor for the development of type 2 diabetes[2,3]. Diabetes is projected to be the 7th most common cause of death by 2030[4]. Ninety percent of diabetes cases are type 2 diabetes (T2D)[5], which is considered mainly a lifestyle disease[6]. Insulin resistance is an intermediary condition of varied etiology, characterized by a reduced responsiveness to the effects of insulin; insulin resistance can often be reversed with diet and lifestyle interventions before the development of the pancreatic dysfunction and insulin insufficiency characteristic of T2D.

CELL SIGNALING PATHWAYS INVOLVED IN INSULIN RESISTANCE

Overview of insulin signaling

In response to postprandial increases in plasma glucose, insulin is secreted into circulation from pancreatic beta cells. Circulating insulin binds the transmembrane insulin receptors embedded in the cell membranes of metabolic tissues (skeletal muscle, adipose tissue and liver primarily). Upon insulin binding to the insulin receptor the tyrosine kinase domain is activated, which in turn phosphorylates tyrosine residues on other target proteins[7]. The tyrosine phosphorylation of cytosolic insulin receptor

substrate (IRS) proteins activates the phosphotidylinositide-3-kinase (PI3K) pathway responsible for most of the metabolic actions of insulin[7-9]. IRS-1 activates PI3K which then adds a third phosphate group to membrane-bound phosphatidylinositol bisphosphate (PIP2) to form phosphatidylinositol triphosphate (PIP3); this reaction recruits protein kinase B (PKB) and 3-phosphoinositide dependent protein kinase-1 (PDK1) from the cytosol to separate membrane PIP3. PIP3-bound PKB can then be activated by phosphorylation carried out by a nearby PIP3-bound PDK1. Activated PKB phosphorylates AS160, resulting in glucose transporter type 4 (GLUT4) translocation from cytosolic vesicles (under low plasma insulin conditions) to the plasma membrane (under higher plasma insulin conditions). GLUT4 is a membrane transport protein responsible for facilitating insulin-stimulated glucose uptake from the blood into metabolic tissues for storage and reducing circulating glucose levels. Interference at any point in insulin signaling can reduce GLUT4 translocation, ultimately reducing the capacity for glucose clearance and contributing to insulin resistance. The activity of IRS-1 can be reduced by serine phosphorylation (classically Ser308) by various agents[10,11]; in fact, it has many tyrosine and serine phosphorylation sites that act together to finely modify IRS signaling[12].

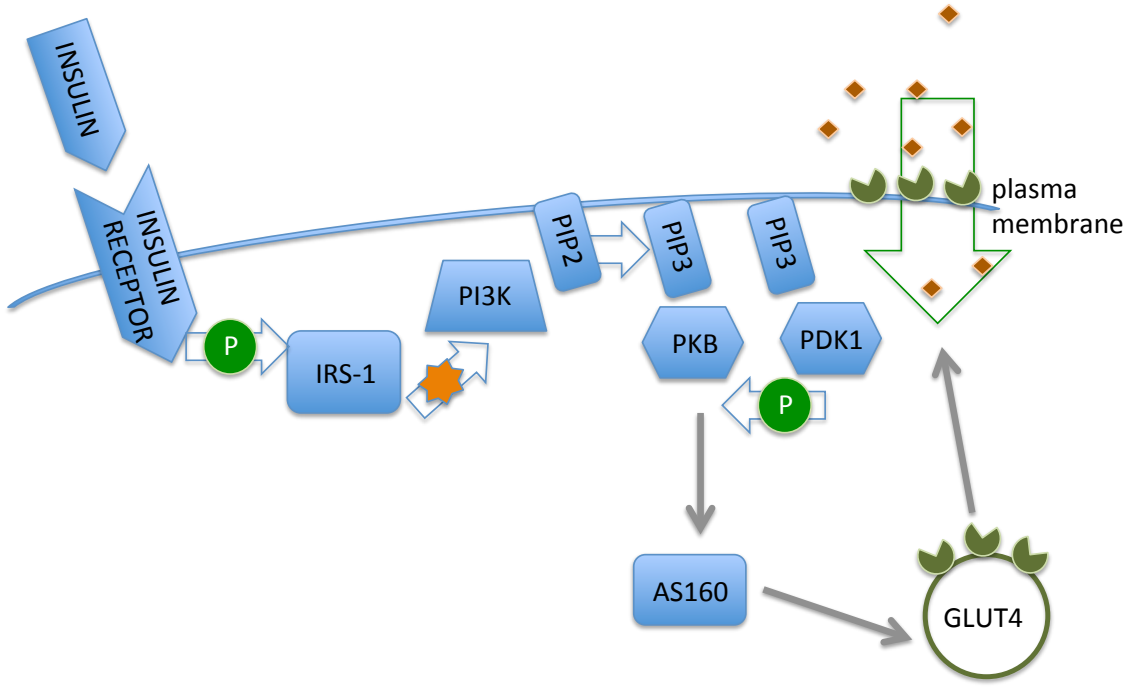

Figure 1: Simplified insulin signaling cascade

Overview of mitogen-activated protein kinase-mediated pathways

Mitogen-activated protein kinases (MAPK) are a family of cytosolic serine/threonine protein kinases that are involved in transducing intracellular signals, and respond to widely varying signals including growth factors and stress mediators such as cytokines, reactive species, radiation, heat shock and electrophilic lipids[13,14]. This enzyme family is characterized by a three-tiered signal transduction cascade (generalized as MAP3K, MAP2K and MAPK) (Figure 2). MAP3 kinases are often localized at the intracellular side of the plasma membrane and are involved in receiving extracellular signals mediated by various membrane receptor complexes[15]. The canonical signal transduction cascade involves the activation of MAP3 kinases by membrane receptor complexes, which then pass the signal on to MAP2 kinases, which ultimately activate MAP kinases resulting in the activation of transcription factors and subsequent changes in gene transcription[15,13].

Although activated MAPKs are also central to intracellular signaling, involved in innumerable cell signaling events, each one having hundreds of potential substrates[16]. The most well-characterized MAPK pathways are those mediated by extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38). The ERK pathway is primarily activated by growth factors, the p38 pathway is primarily activated by stress mediators, and the JNK pathway is intermediate – activated both by growth factors and stress mediators[13,9].

Interference with insulin signaling by mitogen-activated protein kinases

MAPKs can interfere with insulin signaling either directly or indirectly. In their role as mediators of transcription factor activation, MAPKs can propagate wider stress and inflammation processes that can indirectly affect insulin signaling. ERK is somewhat unique among the MAPK pathways as it is insulin responsive, involved in growth and development, and responsible for much of the effect of insulin on gene transcription[13,9]. JNK is involved in the activation of several transcription factors, most notably activator protein-1 (AP-1). Once c-Jun is activated by JNK, it can form half of the heterodimer that makes up AP-1[17]. The other half of AP-1 is c-Fos[18], which can be activated in various ways by any of ERK, JNK or p38, therefore a confluence of intracellular signals converge to mediate AP-1 activation[19]. P-38 is involved in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) – a transcription factor sequestered in the cytosol when bound to inhibitor of nuclear factor kappa B (IκB) under normal conditions. P-38 is involved in the activation of inhibitor of nuclear factor kappa B kinase (IKK), which phosphorylates IκB, releasing NFκB for nuclear translocation[20].

AP-1 and NFκB are generally proinflammatory transcription factors that regulate, but do not initiate, apoptosis[21,17,22].

In their role as intracellular signal transducers, activated MAPKs can directly interfere with insulin signaling. ERK itself can be activated by insulin as the MAP3K in this pathway is downstream of IRS-1 activation[8]. Activated IKK can directly interfere with insulin signaling by serine phosphorylation of insulin receptor or IRS-1; phosphorylation by p-38 is one method of IKK activation[23]. Of the MAPKs, JNK is most often implicated in insulin resistance because activated JNK can directly reduce IRS-1 activity by serine phosphorylation[16]. JNK activity is increased in obese mice and JNK1 knock out mice are protected from obesity induced insulin resistance[24].

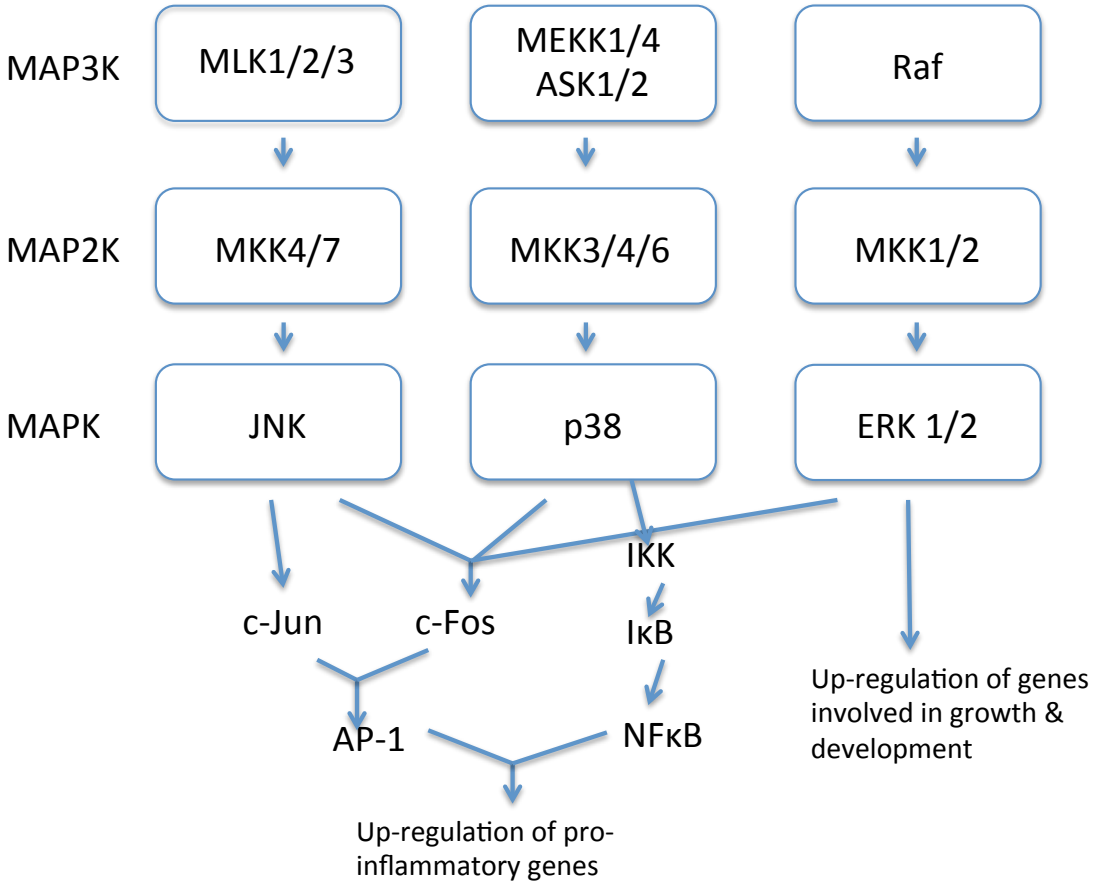

Figure 2: MAPK signaling cascades

HIGH FAT DIET INDUCED INSULIN RESISTANCE AND INFLAMMATION

High fat diet-induced obesity is correlated with the development of insulin resistance in metabolic tissues (adipose tissue, skeletal muscle, liver) and chronic low-grade inflammation. Indices that measure whole body insulin sensitivity, such as the hyperinsulinemic-euglycemic clamp method, show that whole body insulin resistance develops rapidly in response to a high lipid intake and progressively deteriorates over time as the high fat diet continues. However, insulin resistance is a very broad term describing reduced responsiveness to the effects of insulin. Many different factors

arising from different etiologies contribute to insulin resistance, and originate at different time points in the development of high fat diet-related insulin resistance.

Early development of insulin resistance and over-nutrition

The early effects of a high lipid intake results in a decrease in insulin sensitivity at the whole body level and can be detected within hours or days of starting a high fat diet. Skeletal muscle is a major metabolic tissue accounting for ~80% of glucose disposal under normal conditions[25], and reduced skeletal muscle insulin sensitivity is clearly apparent in established obesity, but does not contribute significantly to the early stages of insulin resistance development[26]. Under normal conditions, insulin suppresses endogenous glucose synthesis pathways in the liver. Among the earliest responses to high lipid intake is reduced hepatic suppression of gluconeogenesis[26,27]. A large influx of blood lipids is metabolically challenging for adipocytes, as excess lipids will be stored in adipose tissue using energy-dependent processes. Adipocytes do not have large glucose stores, and the inability to suppress hepatic gluconeogenesis may be an adaptive response to ensure adequate energy availability to adipocytes in the face of this short-term challenge. The endoplasmic reticulum (ER) is a key organelle involved in cellular metabolism. When oversupply of lipids persists, the metabolic demands may outstrip the capacity of the ER, resulting in ER stress[28].

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a key regulator of protein, lipid and cholesterol metabolism. It is the major site of protein synthesis, triglyceride and lipid droplet

formation, calcium storage and is also a major cellular sensor of nutrient status[29]. When metabolic demands exceed the capacity of the ER, such as with nutrient excess, unfolded and misfolded proteins accumulate in the lumen; the signaling pathways initiated by the ER stress response is termed the unfolded protein response (UPR) [30,31]. One factor contributing to ER stress is insufficient availability of the chaperone proteins involved in protein folding, such as binding immunoglobulin proteins (BiP), resulting in a backlog of unfolded proteins[29].

The ER contains three stress-sensing trans-membrane protein-mediated pathways: 1) PKR-like eukaryotic initiation factor 2α kinase (PERK), 2) inositol-requiring enzyme-1 (IRE-1) and 3) activating transcription factor 6 (ATF-6) [29]. Both de novo proteins awaiting post-translational folding and the intraluminal domain of these three trans-membrane proteins are bound to a BiP[29]. Excess protein synthesis reduces the availability of BiP for stress-sensing proteins, which results in their release from the ER membrane to initiate downstream signaling, endeavoring to restore homeostasis[29]. The UPR mediates cell intrinsic stress responses by 1) PERK-mediated activation of antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and pro-inflammatory transcription factor NFκB, 2) IRE-1-mediated JNK activation, and 3) create electrophilic stress by producing excess reactive oxygen species (ROS) [29,32].

MAPKs participate in propagating stress signaling from the ER in various ways. When ER stress extends past the capacity to restore homeostasis, IRE-1 forms a complex with tumor necrosis factor receptor-associated factor (TRAF2) and apoptosis signal-regulating

kinase-1 (ASK1); it is this protein complex that activates JNK in the ER[32]. JNK activation subsequently can directly suppress insulin signaling by serine phosphorylation of IRS-1[33] and JNK activation in the ER is linked to the development of insulin resistance and T2D[34]. Activation of PERK and IRE-1 pathways also activate NFκB by IKK activation mediated by p-38 or IκB degradation[35].

Intermediate development of insulin resistance and increasing adiposity

When a high fat diet continues beyond the short term, excess lipids are stored in enlarging adipocytes and fat mass in general begins to increase. Signs of adipose tissue inflammation develop rapidly in response to high lipid intake and precedes the development of inflammation in other metabolic tissues (skeletal muscle and liver) [28,27,36]. The earliest signs of adipose tissue inflammation appear as an upregulation of proinflammatory genes (TNFα, IL-6, IL-8, MCP-1) apparent in both mature adipocytes and the stromal fraction of adipose tissue, containing preadipocytes, dendritic cells (resident macrophages) and other non-adipocyte components of adipose tissue[28,27]. This is in contrast to obesity-linked inflammation, where proinflammatory gene expression is almost exclusively the domain of the stromal fraction[37].

Insulin resistance progressively worsens as high fat feeding continues and adiposity increases. Adipose tissue insulin resistance emerges prior to the development of reduced glucose uptake in skeletal muscle and liver, and concomitantly with the first appearance of increased reactive lipids[26]. Under normal conditions, insulin stimulates glucose uptake and suppresses lipolysis in adipose tissue; insulin resistance in adipocytes is characterized

by reduced uptake of circulating fatty acids and increased lipolysis, resulting in increased serum free fatty acids[9].

Free fatty acids, insulin resistance and toll-like receptors

Elevated serum free fatty acids are characteristic of obesity[38], and a high fat diet alone increases adipose tissue lipolysis[39]. Increased secretion of FFA into circulation from adipose tissue is a key event in the development of skeletal muscle insulin resistance, which accelerates the decline in whole body glucose homeostasis, and increasing local cell-to-cell pro-inflammatory signaling in adipose tissue.

It is well established that lipid infusion rapidly causes reduced insulin sensitivity in skeletal muscle, causing inflammatory stress[40] and increased circulating TNFα[41]. JNK and p-38 activation are increased in skeletal muscle in response to lipid infusion[40,42]. Extracellular free fatty acid signaling through toll-like receptor (TLR) on the cell surface contributes to the initiation of inflammatory responses, in part by the activation of MAPKs and IKK[43,44]. Lipid-induced insulin resistance in soleus muscle, mediated through TLR4, requires saturated fatty acid (SFA)-induced ceramide production[45].

The role of TLR is perhaps best understood as a pattern recognition receptor that propagates the innate immune response to bacterial lipopolysaccharide (LPS), however TLR also has a role in lipid-induced insulin resistance. TLR4 signaling is potently activated by circulating SFAs resulting in upregulation of IL-6 and TNFα; unsaturated fatty acids do not activate TLR4[46]. In fact, n3 PUFA can completely abrogate TLR4

signaling[46]. *TLR4* interference in mice protects against acute lipotoxicity and high fat diet-induced insulin resistance[43]. *TLR4* knockout mice maintained greater insulin sensitivity and skeletal muscle insulin signaling than wild type mice when challenged with an 8-hour lipid infusion[43]. They also demonstrated reduced JNK phosphorylation and IκBα degradation[43]. Despite developing greater obesity than wild type mice, *TLR4* knockout mice on a HFD were more insulin sensitive and had reduced proinflammatory cytokine production in the liver, but especially in adipose tissue (IL-6, TNFα, MCP-1)[43]. Similarly, mice with a mutation in *TLR4* were more insulin sensitive and had more robust insulin signaling in liver, skeletal muscle and adipose tissue than did wild type mice fed a high fat diet, and did not experience high fat diet-induced increases in JNK or IκBα activation[47]. Therefore, not all high fat diets will be expected to have the same potency to induce insulin resistance, depending on the fatty acid composition.

Later development of insulin resistance and established adiposity

In obesity, resident macrophages are induced to express a pro-inflammatory phenotype that causes the recruitment of infiltrating macrophages[48]. A positive feedback loop mediated by FFA and TLR4 (which is overexpressed in obesity[49]) increase adipose tissue inflammation: increased FFA release from adipocytes triggers TLR4 signaling both in adipocytes, as well as resident and infiltrating macrophages, which upregulates TNFα production by macrophages via MAPK activation, further exacerbating inflammation and insulin resistance in adipocytes[50]. TNFα causes increased lipolysis in adipocytes[51,52], and interferes with the phosphorylation of insulin receptor and IRS-1[53,54] in a p-38 and IKK-

dependent manner[23]. The macrophage population of adipose tissue can rise from 10% of total cells in lean conditions to 50% in obesity[55].

Inflammation, insulin resistance and tumor necrosis factor-α

Early studies showed that *TNFα* interference increased insulin sensitivity in obese rats[56]. Obese women had 2.5 times more TNFα expression in abdominal subcutaneous adipose tissue despite very low or non-detectable plasma amounts; weight loss resulted in increased insulin sensitivity concomitant with reduced adipose TNFα expression[57]. Wellen and Hotamisligil (2003) postulate that the source of serum TNFα is stressed adipocytes, whereas increasing tissue concentrations are due to secretions of TNFα from local activated macrophages that have a paracrine effect on surrounding cells[28]. In ob/ob mice, obesity alone reduced insulin-stimulated tyrosine phosphorylation of insulin receptor by 70% in adipose tissue, 35% in skeletal muscle and 25% in liver[58]. Knocking out *TNFα* restored insulin receptor activation to levels approaching lean controls in adipose tissue and skeletal muscle, but not liver[58], and protects against high fat diet-induced inflammation (systemic and adipose), elevated plasma free fatty acids and insulin resistance[59]. In cultured murine adipocytes, TNFα increased the serine phosphorylation of IRS-1[60] and prolonged exposure reduced mRNA and protein levels of insulin receptor, IRS-1 and GLUT4[61]. TNFα rapidly induces NFκB in adipocytes, along with a proinflammatory or macrophage-like set of genes associated with preadipocytes; TNFα also suppresses genes involved in normal metabolic function of mature adipocytes (GLUT4, HSL, LC-FACoA, PPARγ, C/EBPα) in an NFκB dependent manner[53]. Long-term exposure reduces protein levels of GLUT4, insulin receptor, IRS-1 and PKB[53]. The

main intracellular transducers of TNFR are IKK, JNK, p38 and ERK[16]. Activated MAPKs can interfere with insulin signaling by the serine phosphorylation of IRS proteins[60] and insulin receptor autophosphorylation directly, as well as contribute to the initiation of pro-inflammatory gene expression[62,54].

CONJUGATED LINOLEIC ACIDS

Origins of conjugated linoleic acids

Conjugated linoleic acid (CLA) is a group of geometric and positional isomers of linoleic acid (LA; 18:2 Δ9,12) where the two unsaturated carbon bonds are separated by a single carbon-carbon single bond. CLA cannot be produced by mammalian enzymes and so the only source of CLA for humans is dietary consumption of the meat, milk and derived products of ruminant animals[63]. CLA is produced by bacteria in the rumen of herbivorous mammals when PUFAs are converted to saturated fatty acids by a process termed biohydrogenation[64]. Some of the FA products of this process are absorbed by the animal before complete saturation and make up part of the fatty acid profile of meat and milk produced by these animals[65,66].

The three most abundant CLA isomers in milk and dairy products are the *cis*-9,*tran*s-11, *trans*-7,*cis*-9, and *trans*-10,*cis*-12 isomers that make up approximately 76.5, 6.7 and 1.1% of total CLA, with other isomers present in trace amounts[67,68]. The synthesis of $CLA_{c9,t11}$ is mainly endogenous in ruminants with a small amount synthesized in the rumen, the *trans*-7,*cis*-9 isomer is exclusively endogenous, and $CLA_{t10,c12}$ is exclusively synthesized by bacteria in the rumen[67,68]. The total CLA content of milk increases with the

proportion of forage (high fiber content) in the diet of milk-producing ruminants[66,65,69]. A high grain (high starch content) diet reduces rumen pH and may cause a condition known as milk fat depression (MFD) or if prolonged, subacute ruminal acidosis (SARA)[70]. Low rumen pH alters the fatty acid profile of milk and favors the production $CLA_{t10,c12}$[70]. As $CLA_{t10,c12}$ makes up a higher proportion of the fatty acid profile, it is known to inhibit stearoyl-CoA desaturase (SCD), which depresses the endogenous synthesis of $CLA_{c9,t11}$ and $CLA_{t7,c9}$, further increasing the proportion of $CLA_{t10,c12}$ in milk[71-74].

CLA can also be industrially produced by the alkali isomerization of linoleic acid resulting in an approximately 50:50 ratio of $CLA_{c9,t11}$ and $CLA_{t10,c12}$ (CLA_{mix}) [75-77]. Due to their ease of production, $CLA_{c9,t11}$ and $CLA_{t10,c12}$ are the most widely studied CLA isomers, however CLA_{mix} does not accurately represent naturally occurring ratios of CLA. The health effects of CLA are isomer specific. $CLA_{c9,t11}$ and $CLA_{t10,c12}$ often have opposing health effects in humans, and so studies using CLA_{mix} without taking this into account tend to overemphasize the effects of $CLA_{t10,c12}$.

HEALTH EFFECTS OF CLA

CLA (CLA_{mix}) has successfully been marketed as a weight loss supplement due to the desirable changes in body composition seen in some animal studies that showed dramatic reductions of adipose tissue, sometimes concomitant with an increase in lean body mass[78-80]. However, insulin resistance has also been observed as an outcome of CLA supplementation. Both these effects are linked to the $CLA_{t10,c12}$ isomer.

Effects of CLA on body composition and insulin resistance in at the whole body level in rodent models

A 1997 study was the first to demonstrate the beneficial effects of CLA_{mix} on body composition; CLA-supplemented mice showed up to 60% less fat mass and up to 14% greater lean body mass[78]. However, the dramatic reduction in adipose tissue seen in rodent studies appears to be lipodystrophic and occurs concurrently with an increase in insulin resistance. Additionally, the magnitude of the effect depends on the proportion of total dietary fat intake made up by $CLA_{t10,c12}$.

In mice, high dose CLA-induced reduction in adiposity is due to lipodystrophy, resulting in the complete ablation of some adipose depots. Female mice consuming a diet composed of 10% kcal from fat and containing 0.1 g CLA/100 g diet for 5 months had 40% less WAT than controls, with no apparent effect on insulin sensitivity[79]. Higher doses (1 g CLA/100 g diet) resulted in even greater loss of fat mass (76% retroperitoneal AT and 79% subcutaneous AT) and a dramatic reduction in insulin sensitivity after 9 weeks[79]. When continued for 5 months, there was a complete ablation of BAT, and near ablation of WAT; only parametrial WAT was conserved despite a 27% reduction[80]. This was due both to apoptosis – signs of which appeared as early as day 4 – as well as a reduction in the size of surviving adipocytes, which were 41% smaller after 5 months[80]. This dose resulted in a nearly complete inhibition of GLUT4 mRNA (91%), along with a substantial upregulation of TNFα and UCP-2 in WAT, and an apparent compensatory upregulation of GLUT4 mRNA in skeletal muscle (1.5 fold upregulation in gastrocnemius)[80,79]. Reduced GLUT4 expression in WAT and BAT was detectable by

day 4[80]. These effects were accompanied by a modest deterioration in glucose tolerance, dramatic reduction in insulin sensitivity and elevated plasma insulin[80]. In addition to the absolute dose of CLA, the proportion of CLA to total fat intake can mediate its effects. In a 10% fat diet, this dose of CLA (1 g CLA/100 g diet) makes up 25% of total fat, whereas in a 60% fat diet it represents only 3% of total fat and the effects are moderated[79]. High-fat diet alone reduced GLUT4 expression in WAT by 62% as compared to mice fed a low fat diet, and high-dose CLA only reduced it a further 24%; TNFα and UCP-2 expression were upregulated, but not as dramatically as when the diet contained 10% kcal from fat[79].

Molecular mechanisms of CLA action

$CLA_{t10,c12}$ may exacerbate the effects of a high fat diet on inflammation and insulin sensitivity via MAPK stress kinase signaling. In vitro, CLA can effect cell signaling in the short term and inflammation in the medium and long term.

$CLA_{t10,c12}$ causes an increase in phospholipase C activity (PLC) which cleaves membrane phospholipids releasing a molecule of inositol 1,4,5-trisphosphate (IP3) and a diacyglyceride (DAG)[81,82]. IP3 binds to its receptor on the ER, causing calcium release[81]; DAG is converted by diacylglyceride kinase (DAGK) into phosphatidic acid which also stimulates calcium release from the ER[82]. Increased intracellular calcium occurs within 3 minutes of $CLA_{t10,c12}$ treatment in primary human adipocytes[81]. This effect is followed by increased expression of chaperone proteins (heat shock proteins) which suggests the UPR has been initiated by ER stress[81]. ER stress ultimately activates MAPKs (ERK,

JNK, c-Jun) and increases expression of inflammatory genes, while decreasing the expression of genes involved in energy metabolism (PPARγ, GLUT4, ACC, SCD1) [81,82]. This is followed by a reduction in insulin-stimulated glucose uptake[81]. PLC inhibitors only partially attenuate these effects, which suggests CLA-induced inflammation is only partially mediated by PLC[81]. DAGK inhibitors also attenuate intracellular calcium accumulation and changes in gene expression, reducing inflammatory markers and increasing insulin sensitivity[82]. Additionally, the inhibition of intracellular calcium accumulation initiated by $CLA_{t10,c12}$ results in a blunted increase of ROS, inflammatory gene expression, and ERK and JNK activation[83]. Calcium inhibitors block the binding of NFκB to the promoter regions of IL-8 and COX-2 that is upregulated by $CLA_{t10,c12}$ due to the release of cytosol-bound NFκB [83].

Microarray analysis of the effect of $CLA_{t10,c12}$ -treated adipocytes demonstrated that among the earliest effect of $CLA_{t10,c12}$ is to cause ER stress, which precedes other changes in inflammatory gene expression[84]. Oversupply of nutrients in high fat feeding and obesity causes ER stress in metabolic tissues resulting in cellular stress due to MAPK activation and increasing reactive species[34,29]. The combination of MAPK signaling and increasing electrophilic stress causes an upregulation first of antioxidant enzymes, and if electrophilic stress continues, of proinflammatory NFκB and AP-1 that initiate a broader proinflammatory response[29].

$CLA_{t10,c12}$ has other effects that are more directly linked to insulin sensitivity. PPARγ is a key transcription factor in the adipocyte phenotype regulating fatty acid and glucose

metabolism, which causes increased fatty acid uptake and adipogenesis. It is the molecular target of insulin sensitizing thiazolidinedione drugs used in the treatment of T2D. $CLA_{t10,c12}$ indirectly inhibits PPARγ expression in adipocytes and reduces adipogenesis via the activation of NFκB, which increases IL-6 production responsible for PPARγ suppression[85]. Additionally, $CLA_{t10,c12}$ increased the serine phosphorylation of IRS-1 in the liver of rats fed a high fat diet for 8 weeks[86].

$CLA_{t10,c12}$ increases lipolysis and reduces fatty acid uptake into adipocytes. Reduced glucose and fatty acid uptake in adipocytes is dependent on ERK and NFκB activation, as well as pro-inflammatory cytokines (IL-6, IL-8 TNF-α)[87]. In human adipocytes, $CLA_{t10,c12}$ caused a strong and sustained activation of ERK, concomitant with increased IL-6 and IL-8 secretion, which preceded $CLA_{t10,c12}$ -induced delipidation, decreased insulin-stimulated glucose uptake, fatty acid metabolism, and downregulation of PPARγ[87]. CLA_{mix} reduces body fat by increasing lipolysis, and reduces lipid uptake by inhibiting lipoprotein lipase in adipocytes[88,89].

Health effects of CLA in human clinical trials

In human clinical trials, $CLA_{t10,c12}$ seems to result in a very small improvement of body composition in people with pre-existing obesity, usually limited to a small reduction in fat mass and often accompanied by a worsening of insulin resistance[90-94]. These effects seem to occur at relatively low doses, with no additional beneficial effects on body composition with increased amounts[90]. In fact, CLA is also associated with increased inflammation and so higher doses may not be advisable[95]. When normalized to body

weight, the doses used in human studies are far lower than those used in animal studies which produced dramatic results.

Human dietary intake of CLA

Food frequency questionnaires and food diaries can be used to estimate dietary intake of nutrients by calculating intake based on nutritional databases. Ritzenthaler et al. (2001) created actual food duplicates of CLA-containing items reported in food frequency questionnaires and food diaries from 93 adults in order to directly measure CLA content of food items to get a more accurate estimate of dietary CLA intake[96]. They concluded that these types of estimates slightly underreport dietary intake of CLA and suggest that typical dietary intake is on average 212 mg/day for men, and 151 mg/day for women[96]. This amounts to approximately 19 mg/day of $CLA_{t10,c12}$ and 193 mg/day of $CLA_{c9,t11}$ for men, and 11 mg/day $CLA_{t10,c12}$ and 140 mg/day of $CLA_{c9,t11}$ for women[96]. The effective daily dose of $CLA_{t10,c12}$ works out to 0.235 mg/kg for men and 0.177 mg/kg for women based on the average body weight reported in the study[96]. This is far lower than amounts consumed by CLA supplementation and lower by orders of magnitude than the doses used in most animal studies.

Effect of $CLA_{t10,c12}$ in healthy adults

The effects of $CLA_{t10,c12}$ tend to be more pronounced in those who are already overweight or obese. Healthy postmenopausal women consuming either 2.2 or 0.4 g $CLA_{t10,c12}$ per day had increased markers of inflammation and lipid peroxidation in the higher dose group[97]. A study of healthy men receiving either $CLA_{t10,c12}$ or $CLA_{c9,t11}$ in

doses ranging from 0.5 to 2.5 g/day found no effect of either isomer on insulin sensitivity or resistance[98]. Plasma triglycerides, cholesterol and low density lipoprotein were slightly elevated among men receiving $CLA_{t10,c12}$, but the differences were very small, despite being statistically different[98]. In healthy subjects, the effects of CLA were negligible, however the doses used were lower than those typically used in studies of pre-existing obesity.

Effect of $CLA_{t10,c12}$ in obese adults

The effects of CLA are more pronounced in overweight and obese subjects than in healthy ones. Obese men presenting with metabolic syndrome receiving 3.4 g/day of $CLA_{t10,c12}$ experienced a 578% increase in lipid peroxidation and 110% increase in circulating C-reactive protein[95]. Subjects of both genders who were overweight or obese receiving the same dose for 12 weeks had a small reduction in body fat mass, but no difference in lean body mass or plasma lipids[90]. This study showed no added benefit on body composition of doses exceeding 3.4 g/day[90]. Similar results were seen in obese men receiving 4.2 g/day CLA_{mix}[91]. These men experienced a small (~1%) but statistically significant reduction in abdominal fat, with no other changes in body composition[91]. CLA may also interfere with insulin sensitivity. Obese men were administered 3.4 g/day of either $CLA_{t10,c12}$ or CLA_{mix}; hyperinsulemic-euglycemic clamp results showed that $CLA_{t10,c12}$ caused a 15% reduction in insulin sensitivity, while the lower $CLA_{t10,c12}$ dose contained in CLA_{mix} had no effect[92]. $CLA_{t10,c12}$ treatment resulted in increased fasting glucose and circulating C-reactive protein, while CLA_{mix} had no effect[92]. $CLA_{t10,c12}$ also resulted in increased proinsulin – an indication of T2D and CVD risk – while the effect of

CLA_{mix} was not significant[92]. Thrush et al. (2007) found no change in body composition of overweight, non-diabetic subjects after 12 weeks consuming 4 g/day of CLAmix, however whole body glucose tolerance was decreased and ceramides accumulated in skeletal muscle (vastus lateralis)[99]. Overall, a meta-analysis of 18 clinical trials concluded that 3.2 g CLA_{mix} /day resulted in a 0.2 lb loss of body weight per week without any accompanying dietary changes[93]. A separate meta-analysis similarly concluded that CLA supplementation also resulted in a small increase in lean body mass[94].

Chapter Two: Introduction

Conjugated linoleic acid (CLA) is a group of geometric and positional isomers of linoleic acid (LA; 18:2 Δ9,12) where the two unsaturated carbon bonds are separated by a single carbon-carbon single bond. CLA cannot be produced by mammalian enzymes and so the only source of CLA for humans is dietary consumption of the meat, milk and derived products of ruminant animals[63]. The most abundant, and well studied, CLA isomers are $CLA_{c9,t11}$ and $CLA_{t10,c12}$ which make up approximately 76.5% and 1.1% of total naturally occurring CLA, respectively[67,68]. These isomers have different metabolic effects and $CLA_{t10,c12}$ has been shown to reduce fat mass in rodents and humans, as well as increase insulin resistance and inflammation[80,100,97].

In order to increase productivity, Canadian dairy cows are fed a high grain, low forage ration, that can sometimes produce a condition known as subacute ruminal acidosis (SARA)[101-103]. SARA changes the rumen pH resulting in increased production of the $CLA_{t10,c12}$ isomer[70,104]. In light of the evidence that high $CLA_{t10,c12}$ intake can cause insulin resistance and inflammation, we endeavored to discover whether there was a health risk to consumers relating to changes in insulin sensitivity or inflammation associated with elevated $CLA_{t10,c12}$ in dairy products. To that end, a 60% fat rodent diet was produced, composed entirely of butter manufactured from milk collected from dairy cows experiencing SARA. This butter represents the most $CLA_{t10,c12}$-enriched source of naturally occurring dietary fat for humans.

Using an established model of high fat diet-induced insulin resistance, female Sprague-Dawley rats were fed either a low (10%) or high (60%) fat diet for 8 weeks. SARA butter was compared against a commercially available butter and a custom non-SARA butter was included to account for any variability arising from the production of the custom SARA butter. The 5 treatment groups in this study included the 10% low fat diet (LFD; negative control) and 60% high fat diets (HFD): lard-based diet (LARD; positive control), $CLA_{t10,c12}$–enriched butter (SARA), non-SARA butter (NonSARA), and commercial butter (COM). The effects of the diet on whole body glucose homeostasis and inflammation, indicated by MAPK activation, were evaluated after 8 weeks.

We hypothesized that rodents consuming a high fat diet for 8 weeks would gain fat mass. Subsequently, we expected that all high fat diets would result in increased insulin resistance as compared to low fat-fed controls, and that of the butter-based diets, the SARA group would show the greatest deviation from normal whole body glucose homeostasis. We also expected to see indication of cellular or tissue inflammation in the high fat-fed groups; assuming that adipose tissue would be the initiator of inflammation, we expected to see the greatest degree of MAPK activation in adipose tissue, followed by skeletal muscle and liver depending on how far the high fat diet-induced pathology had developed[105].

Chapter Three: Methods

DIET PREPARATION

In order to evaluate the potential risk of high dietary levels of $CLA_{10t,12c}$, a custom butter was produced at the University of Guelph containing the maximum possible natural amount of this isomer. SARA was induced in dairy cows with a high grain, low forage diet, and the milk harvested was used to produce a high $CLA_{10t,12c}$ butter (SARA) as well as a control butter produced from the milk of healthy cows (NonSARA). The NonSARA group was included in the study to account for any potential differences between custom and commercially produced butters. A popular local consumer brand of butter was purchased commercially (COM; Gaylea Brand unsalted, Ontario, Canada).

Five rodent diets were used in the study (Table 1). A negative control low fat diet (LFD) containing 10% kcal from fat (5.5% soybean oil and 4.5% lard) and a positive control high fat diet (LARD) containing 60% kcal from fat (5.5% soybean oil and 54.5% lard) were obtained from Research Diets, New Jersey, USA (D12450B, D12492). A premix identical in composition to the LARD excluding lard (D12492px) was used to blend custom butter-based diets with 60% kcal from fat (5.5% soybean oil and 54.5% butter). Due to the higher water content of butter as compared to lard, the amount of butter added to make up 60% kcal from fat was based on the analysis of custom butters (fat content 83.2% ± 0.45). Butter diets were prepared by blending 295 g of butter with 528.8 g of diet premix in a food mixer (Hobart Canada, Ontario, Canada).

Table 1: Diet compositions

	LFD		Butter		HFD	
	g	kcal	g	kcal	g	kcal
Casein	200	800	200	800	200	800
L-Cyteine	3	12	3	12	3	12
Cornstarch	315	1260	0	0	0	0
Maltodextrin	35	140	125	500	125	500
Sucrose	350	1400	68.8	275.2	68.8	275.2
Cellulose	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225
Lard	20	180	0	0	245	2205
Butter	0	0	295	2165	0	0
Mineral Mix	10	0	10	0	10	0
Dicalcium phosphate	13	0	13	0	13	0
Calcium phosphate	5.5	0	5.5	0	5.5	0
Potassium citrate	16.5	0	16.5	0	16.5	0
Vitamin mix	10	40	10	40	10	40
Choline bitartate	2	0	2	0	2	0
Dye	0.05	0	0	0	0.05	0
Total	1055.05	4057	823.8	4017.2	773.85	4057.2
kcal/g	3.85		4.88		5.24	
% kcal from fat	10		60		60	

ANIMAL, HOUSING & EXPERIMENTAL GROUPS

Twenty five female Sprague Dawley rats (mean body weight 137.6 g ± 1.337) were obtained from Charles River (Quebec, Canada) and individually housed in a 12-hour reversed light/dark cycle with free access to water and a standard chow diet for a 1 week acclimation period. Female Sprague-Dawley rats were selected to stay consistent with previous work on the effects of SARA butter (unpublished). Rats were randomly assigned to one of five dietary treatment groups (LFD, COM, NonSARA, SARA or LARD) and received food and water ad libitum for 8 weeks (Figure 3). Body weight and food intake were recorded several times per week for the experimental period. All ethical protocols as outlined by the Animal Care Committee in compliance with the guidelines outlined by the Canadian Council on Animal Care at the University of Guelph were followed.

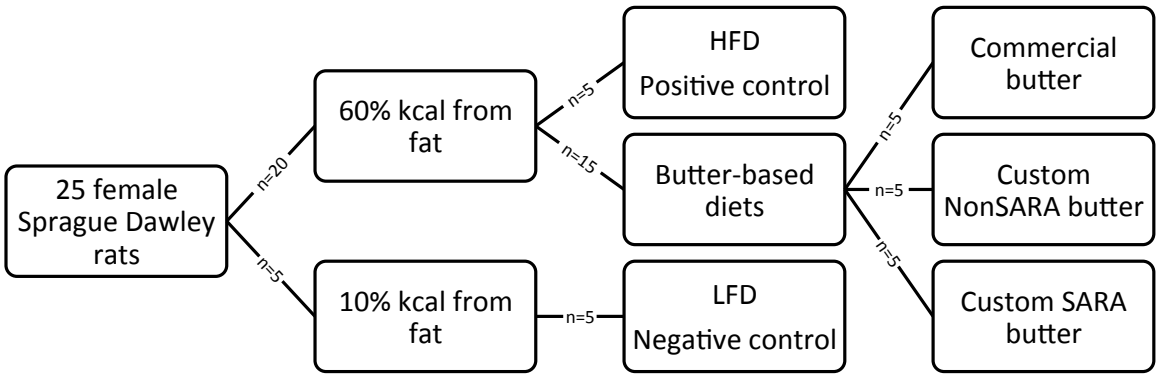

Figure 3: Experimental design of the current study

WHOLE BODY INSULIN SENSITIVITY TESTS

During the final week, whole body glucose homeostasis was evaluated with three types of tolerance tests to evaluate systemic glucose tolerance, insulin sensitivity and liver-specific insulin sensitivity. Glucose, insulin and pyruvate tolerance tests were performed in the same week with a day of rest between each test.

Glucose tolerance test

Glucose tolerance testing (GTT) measures insulin resistance by estimating the rate of systemic glucose disposal. Equalized glucose doses are administered by injection into the intraperitoneal body cavity and blood glucose levels are subsequently measured at

established time points. Under normal conditions, the glucose bolus results in a brief spike in blood glucose levels that are quickly returned to normal. Glucose intolerance is evidenced by a reduced capacity to clear the glucose bolus, which demonstrates a degree of insulin resistance or alternatively insulin insufficiency.

A 20% D-glucose solution was prepared in pure H20. Rats were fasted for 6 hours and basal blood glucose was determined with blood from the tail tip using standard glucose strips and glucometer (OneTouch Ultra 2, LifeScan Inc., Milpitas, CA, USA). A glucose bolus (2 g glucose/kg body weight) was administered by intraperitoneal injection and blood glucose levels were evaluated at 15, 30, 45, 60, 90 and 120 minutes post injection.

Insulin tolerance test

Insulin tolerance testing (ITT) measures insulin sensitivity by evaluating the magnitude of response to an equalized insulin bolus administered as described above. Under normal conditions of insulin responsiveness, blood glucose drops quickly to a negative peak and is slowly normalized as metabolic glucose homeostasis is restored. When sensitivity to insulin is reduced, the drop in blood glucose is lessened and the return to basal glucose levels more rapid, and suggests insulin resistance.

Stock insulin solution was diluted in saline to obtain a 0.025U/100µl solution. Basal blood glucose was determined in non-fasted rats with blood from the tail tip as described above. Blood glucose levels were determined at 10, 20, 30, 45, 60, 90 and 120 minutes after an intraperitoneal insulin injection (0.75 U insulin/kg body weight).

Pyruvate tolerance test

Pyruvate tolerance testing (PTT) evaluates the efficiency of hepatic glucose output. Equalized pyruvate doses are administered and blood glucose evaluated as described above. Under normal conditions of insulin sensitivity, pyruvate – a substrate for hepatic gluconeogenesis – stimulates a rise in blood glucose corresponding to an increase in hepatic gluconeogenesis. The rise in blood glucose would be expected to stimulate insulin secretion to restore glucose homeostatsis.

A 40% pyruvate solution in saline was prepared and the pH was adjusted to 7.35. Rats were fasted for 6 hours and basal blood glucose was determined as described above. A pyruvate bolus (2 g pyruvate/kg body weight) was administered by intraperitoneal injection and blood glucose levels were evaluated at 15, 30, 45, 60, 90 and 120 minutes post injection.

Group means were compared using the area under the curve (AUC) taken from baseline blood glucose (mmol/L) for each animal; the ITT used the AUC below baseline blood glucose. Additionally, each time point was compared within each tolerance test to clarify at which point the group means diverged, and in order to more clearly display the variability and significance.

TISSUE HARVESTING

Prior to surgeries, animals were fasted for 6 hours and anesthetized with sodium pentobarbital (1 mg/kg). Two skeletal muscles were extracted to represent potential

differences in metabolism between fiber types (type I slow twitch soleus muscle and type II fast twitch tricep muscle); two types of adipose tissue were harvested to evaluate potential differences between depots (gonadal adipose tissue is more highly correlated with insulin resistance than subcutaneous adipose tissue) and frozen immediately in liquid nitrogen and stored at -80°C awaiting subsequent analysis. The liver was also removed and frozen immediately in liquid nitrogen; samples from the left lobe were used in subsequent Western blot analysis.

WESTERN BLOT ANALYSIS

All tissues were prepared for Western blotting from whole cell lysates. Protein was extracted from tissue subsamples by submerging in an ice-cold cell lysis buffer supplemented with protease inhibitors to stabilize proteins and their phosphorylation states. Samples were homogenized for 2 1-minute cycles using a Fast Prep® tissue homogenizer (MP Biomedicals, LLP, Quebec, Canada). Samples were centrifuged at 15,000 rpm for 10 minutes and the supernatant removed by pipette, avoiding the surface lipid layer. This was repeated twice to minimize lipid contamination of the samples. The protein concentration of the samples was determined spectrophotometrically on a 96-well plate using a BSA assay. Protein content of samples were equalized in the preparation of samples for Western blotting by diluting samples to the same concentration with cell lysis buffer; final samples were prepared with a 1:1 of sample + cell lysis buffer to LPS solution. Proteins were separated by electrophoresis on 1.5 mm 10% SDS-PAGE acrylamide gels (1.5 hours at 100-140 mV). Protein bands were transferred onto nitrocellulose membranes in a methanol-containing transfer buffer at 4°C (1.5 hours at

0.2 amps). Equal loading was verified with nonspecific protein staining with Ponceau-S stain (Sigma Aldrich, Missouri, USA). Membranes were incubated for 1 hour in a 5 % skim milk powder solution in a Tris-buffered solution with Tween (TBST) to block non-specific protein binding, and then incubated with primary antibodies (~1:1000 dilution in a 5% BSA TBST solution) overnight at 4°C (SAPK/JNK, P-SAPK/JNK, ERK1/2, P-ERK1/2, p38 MAPK, P-p38 MAPK; Cell Signaling, Massachusetts, USA). Membranes were washed in TBST to remove excess primary antibody, incubated 1 hour with a horseradish-peroxidase-linked secondary anti-rabbit antibody at room temperature, and subsequently rewashed in TBST followed by TBS in preparation for imaging. Membranes were briefly incubated with an electrochemiluminescence solution (Western Lightning with ECL, Perkin Elmer, Massachusetts, USA) and detected via chemiluminescence and quantified via densitometry with an imaging system.

STATISTICAL AND OTHER ANALYSIS

Statistical analysis used in this study compared means with one-way ANOVA to determine group effects of diet treatments[106]. The Dunnett post-hoc test was used compare treatment groups against control group (LFD) [106]. All statistical analysis was calculated using Prism 5.0 software (GraphPad Software Inc. 2008, San Diego, USA). Two-tailed, unpaired Student's t-test statistical analysis was used to compare blood glucose values between COM and NonSARA tolerance tests[106]. Statistical significance was accepted at $p \leq 0.05$.

The equation used to calculate percent change is as follows:

$$= \frac{final\ value - initial\ value}{initial\ value} \times 100$$

The equation used to normalize food intake to body size (Figure 7) is as follows:

$$= \frac{mean\ daily\ food\ intake\ (g)\ \times\ kcal\ of\ the\ diet\ (kcal/g)}{mean\ body\ weight\ (g)}$$

Chapter 4: Results

RODENT DIETS

The fatty acid profile of butters can be seen in Table 2. There were no substantial differences in the fatty acid profile between COM and NonSARA butters. Notably, COM butter contained twice the amount of $CLA_{10t,12c}$ than butter produced from verifiably healthy cows (NonSARA), which may be due to the current incidence rate of SARA in the commercial dairy industry (0.008 g/100 g NonSARA and 0.016 g/100 g COM). SARA butter exhibited the characteristic fatty acid type increases; 12-fold more $CLA_{10t,12c}$ and 14-fold more *trans*-10 18:1 from NonSARA butter. This shift in the fatty acid profile is consistent with the literature, but interestingly, SARA butter also contained more than twice the amount of $CLA_{c9,t11}$ than NonSARA butter.. Commercial butter contained twice as much $CLA_{10t,12c}$ as NonSARA butter (0.016 g/100 g and 0.008 g/100g, respectively), and SARA butter contained nearly 10 times more (0.097 g/100 g $CLA_{10t,12c}$). SARA butter also contained more total CLA than both COM and NonSARA butters (1.44, 0.62 and 0.55 g/100 g, respectively).

Table 2: Fatty acid profile comparison of butters (g/100 g)

Fatty acid	NonSARA		SARA		COM	
	Mean	SD	Mean	SD	Mean	SD
4:0	1.78	0.1191	1.42	0.2136	2.32	0.1061
6:0	1.79	0.1969	1.07	0.0890	2.09	0.0354
8:0	1.26	0.1239	0.68	0.0336	1.31	0.0707
10:0	3.07	0.1923	1.62	0.0743	3.03	0.0071
11:0	0.04	0.0078	0.05	0.0169	0.09	0.0120
12:0	3.62	0.1002	2.33	0.0579	3.38	0.0778
13:0	0.18	0.0150	0.18	0.0167	0.22	0.0156
13:0 iso	0.03	0.0013	0.02	0.0013	0.03	0.0014
13:0 anteiso	0.10	0.0082	0.08	0.0053	0.10	0.0085
14:0	12.94	0.0970	9.61	0.5116	10.90	0.1414
14:0 iso	0.17	0.0323	0.06	0.0092	0.16	0.0658
14:1 9c	1.09	0.0380	1.48	0.0579	1.03	0.0071
15:0	1.02	0.0855	1.15	0.1712	1.19	0.0212
15:0 iso	0.20	0.0060	0.13	0.0020	0.24	0.0651
15:0 anteiso	0.46	0.0315	0.43	0.0034	0.45	0.0247
16:0	32.39	0.2071	24.69	1.8768	32.10	0.1414
16:0 iso	0.38	0.0204	0.17	0.0133	0.29	0.0007
16:1 9c	1.25	0.2695	1.70	0.1186	1.61	0.0283
17:0	0.63	0.0174	0.57	0.0408	0.78	0.1096
17:0 iso	0.31	0.0252	0.55	0.0353	0.32	0.0092
17:0 anteiso	0.15	0.0072	0.17	0.0148	0.17	0.0247
18:0	9.75	0.6879	8.05	0.2376	9.11	0.0283

Fatty acid	NonSARA		SARA		COM	
	Mean	SD	Mean	SD	Mean	SD
18:0 iso	0.05	0.0050	0.05	0.0047	0.06	0.0170
18:0 anteiso	0.16	0.0313	0.24	0.0057	0.24	0.0113
18:1 9c	19.05	0.7448	21.66	2.2448	19.60	0.0000
18:1 11c	0.53	0.0257	1.27	0.0626	0.65	0.0580
18:1 12c	0.36	0.0208	0.74	0.1293	0.47	0.0120
18:1 13c	0.06	0.0059	0.16	0.0302	0.10	0.0184
18:1 4t	0.02	0.0032	0.04	0.0057	0.03	0.0021
18:1 5t	0.02	0.0013	0.05	0.0061	0.03	0.0078
18:1 6t-8t	0.37	0.0237	1.23	0.0638	0.33	0.0064
18:1 9t	0.38	0.0300	0.99	0.0345	0.35	0.0099
18:1 10t	0.46	0.0184	6.56	0.7763	0.48	0.0014
18:1 11t	1.04	0.0387	2.07	0.3716	1.00	0.0071
18:1 12t	0.41	0.0179	0.97	0.0634	0.40	0.0085
18:1 13t-14t	0.61	0.0308	1.68	0.1331	0.68	0.0177
18:1 16t-18t	0.30	0.0253	0.44	0.0560	0.35	0.0325
18:2 9c,12c	1.72	0.0641	2.50	0.4502	2.23	0.0919
18:2 11t,15c	0.07	0.0055	0.39	0.0338	0.14	0.0629
18:2 9c,11t	0.49	0.0285	1.16	0.2881	0.49	0.0325
18:2 9t,11c	0.01	0.0024	0.08	0.0082	0.02	0.0014
18:2 10t,c12	0.01	0.0009	0.10	0.0058	0.02	0.0014
18:2 9t,11t&10t,12t	0.03	0.0078	0.08	0.0083	0.06	0.0106
18:2 11t,13t	0.01	0.0040	0.03	0.0088	0.03	0.0021
18:3 n3	0.33	0.0030	0.40	0.0591	0.48	0.0127
18:3 n6	0.03	0.0029	0.04	0.0076	0.04	0.0021

Fatty acid	NonSARA		SARA		COM	
	Mean	SD	Mean	SD	Mean	SD
18:4 n3	0.03	0.0045	0.04	0.0154	0.02	0.0007
20:0	0.16	0.0029	0.11	0.0043	0.09	0.0233
20:1 11c	0.14	0.0080	0.12	0.0186	0.10	0.0226
20:2 n6	0.03	0.0058	0.04	0.0058	0.04	0.0035
20:3 n3	0.01	0.0028	0.03	0.0012	0.02	0.0092
20:3 n6	0.09	0.0153	0.09	0.0038	0.10	0.0028
20:4 n3	0.03	0.0095	0.03	0.0069	0.03	0.0021
20:4 n6	0.11	0.0032	0.07	0.0051	0.14	0.0000
20:5 n3	0.04	0.0097	0.03	0.0032	0.04	0.0007
22:0	0.05	0.0044	0.02	0.0052	0.03	0.0049
22:3 n3	0.03	0.0155	0.12	0.0966	0.05	0.0191
22:4 n6	0.01	0.0033	0.03	0.0025	0.02	0.0021
22:5 n3	0.06	0.0039	0.05	0.0064	0.06	0.0028
22:6 n3	0.03	0.0041	0.04	0.0022	0.02	0.0014
23:0	0.01	0.0033	0.02	0.0087	0.02	0.0057
24:0	0.02	0.0062	0.03	0.0171	0.02	0.0141
26:0	0.01	0.0020	0.02	0.0066	0.02	0.0028

BODY WEIGHT AND FOOD CONSUMPTION

Following 8 weeks of high-fat feeding, the growth curves were similar between groups (Figure 4); there was no statistical difference between the group means of final body weight ($p = 0.1938$), although the LFD group was the lowest (Figure 4 & 5). The LFD group had the highest mean daily food intake by weight (Figure 6), but when the total caloric value of mean daily food intake was normalized to body weight, all groups consumed nearly identical calories relative to their body size (Figure 7).

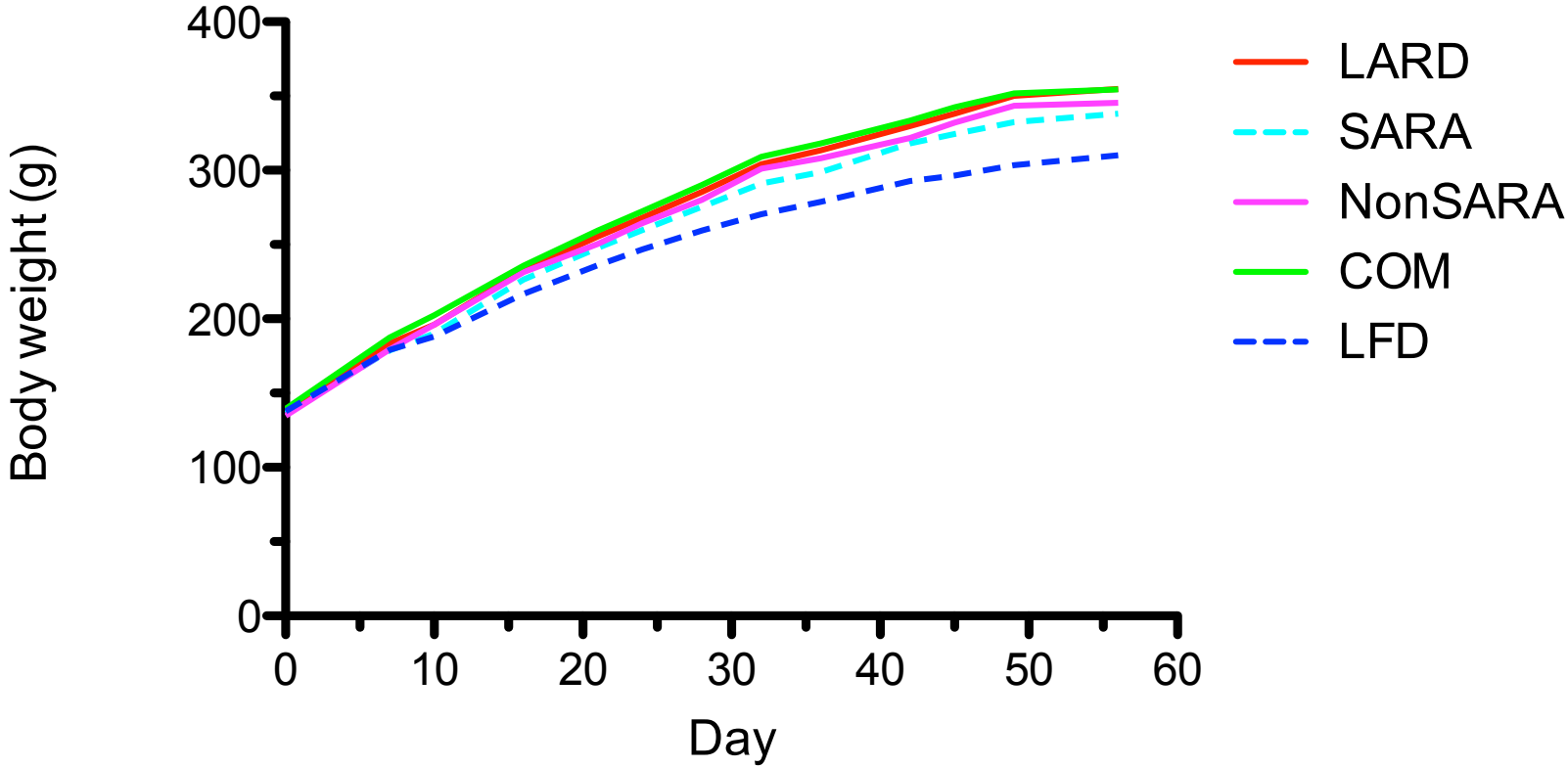

Figure 4: Growth curve of rats fed either low or high fat diets for 8 weeks

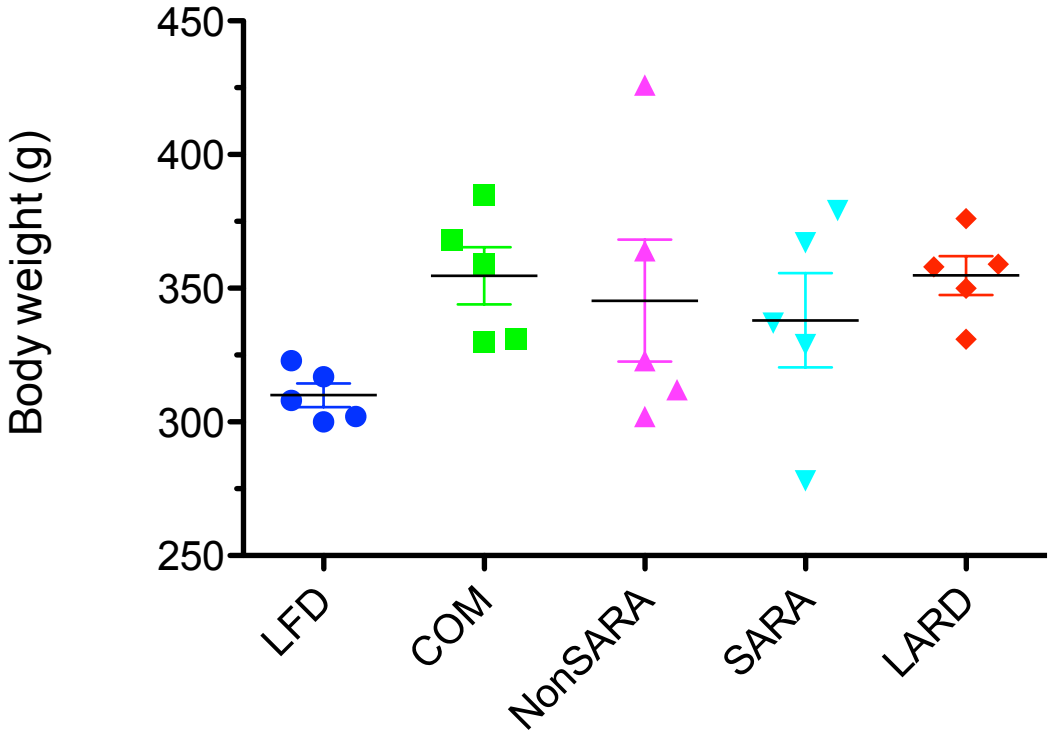

Figure 5: Final body weight of rats fed either low or high fat diets for 8 weeks

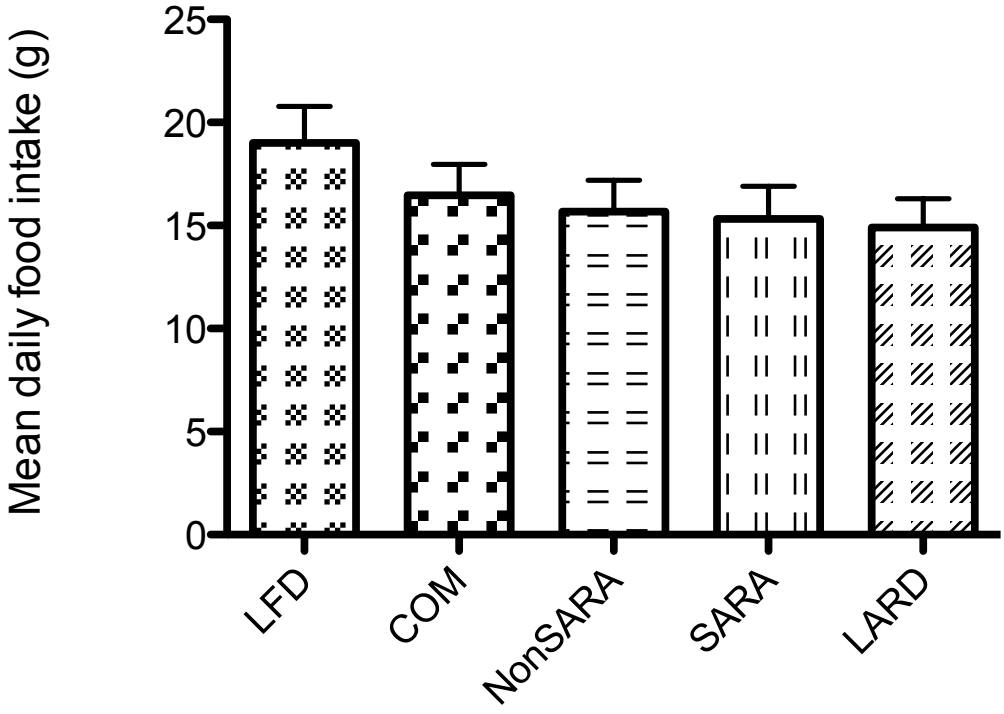

Figure 6: Mean daily food intake of rodents fed either a low or high fat diet for 8 weeks

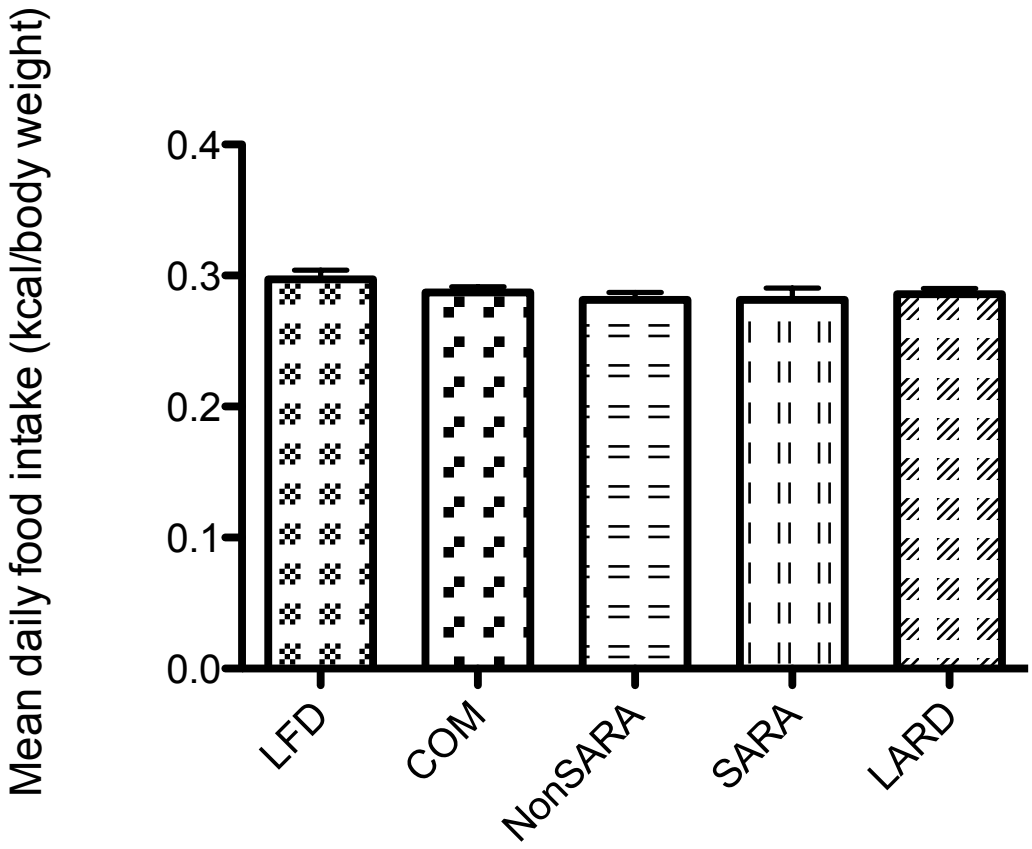

Figure 7: Mean daily food intake in kcal of rats fed either low or high fat diets for 8 weeks

WHOLE BODY GLUCOSE TOLERANCE

There was no significant difference in the area under the curve (AUC) between groups for any of the tolerance tests, therefore tables have been included for each time point of each tolerance test in order to more clearly express the variability and significance of the differences between groups. Despite the lack of statistical significance, there was a consistent trend across all the tolerance tests. The lard-based diet resulted in the greatest deviation of whole body insulin sensitivity relative to the low fat diet; of the butter-fed groups, the SARA group demonstrated the greatest deviation in whole body insulin sensitivity relative to the low fat diet (Figure 8, 10 &12).

Glucose tolerance test results

ANOVA statistical analysis did not uncover a statistical difference between groups either by comparing AUCs ($p = 0.5041$) or at any individual time point of the GTT (Figure 9, Table 3). The total AUC was 1.9%, 24.4%, 62.2% and 69.9% greater than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 9). Peak blood glucose measured 15 minutes after glucose injection was 12.8%, 16.9%, 28.6% and 47.9% greater than LFD for COM, NonSARA, SARA and LARD, respectively (see Table 3 for values).

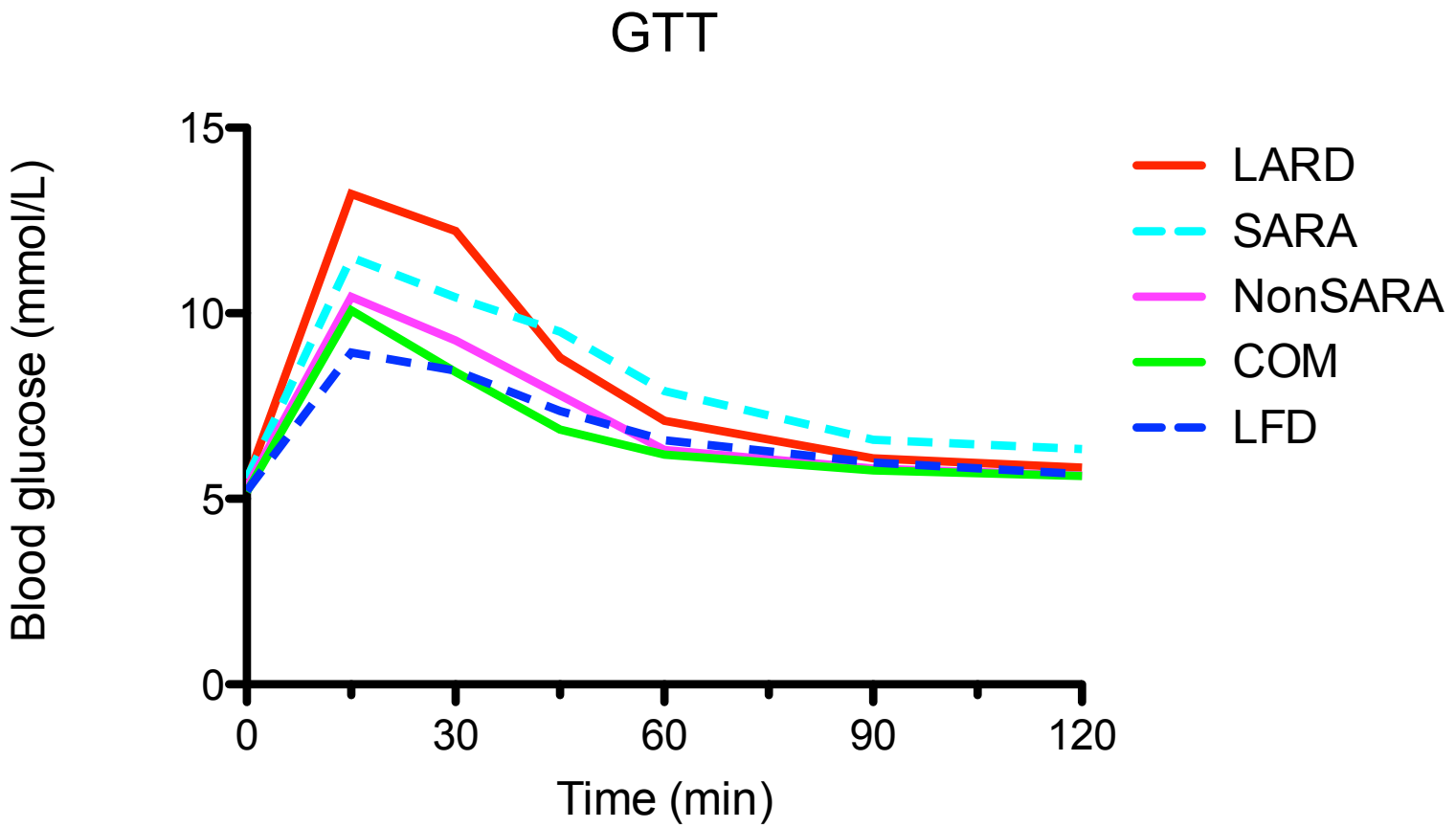

Figure 8: Glucose tolerance test results for rats fed either a low or high fat diet for 8 weeks

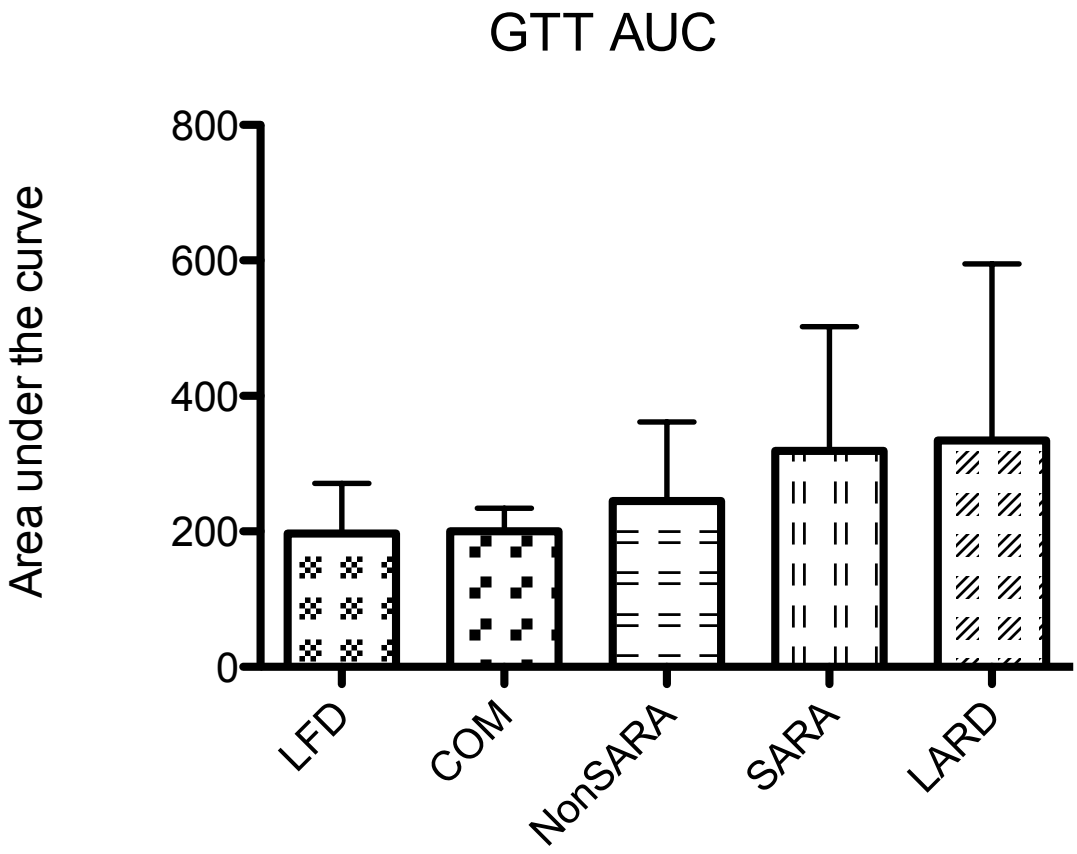

Figure 9: Total area under the curve for glucose tolerance test

Table 3: Blood glucose values (mmol/L) at each time point of a glucose tolerance test

Time	LFD		COM		NonSARA		SARA		LARD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	5.22	0.1881	5.20	0.2470	5.30	0.1140	5.54	0.1568	5.48	0.2691
15	8.94	0.4351	10.08	0.7996	10.45	1.3970	11.50	1.7200	13.22	2.6720
30	8.46	0.3954	8.42	0.3760	9.27	1.1700	10.43	1.7250	12.22	2.0330
45	7.37	0.1908	6.87	0.3680	7.79	0.7574	9.51	1.1170	8.81	1.1380
60	6.58	0.2267	6.20	0.2345	7.04	0.6169	7.90	0.5650	7.11	0.6936
90	5.98	0.2107	5.77	0.3015	5.82	0.3856	6.59	0.2985	6.09	0.3964
120	5.67	0.2154	5.62	0.2596	5.63	0.3992	6.34	0.4007	5.85	0.0775

Statistically significant results are indicated with a '*' (p-value < 0.05)

Insulin tolerance test results

ANOVA statistical analysis did not uncover a statistical difference between groups mean AUC ($p = 0.3682$) (Figure 10 & 11). The total AUC was 21.7%, 31.9%, 35.2% and 45.3% lower than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 11). However when evaluated at individual time points, the LARD group was significantly different from LFD at the 10, 20, 30, 45 and 120-minute time points; SARA differed significantly from LFD only at the 10-minute time point and NonSARA differed significantly from LFD only at the 20-minute time point (Table 4). Peak drop in blood glucose measured 20 minutes after glucose injection was 21.6%, 41.0%, 29.8% and 52.3% greater than LFD for COM, NonSARA, SARA and LARD, respectively (see Table 4 for values).

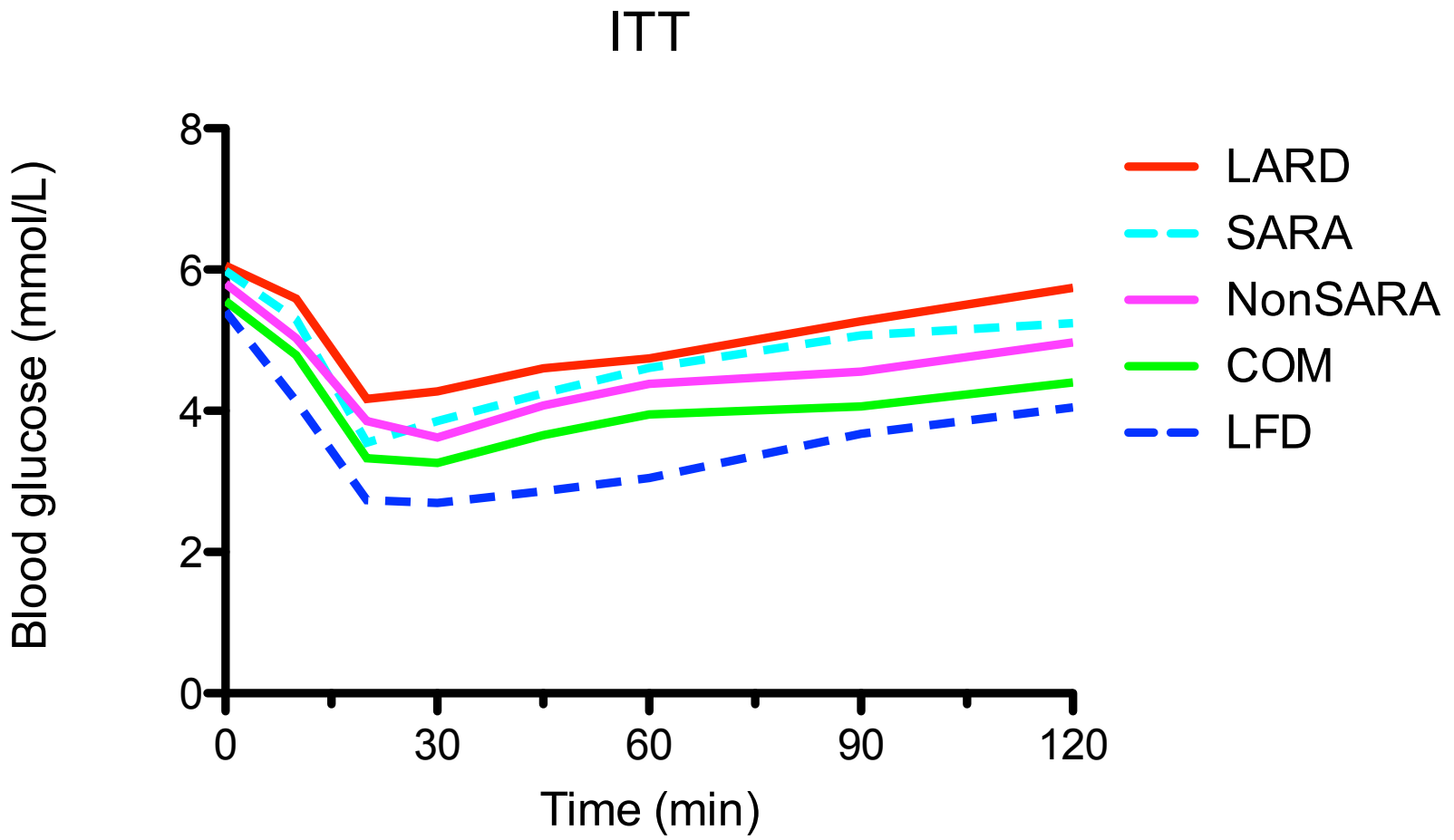

Figure 10: Insulin tolerance test results for rats fed either a low or high fat diet for 8 weeks

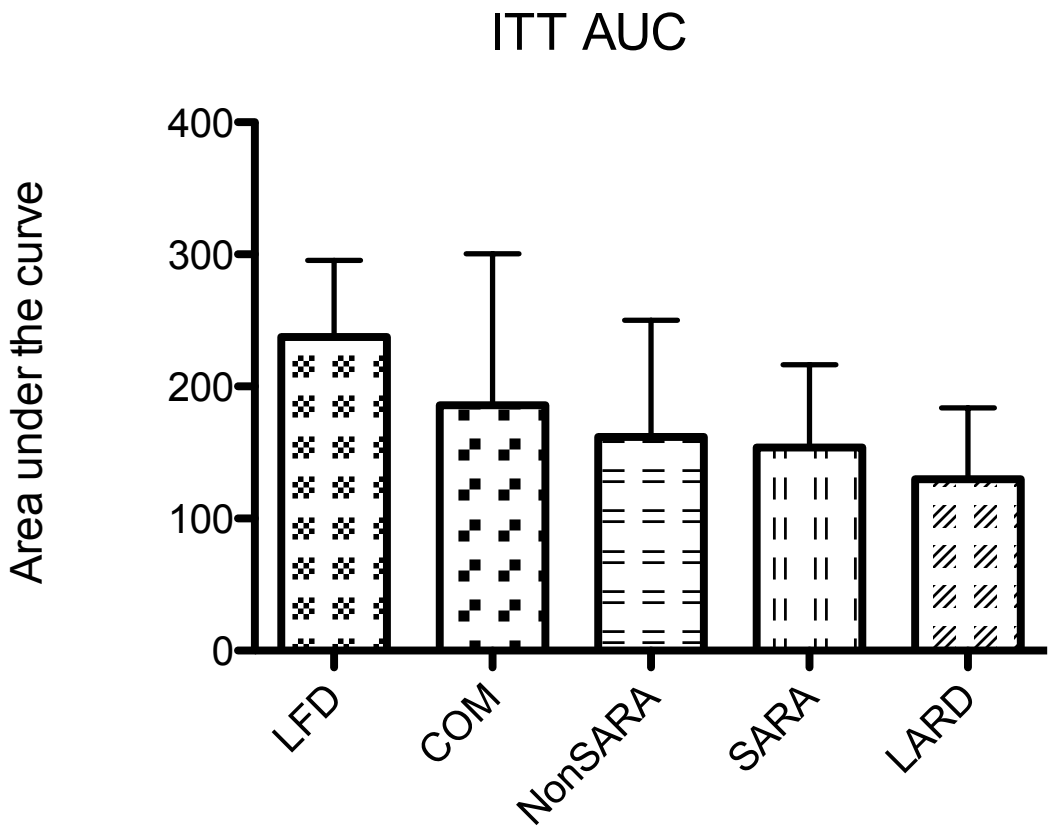

Figure 11: Total area under the curve for insulin tolerance test

Table 4: Blood glucose values (mmol/L) of each time point of an insulin tolerance test

Time	LFD		COM		NonSARA		SARA		LARD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	5.41	0.2258	5.56	0.2227	5.80	0.1304	5.98	0.3247	6.06	0.2522
10	4.14	0.3965	4.79	0.0980	5.03	0.2396	*5.29	0.3747	*5.59	0.1735
20	2.74	0.1796	3.33	0.2289	*3.86	0.3326	3.55	0.2940	*4.17	0.2750
30	2.70	0.1882	3.26	0.4377	3.62	0.3747	3.86	0.3326	*4.28	0.1032
45	2.86	0.2672	3.66	0.5566	4.08	0.4994	4.25	0.2564	*4.60	0.0932
60	3.05	0.4699	3.95	0.6042	4.38	0.5238	4.61	0.3648	4.74	0.2255
90	3.68	0.5648	4.06	0.6028	4.56	0.5016	5.07	0.1625	5.27	0.1091
120	4.05	0.4628	4.40	0.4324	4.97	0.3382	5.24	0.1826	*5.74	0.4331

Statistically significant results are indicated with a '*' (p-value < 0.05)

Pyruvate tolerance test results

ANOVA statistical analysis of the group mean AUC for the PTT uncovered a statistical difference ($p = 0.0466$) only between LFD and LARD groups using the Dunnett post-hoc test (Figure 12 & 13), however this effect was due to large responses of two individuals in the LARD group (AUC for LARD group were 54.8, 69.8, 136.1, 514.5 and 515.0). The PTT response curves were consistent across all subjects with the exception of these two subjects from the LARD group (see Appendix B). The total AUC was 0.9%, 16.5%, 25.7% and 302.7% greater than LFD for COM, NonSARA, SARA and LARD, respectively (Figure 13). Peak blood glucose measured 15 minutes after glucose injection was 0.3% lower than LFD for COM, and 1.8%, 4.6% and 25.8% greater than LFD for NonSARA, SARA and LARD, respectively (see Table 5 for values). Statistically significant differences appeared only between LFD and LARD groups at the 30 and 45-minute time points (Table 5).

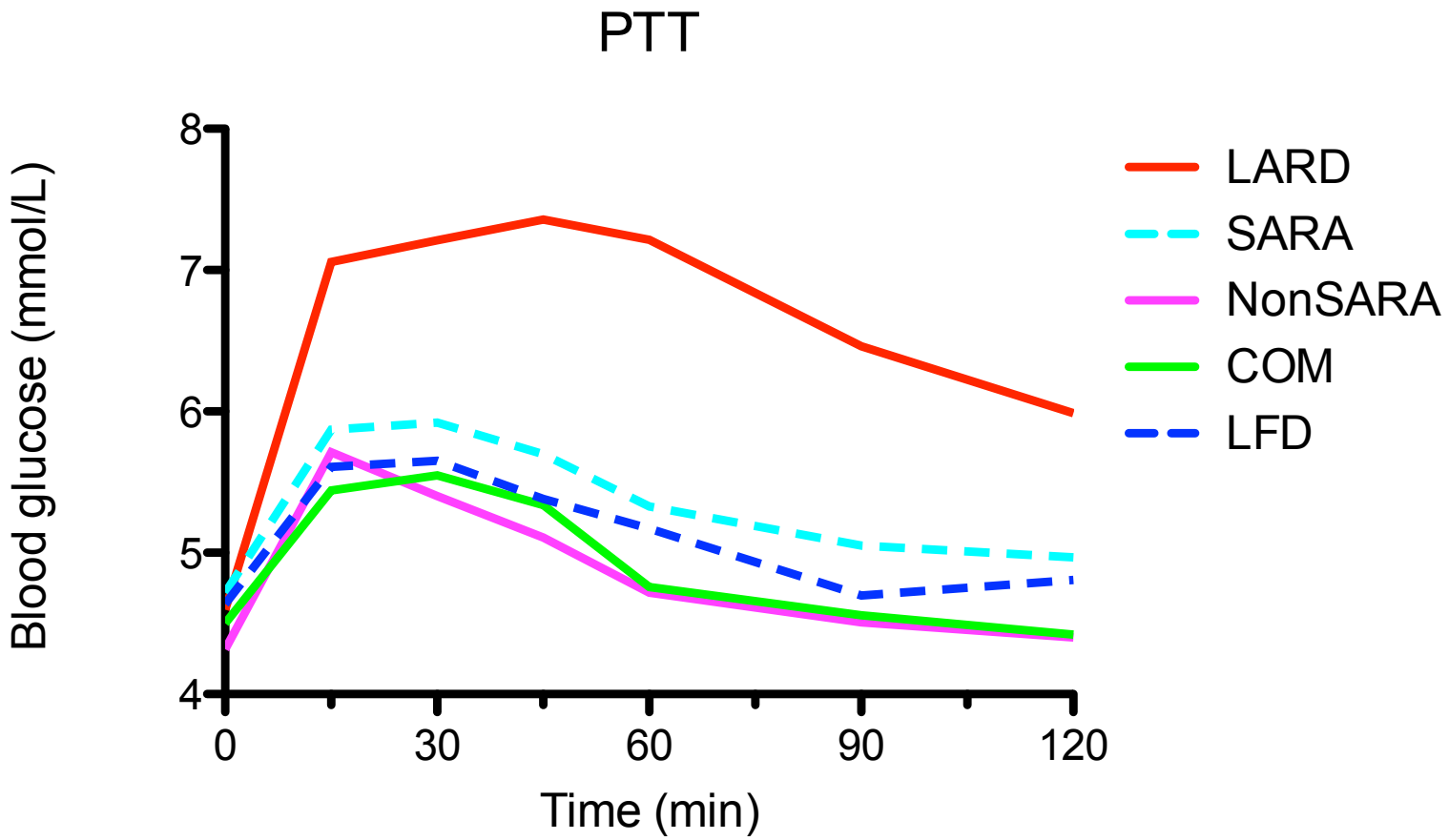

Figure 12: Pyruvate tolerance test for rats fed either a low or high fat diet for 8 weeks

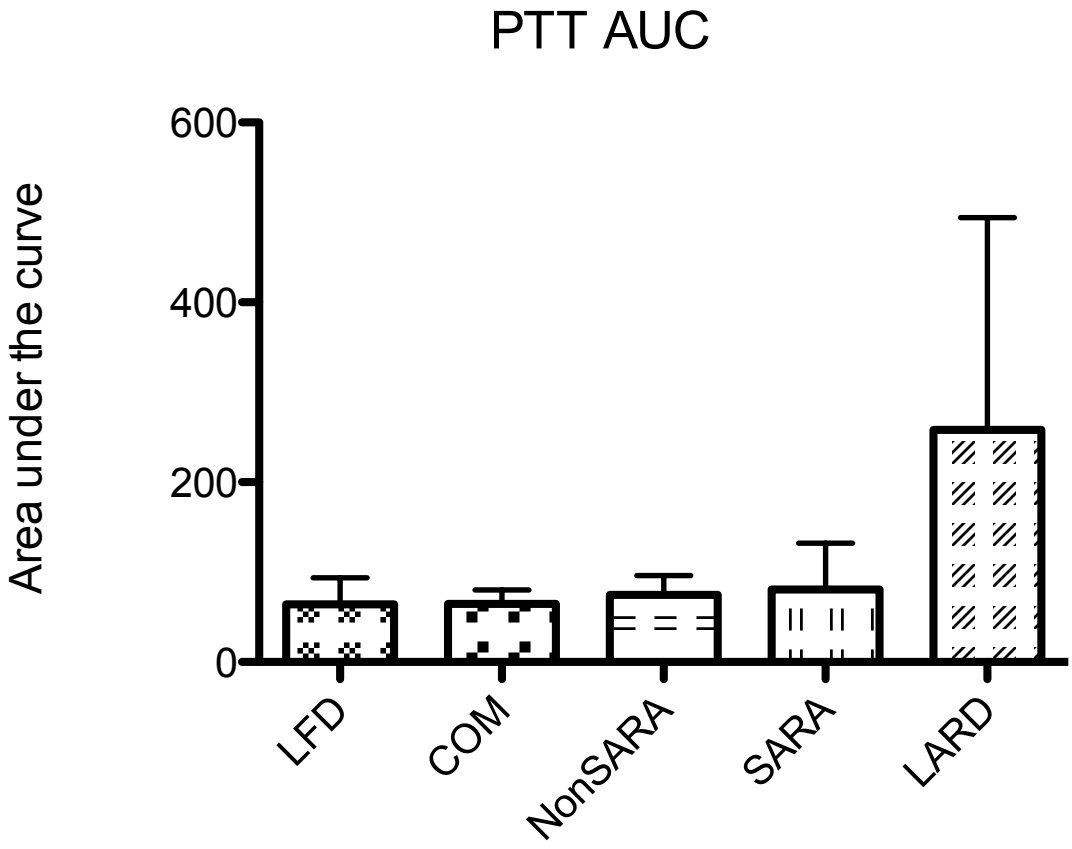

Figure 13: Total area under the curve for pyruvate tolerance test

Table 5: Blood glucose values (mmol/L) of each time point of a pyruvate tolerance test

Time	LFD		COM		NonSARA		SARA		LARD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	4.64	0.1166	4.50	0.1265	4.32	0.1934	4.72	0.3800	4.60	0.0837
15	5.61	0.1735	5.44	0.0980	5.71	0.2891	5.87	0.3192	7.06	0.8291
30	5.65	0.0671	5.55	0.0837	5.40	0.1796	5.92	0.2239	*7.21	0.8533
45	5.38	0.1985	5.34	0.1065	5.11	0.0927	5.70	0.2043	*7.36	0.9351
60	5.17	0.1700	4.76	0.2159	4.72	0.1393	5.33	0.1625	7.22	1.2020
90	4.70	0.1949	4.56	0.1568	4.51	0.1453	5.05	0.1904	6.46	1.1340
120	4.81	0.1453	4.42	0.1393	4.40	0.2408	4.97	0.2824	5.99	0.8171

Statistically significant results are indicated with a '*' (p-value < 0.05)

Table 6: Results of unpaired Student's t-test comparison of blood glucose values between commercial butter and NonSARA butter-fed rodents

Tolerance test	*P*-value
GTT	0.6610
ITT	0.2881
PTT	0.8380

MOLECULAR MARKERS OF CELL STRESS AND INFLAMMATION

There was no substantial activation of any of the MAP kinase pathways that were evaluated (ERK, JNK, p38) by any of the high fat diets in this study (Figures 14-18). In gonadal adipose tissue, there was a statistically significant increase in the phosphorylation of ERK (2.8 fold relative to LFD) and no detectable phosphorylation of p38 (Figure 14). Whereas in subcutaneous adipose tissue, p-38 activation was greater in all the high fat groups, but only the SARA group reached statistical significance (5.8 fold increase relative to LFD) (Figure 15). In skeletal muscle, there was a 2-fold increase in p38 activation in the tricep muscles of the LARD group, and 3.1 and 3.3-fold increase in COM and SARA groups, respectively (Figure 16 & 17). There was no detectible activation of JNK in either skeletal muscle or liver (Figures 16-18).

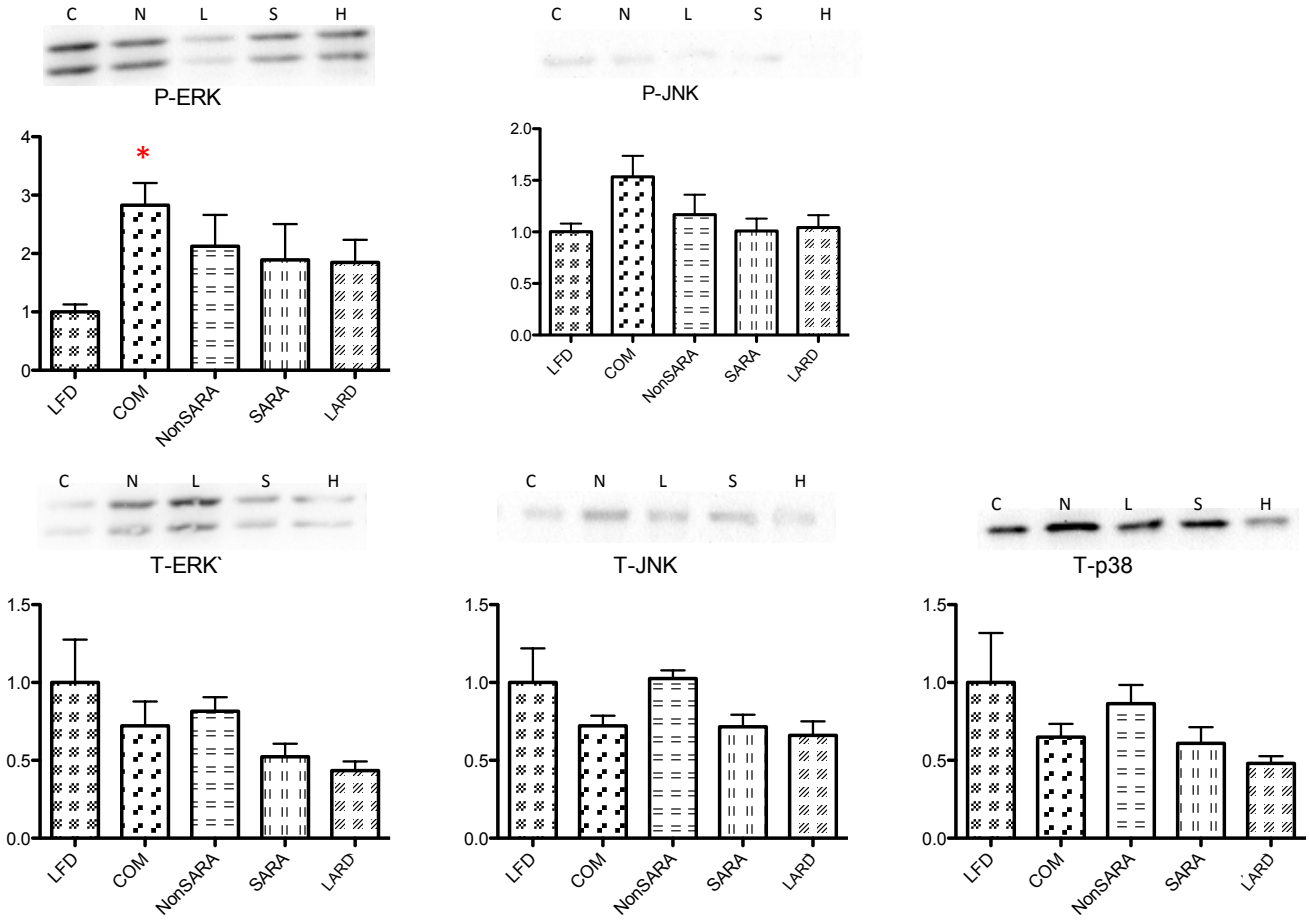

Figure 14: Markers of inflammation in gonadal adipose tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a '*'.

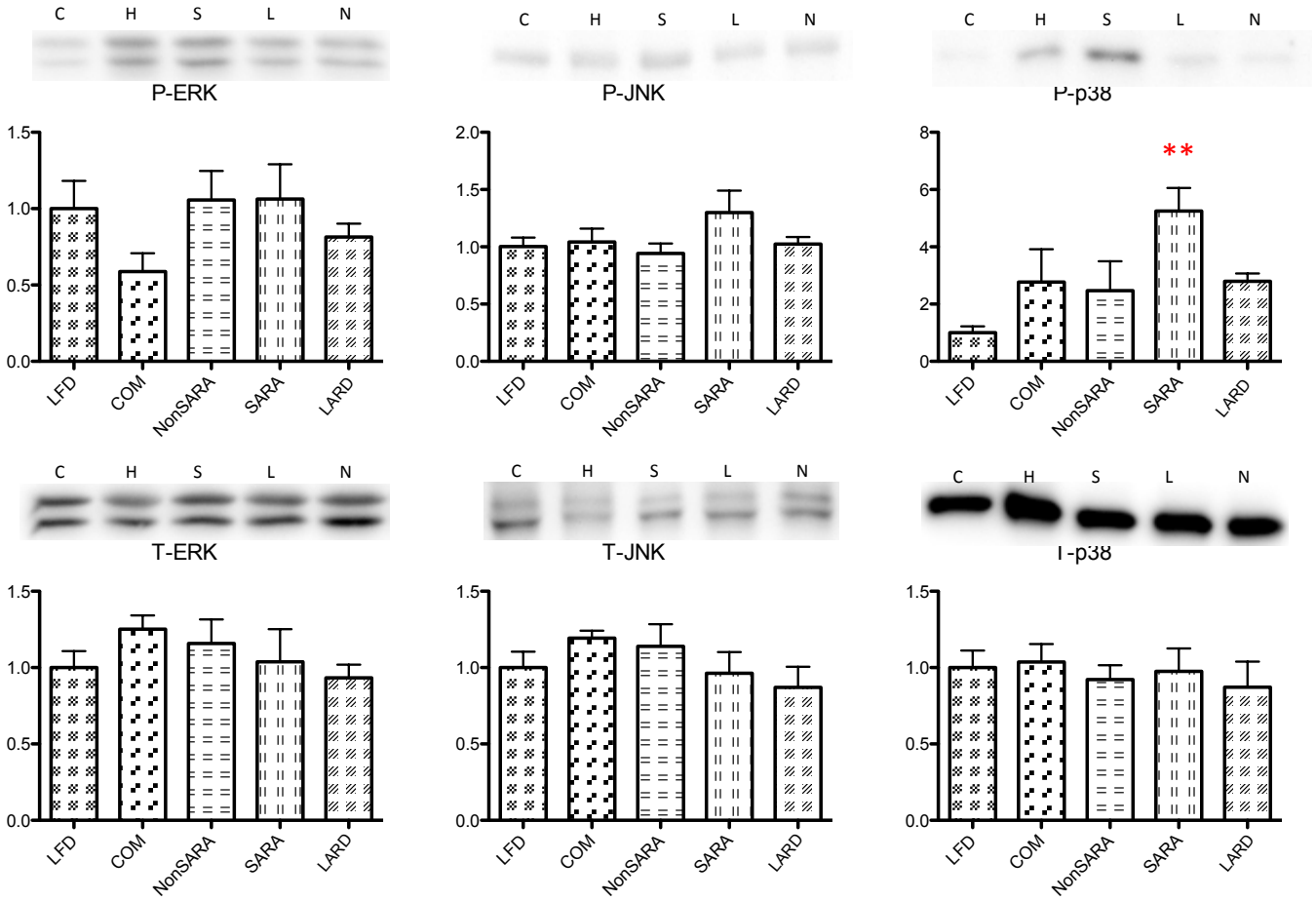

Figure 15: Markers of inflammation in the subcutaneous adipose tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a '*'.

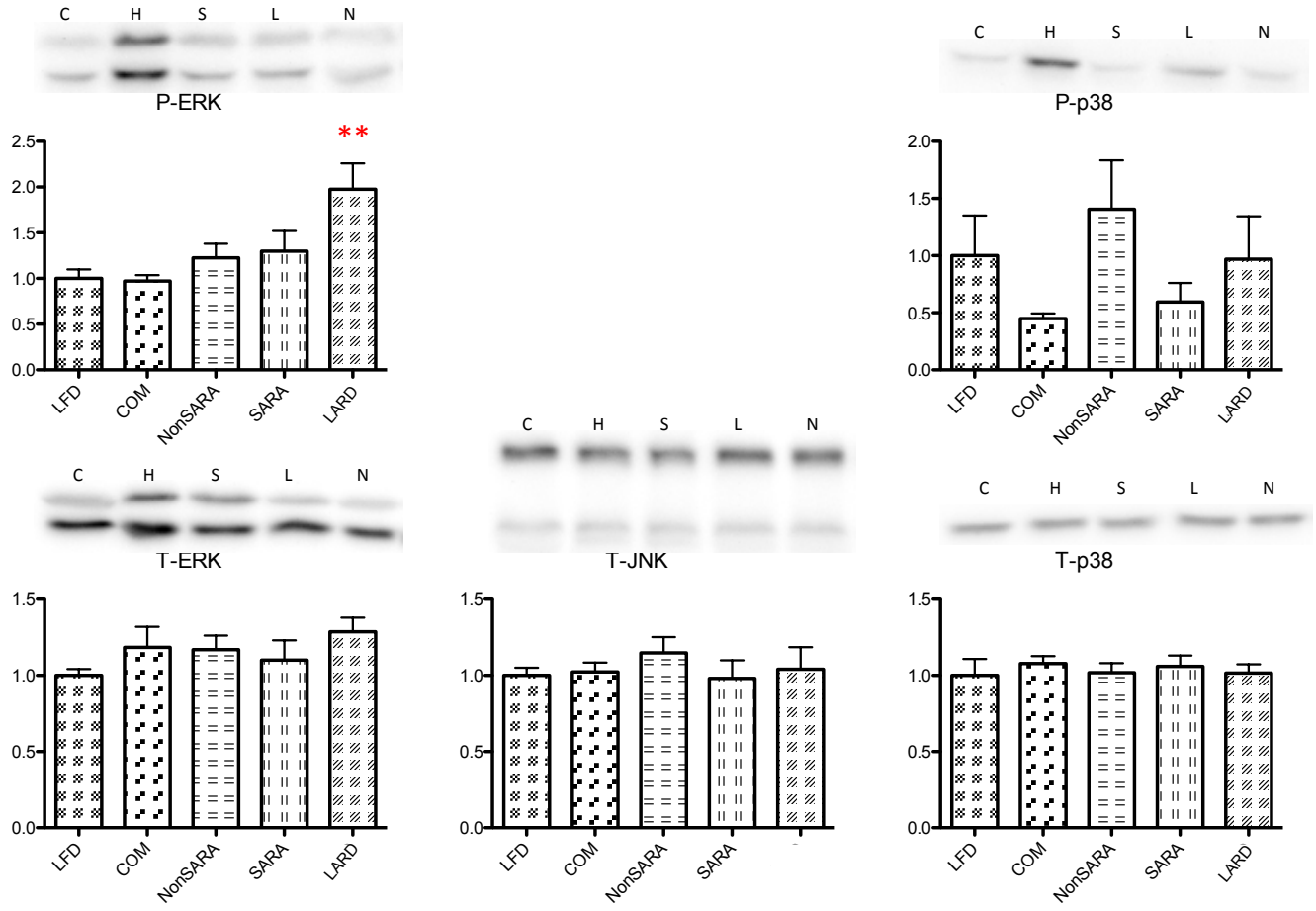

Figure 16: Markers of inflammation in the tricep muscle tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a '*'.

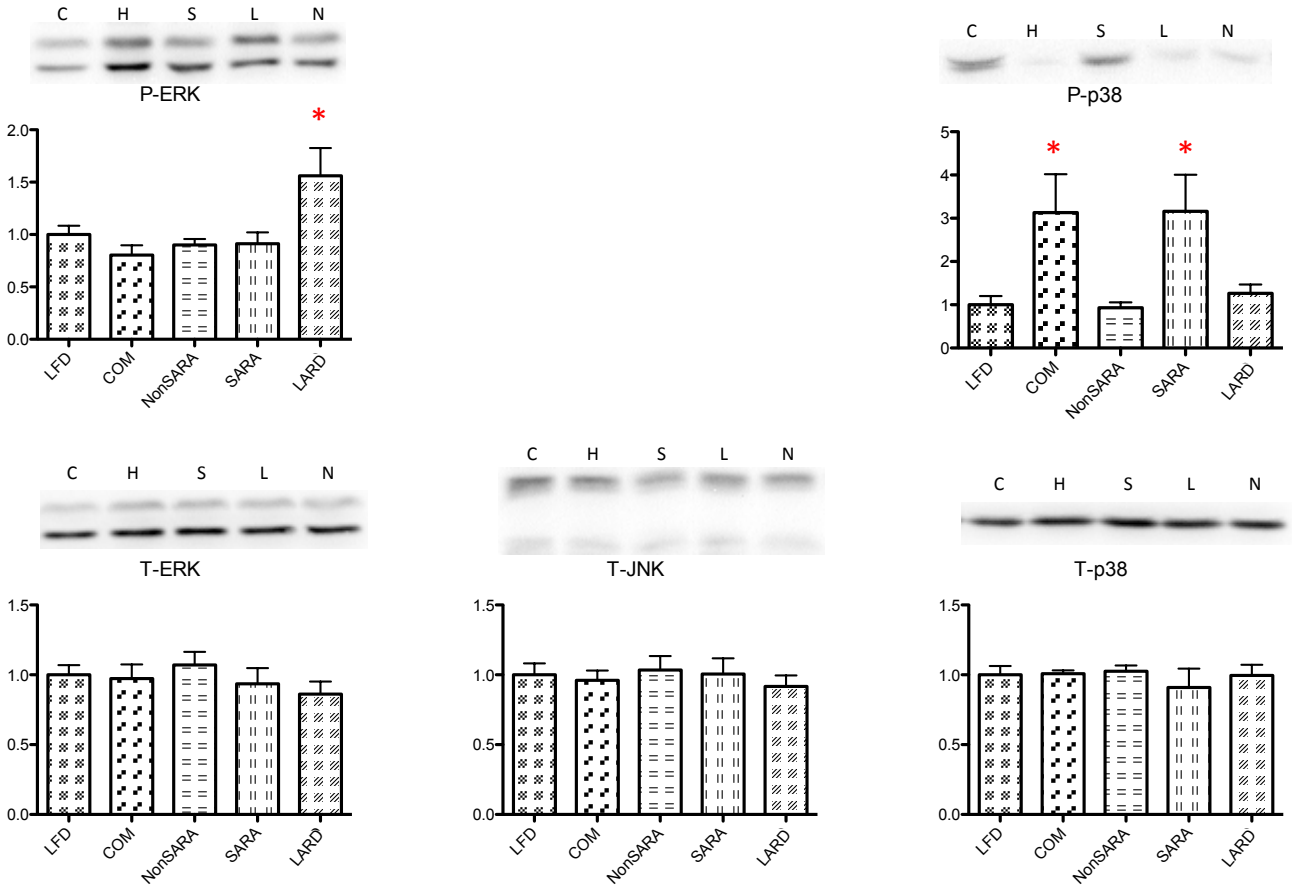

Figure 17: Markers of inflammation in the soleus muscle tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a '*'.

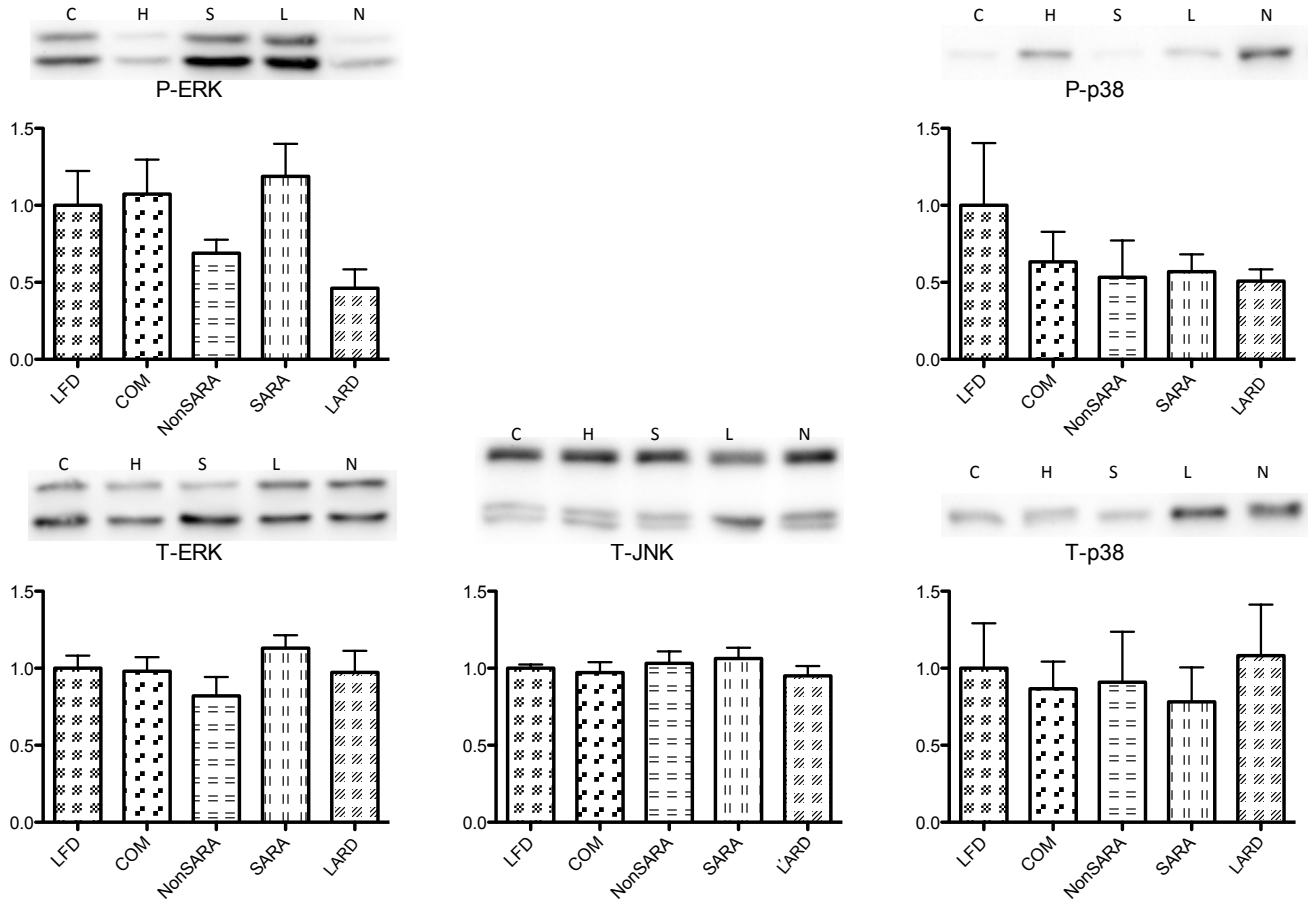

Figure 18: Markers of inflammation in the liver tissue of rodents fed either a low or high fat diet for 8 weeks

Y-axis units express fold change relative to LFD. Blots are labeled L) low fat diet, H) high fat diet, C) commercial butter-based diet, N) NonSARA butter-based diet and S) SARA butter-based diet. Groups that are statistically significant are indicated with a '*'

Chapter Five: Discussion

This study was conducted to evaluate potential negative health effects relating to insulin sensitivity and inflammation of naturally occurring dietary $CLA_{t10,c12}$, given the studies showing that supplemental doses of this isomer has a negative impact on insulin resistance[90-94]. To that end, we produced a 60% high fat diet with the fat component composed entirely of butter produced from milk collected from dairy cows suffering from SARA – a condition that can sometimes result from the dairy industry production practice of feeding a high grain, low forage diet ration to milk-producing cows[101-103]. This butter represents the most $CLA_{t10,c12}$-enriched source of naturally occurring dietary fat for humans. We compared the effects of the high $CLA_{t10,c12}$ butter against a commercially available butter, and included a custom non-SARA butter as control against differences that may arise from custom versus commercially produced butter products.

This study did not produce statistically significant differences in the AUC between any treatment groups in the evaluation of whole body glucose homeostasis using glucose, insulin and pyruvate tolerance tests. The single exception was a statistically significant ($p < 0.05$) effect of the LARD diet in the pyruvate tolerance test due to an exceptionally strong response by two of the five subjects in that group (AUC for the lard group were 54.8, 69.8, 136.1, 514.5 and 515.0). However, this experiment had a small sample size (n=5 per group) and 5 treatment groups, which reduced the power of the statistics produced by this experiment[106]. Despite the low power of the statistics and their inconclusive nature, there were some consistent trends across the tolerance tests.

At the end of 8 weeks, all rats were euglycemic. After 8 weeks of high-fat feeding, there was a consistent trend across all the tolerance tests. The lard-based diet resulted in the greatest deviation of whole body insulin sensitivity relative to the low fat diet in each of the glucose (Figure 8), insulin (Figure 10) and pyruvate tolerance tests (Figure 12). That the lard-based diet had the greatest impact on whole body glucose homeostasis was expected, and served as the positive control in this experiment. The small sample size (n=5 per group) and large number of treatment groups,rendered any comparison to the other treatment groups inconclusive. Despite this fact, there was another trend among the butter-based diets that was consistent across all three tolerance tests. The SARA group demonstrated the most glucose intolerance in the GTT (Figure 8), the least insulin sensitivity in the ITT (Figure 9), and had the greatest hepatic glucose production in the PTT (Figure 10) of the butter-fed groups; however, these results only reached statistical significance at the single time point of the ITT where there was the greatest drop in blood glucose (Table 4). No statistical differences were demonstrated in whole body glucose homeostasis between commercial and non-SARA butter in any of the tolerance tests (Table 6). Due to the low power of the statistics resulting from group mean analysis, a Student's t-test was performed between the commercial and NonSARA butters to more clearly analyze the difference between these groups, since the NonSARA group was included as a control against potential variability due to custom, small-batch butters (Table 6). There were no significant differences between the blood glucose values of the COM and NonSARA groups in any tolerance test (p=0.6610 for the GTT, p=0.2881 for the ITT, p=0.830 for the PTT). In future studies, it may be possible to eliminate the non-

SARA group. All groups had nearly identical food intake (Figure 4) and there was no significant difference in the final mean body weight of the groups, though the LFD group had the lowest final body weight (Figure 2). Caloric intake and fat mass are unlikely to be responsible for the effects on whole body glucose homeostasis seen in high fat-fed rodents. Though the statistics in this experiment were inconclusive, the consistent trends across all three tolerance tests alludes to the possible existence of a treatment effect that may have the potential to be more robust with a larger sample size.

Generally, a 60% high fat diet composed mainly of lard is a commonly used model, and will typically produce systemic and tissue-specific insulin resistance after 8 weeks. In the current study, not even the positive control (LARD) produced the expected degree of insulin resistance. This is likely due to the small sample size (n=5) used in this experiment, gender may have a confounding effect as well. Most studies examining high fat diet-induced insulin resistance use male rats, although the Sprague-Dawley strain is commonly used in metabolic studies[107]. In fact, a replicate of this study failed to produce any difference in body weight or whole body glucose homeostasis between any group (including LFD), even after 22 weeks of high fat feeding (See Appendix A).

MAPKs are thought to be centrally involved in the signaling pathways that mediate interference with insulin signaling. MAPKs - and JNK in particular - can directly interfere with insulin signaling by reducing the activity of IRS-1 via serine phosphorylation, and can be involved in upregulating pro-inflammatory genes that can negatively impact insulin sensitivity[33,12,52]. MAPK activation may therefore indicate 1)

cell intrinsic stress involved in early insulin resistance development (ER stress), 2) the potential inflammatory nature of the fatty acid profile of the diet by mediating signaling through TLR4, and 3) the intensity of tissue inflammation by transducing pro-inflammatory cytokines commonly involved in insulin resistance such as TNFα. We examined MAPK quantity and activation in five metabolic tissues: gonadal and subcutaneous adipose tissue, tricep and soleus muscle, and in the liver. We expected to see the greatest activation of MAPKs in adipose tissue, anticipating that both cell intrinsic metabolic stress and inflammation would originate there. However, we saw limited evidence for the involvement of MAPKs in any of the five tissues we examined. The mechanism underlying the modest insulin resistance induced by high fat feeding in this study is unclear, based on the measurements that were taken.

High dairy intake has been associated with a reduced risk for T2D[108,109] without increasing the risk of cardiovascular disease[110]; however, a meta-analysis concluded that low fat dairy was more effective than high fat dairy consumption[111]. A recent study demonstrated that high consumption of low fat dairy improved fasting insulin by 9% and insulin resistance by 11% in healthy obese individuals[112]. Few studies focus on butter as the sole source of dietary fat. One interesting study demonstrated that healthy men consuming a high fat, butter-based diet supplemented with 5.5 g/day CLA_{mix} showed elevated markers of lipid peroxidation compared to butter alone, but no significant differences in fasting insulin, glucose or insulin resistance; however, there was no non-butter control used in this study[113].

Dairy and butter fat are complex mixtures, and which components impact insulin sensitivity and metabolism remains unknown. The simple addition of milk to the diet in a high sucrose-induced model of insulin resistance was sufficient to improve the insulin sensitivity of rats[114]. There are other components of butter fat that have a marked effect on insulin resistance and inflammation. The supplementation of butyric acid (a short-chain fatty acid found in milk fat) in the diet of obese, high fat-fed mice improved insulin sensitivity (reduced fasting glucose, insulin and improved insulin tolerance) as well as increased thermogenesis, fatty acid oxidation, mitochondrial function and biogenesis in skeletal muscle[115]. Butyrate attenuated proinflammatory signaling (reduced activation of MAPKs and activity of NFκB) and the production of proinflammatory cytokines (TNFα, MCP-1, IL-6) in adipocytes co-cultured with macrophages[116]. These mechanisms were mediated by the butyrate-induced suppression of lipolysis by down-regulating triglyceride lipase, hormone-sensitive lipase and fatty acid binding protein-4 in adipocytes[116]. Additionally, butyrate enhanced the production of anti-inflammatory cytokines (IL-10 and IL-4) and reduced proinflammatory IL-2 and IFN-γ in stimulated human peripheral blood monocytes[117].

In conclusion, the outcome of this study does not suggest an increased risk of insulin resistance in healthy rodents from the higher than normal $CLA_{t10,c12}$ levels that may result from dairy industry production practices. The decreasing impact on insulin resistance of $CLA_{t10,c12}$ as the dose used makes up a smaller proportion of total dietary fat[79,80], and the modest effects on insulin resistance of pharmaceutical doses in human clinical trials[90-94], indicate that slightly elevated $CLA_{t10,c12}$ content of dairy products are unlikely to pose a

health risk to healthy individuals. However, clinical trials show that the effects of $CLA_{t10,c12}$ are more potent in those already overweight or obese than in lean individuals[93,94,97,98]. Therefore it may be prudent to evaluate the effects of SARA butter in a model of established obesity, or for high risk groups such as those with existing insulin resistance or type 2 diabetes.

Chapter Six: Integrative Discussion

The purpose of the study was to evaluate potential negative health effects relating to insulin sensitivity and inflammation of consuming dairy products with increased $CLA_{t10,c12}$ content by evaluating whole body glucose homeostasis and MAPK activation. Elevated $CLA_{t10,c12}$ content of dairy products can result from production practices that increase the incidence of SARA in milk-producing dairy cows. This study was able to evaluate the general question of the hypothesis, but also had some significant shortcomings that should be accounted for in future studies of this type.

Aside from the small sample size (n=5), the main shortcoming in this study was that the positive control group did not produce the anticipated results, which complicates the interpretation of the lack of MAPK activation. In theory, MAPK activation should be able to capture changes in cell metabolism occurring at various points from earlier to later stages of high fat diet-induced insulin resistance: ER stress, FFA signaling through TLR4, pro-inflammatory cytokine signaling and inflammation. Further analysis would be required to determine which factors were responsible for MAPK activation, however due to the lack of MAPK activation detected in the current study, no further analysis was carried out.

High fat feeding is a widely used model to induce insulin resistance in metabolism studies; the effects of a high fat diet develop rapidly[118,119]. Deterioration of glucose

tolerance in mice is measurable by the third day of a high fat diet[27,26,118,119]; hepatic insulin resistance appears as early as one week and skeletal muscle insulin resistance as early as three weeks after the initiation of a high fat diet varying from 45% to 60% kcal from fat[26,27,119,120-122]. In humans, a high fat diet composed of 45% of kcal from fat reduced insulin sensitivity by 11% in less than one month[123]; a 60% fat diet resulted in elevated fasting glucose and reduced suppression of hepatic gluconeogenesis after only 5 days[124]. In the current study, the positive control (LARD) diet did not produce the degree of whole body insulin resistance that was expected after eight weeks of high fat feeding.

Males are represented more frequently than females as the animal and human subjects in metabolism studies, although the Sprague-Dawley strain of rat is commonly used[125,107]. The blunted effects of the LARD diet on whole body glucose homeostasis in the current study may be due to gender differences in fat storage and adipose tissue metabolism. In humans, as in rats, females generally have a greater fat mass as a percent of total body weight than males[126,127]. Females may have metabolic adaptations to manage higher adiposity that makes them less susceptible to small perturbations of adipose tissue dysfunction[128-130]. As a result, females may have a higher threshold of tolerance to high fat intake and/or increasing adiposity before the negative impacts become apparent[128-130]. Since the current study was looking at relatively short-term effects, there may not have been sufficient time to allow significant metabolic dysfunction to develop. Additionally, the pattern of fat storage is different between genders. Females tend to store excess fat in subcutaneous adipose tissue (SAT) depots, whereas males tend to store excess fat in visceral adipose tissue (VAT) [127,131]. Abdominal adiposity, and VAT in particular, is

more highly correlated with T2D risk and the development of insulin resistance than any other adipose tissue depot[3,127]. These results highlight the importance of acknowledging the sex differences in health studies. Applying knowledge gained from health studies focused on a single gender may not serve the entire population. In order to obtain results more comparable to the body of scientific literature, it may be helpful to repeat this study using male rats – if indeed gender is the variable that accounts for the discrepancy in the current study.

Two alternate modifications of this study could produce clearer results in the future. Firstly, a larger sample size would be useful in order to evaluate molecular mechanisms of involved in the very early stages of insulin resistance. For instance, tissue harvesting could be divided into 2 groups: one control group sacrificed in a fasted state and a treatment group that received an insulin injection prior to sacrifice. This design would allow for comparisons in endogenous insulin signaling by examining targets in the phosphorylation cascade that transduces the effects of extracellular insulin, such as the activation of IRS-1 or downstream targets including PKB, AS160 or GLUT4 translocation. Such a design would allow for the evaluation of any tissue type chosen (adipose depots, different skeletal muscles, liver, cardiac muscle). Alternatively, muscle strip explants or cultured adipose tissue explants from each treatment group could be employed for similar purposes.

Additional measurements need to be taken in future studies to identify the cause of MAPK activation once the model has been adjusted to produce insulin resistance and

increasing adiposity. For instance, IRE-1 activation or intracellular calcium levels could indicate ER stress. A measure of plasma cytokines, adipokines, and free fatty acids would also be useful in interpreting potential causes of MAPK activation, as would TLR and TNFR activation.

Although the results of this study do not suggest that high dietary intake of $CLA_{t10,c12}$ pose a health risk to healthy individuals, further study may be warranted. Clinical trials show that the effects of $CLA_{t10,c12}$ are more potent in those already overweight or obese than in lean individuals. Therefore it may be prudent to evaluate the effects of SARA butter in a model of established obesity, or for high-risk groups such as those with existing insulin resistance or type 2 diabetes.

References

1. World Health Organization: WHO Fact Sheet N°311: Obesity and overweight. www.who.int 2013.

2. Edelstein SL, Knowler WC, Bain RP, Andres R, Barrett-Connor EL, Dowse GK, Haffner SM, Pettitt DJ, Sorkin JD, Muller DC, Collins VR, Hamman RF: Predictors of progression from impaired glucose tolerance to NIDDM: an analysis of six prospective studies. Diabetes 1997, 46:701–710

3. Carey VJ, Walters EE, Colditz GA, Solomon CG, Willett WC, Rosner BA, Speizer FE, Manson JE: Body fat distribution and risk of non-insulin-dependent diabetes mellitus in women: The Nurses' Health Study. American Journal of Epidemiology 1997, 145:614–619

4. World Health Organization: Global status report on noncommunicable diseases 2010. www.who.int 2010.

5. World Health Organization: Definition, diagnosis and classification of diabetes mellitus and its complications. www.who.int 1999.

6. Tuomilehto J: Type 2 diabetes is a preventable disease: Lifestyle is the key. Journal of Medical Sciences 2010, 3:82–86

7. Boura-Halfon S, Zick Y: Phosphorylation of IRS proteins, insulin action, and insulin resistance. AJP: Endocrinology and Metabolism 2009, 296:E581–E591

8. Taniguchi CM, Emanuelli B, Kahn CR: Critical nodes in signalling pathways: insights into insulin action. Nature Reviews: Molecular Cell Biology 2006, 7:85–96

9. de Luca C, Olefsky JM: Inflammation and insulin resistance. FEBS Letters 2008, 582:97–105

10. Carlson CJ, White MF, Rondinone CM: Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. Biochemical and Biophysical Research Communications 2004, 316:533–539

11. Gual P, Gr meaux T, Gonzalez T, Le Marchand-Brustel Y, Tanti JF: MAP kinases and mTOR mediate insulin-induced phosphorylation of Insulin Receptor Substrate-1 on serine residues 307, 612 and 632. Diabetologia 2003, 46:1532–1542

12. Tanti J-F, Jager J: Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. Current Opinion in Pharmacology 2009, 9:753–762

13. Katz M, Amit I, Yarden Y: Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 2007, 1773:1161–1176

14. Rudolph TK, Freeman BA: Transduction of Redox Signaling by Electrophile-Protein Reactions. Science Signaling 2009, 2:re7–re7

15. Zarubin T, Han J: Activation and signaling of the p38 MAP kinase pathway. Cell Research 2005, 15:11–18

16. Schröfelbauer B, Hoffman A: How do pleiotropic kinase hubs mediate specific signaling by TNFR superfamily members? Immunological Reviews 2011, 244:29–43

17. Lin A: Activation of the JNK signaling pathway: Breaking the brake on apoptosis. BioEssays 2002, 25:17–24

18. Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, Karin M: The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 1988, 54:541–552

19. Monje P, Hernández-Losa J, Lyons RJ, Castellone MD, Gutkind JS: Regulation of the transcriptional activity of c-Fos by ERK: a novel role for the prolyl isomerase PIN1. Journal of Biological Chemistry 2005, 280:35081–35084

20. Nishikori M: Classical and Alternative NF-kB Activation Pathways and Their Roles in Lymphoid Malignancies. Journal of Clinical and Experimental Hematopathology 2005, 45:15–24

21. Zhang X, Huang X, Olumi AF: Repression of NF-κB and activation of AP-1 enhance apoptosis in prostate cancer cells. International Journal of Cancer 2009, 124:1980–1989

22. Meyer M, Pahl HL, Baeuerle PA: Regulation of the transcription factors NFkB and AP-1 by redox changes. Chemico-Biological Interactions 1994, 91:91–100

23. de Alvaro C, Teruel T, Hernandez R, Lorenzo M: Tumor Necrosis Factor Produces Insulin Resistance in Skeletal Muscle by Activation of Inhibitor B Kinase in a p38 MAPK-dependent Manner. Journal of Biological Chemistry 2004, 279:17070–17078

24. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. Nature 2002, 420:333–338

25. Baron AD, Brechtel G, Wallace P, Edelman SV: Rates and tissue sites of non-insulin and insulin-mediated glucose uptake in humans. American Journal of Physiology 1988, 255:E769–74

26. Turner N, Kowalski GM, Leslie SJ, Risis S, Yang C, Lee-Young RS, Babb JR, Meikle PJ, Lancaster GI, Henstridge DC, White PJ, Kraegen EW, Marette A, Cooney GJ,

Febbraio MA, Bruce CR: Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding. Diabetologia 2013, 56:1638–1648

27. Lee YS, Pingping L, Huh JY, Hwang IJ, Lu M, Kim JI, Ham M, Talukdar S, Chen A, Lu WJ, Bandyopadhyay GK, Schwendener R, Olefsky J, Kim JB: Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance. Diabetes 2011, 60:2474–2483

28. Wellen KE, Hotamisligil GS: Obesity-induced inflammatory changes in adipose tissue. Journal of Clinical Investigation 2003, 112:1785–1788

29. Gregor MF, Hotamisligil GS: Adipocyte stress: the endoplasmic reticulum and metabolic disease. The Journal of Lipid Research 2007, 48:1905–1914

30. Cnop M, Foufelle F, Velloso LA: Endoplasmic reticulum stress, obesity and diabetes. Trends in Molecular Medicine 2012, 18:59–68

31. Schröder M: Endoplasmic reticulum stress responses. Cellular and Molecular Life Sciences 2007, 65:862–894

32. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D: Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1. Science 2000, 287:664–666

33. Aguirre V, Uchida T, Yenush L, Davis R, White MF: The c-Jun NH2-terminal Kinase Promotes Insulin Resistance during Association with Insulin Receptor Substrate-1 and Phosphorylation of Ser307. Journal of Biological Chemistry 2000, 275:9047–9054

34. Özcan U, Cao Q, Yilmaz E, Lee A-H, Iwakoshi NN, Özdelen E, Tuncman G, Görgün CZ, Glimcher LH, Hotamisligil GS: Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes. Science 2004, 306:457–461

35. Hotamisligil GS: Endoplasmic Reticulum Stress and the Inflammatory Basis of Metabolic Disease. Cell 2010, 140:900–917

36. Mathur SK, Jain P, Mathur P: Microarray Evidences the Role of Pathologic Adipose Tissue in Insulin Resistance and Their Clinical Implications. Journal of Obesity 2011, 2011:1–16

37. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. Journal of Clinical Investigation 2003, 112:1821–1830

38. Delarue J, Magnan C: Free fatty acids and insulin resistance. Current Opinion in Clinical Nutrition and Metabolic Care 2007, 10:142–148

39. Howe HR III, Heidal K, Choi MD, Kraus RM, Boyle K, Hickner RC: Increased adipose tissue lipolysis after a 2-week high-fat diet in sedentary overweight/obese men. Metabolism 2011, 60:976–981

40. Kim BS, Cha H-N, Kim Y-W, Kim J-Y, Dan J-M, Park S-Y: Inhibition of Lipid Infusion–Induced Skeletal Muscle Insulin Resistance by Cotreatment With Tempol and Glutathione in Mice. Journal of Pharmacological Sciences 2009, 110:370–380

41. Kopecky J, Krusinova E, Klementova M, Kazdova L, Mlejnek P, Pravenec M, Hill M, Pelikanova T: Plasma concentrations and adipose tissue expressions during 24-hour lipid infusion in healthy men. Physiological Research 2010, 59:86–96

42. Ragheb R, Shanab GML, Medhat AM, Seoudi DM, Adeli K, Fantus IG: Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: Evidence for PKC activation and oxidative stress-activated signaling pathways. Biochemical and Biophysical Research Communications 2009, 389:211–216

43. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS: TLR4 links innate immunity and fatty acid–induced insulin resistance. Journal of Clinical Investigation 2006, 116:3015–3025

44. Davis JE: Development of obesity-induced inflammation and insulin resistance: The role of adipose tissue, fatty acids, and toll-like receptors. 2008, 1–171

45. Holland WL, Bikman BT, Wang L-P, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, Pagliassotti MJ, Scherer PE, Summers SA: Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid–induced ceramide biosynthesis in mice. Journal of Clinical Investigation 2011, 121:1858–1870

46. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chanmugam P, Bray G, Hwang DH: Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids. The Journal of Lipid Research 2002, 44:479–486

47. Tsukumo DML, Carvalho-Filho MA, Carvalheira JBC, Prada PO, Hirabara SM, Schenka AA, Araujo EP, Vassallo J, Curi R, Velloso LA, Saad MJA: Loss-of-Function Mutation in Toll-Like Receptor 4 Prevents Diet-Induced Obesity and Insulin Resistance. Diabetes 2007, 56:1986–1998

48. Lumeng CN, Bodzin JL, Saltiel AR: Obesity induces a phenotypic switch in adipose tissue macrophage polarization. Journal of Clinical Investigation 2007, 117:175–184

49. Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE: The Lipopolysaccharide-activated Toll-like Receptor (TLR)-4 Induces Synthesis of the Closely Related Receptor TLR-2 in Adipocytes. Journal of Biological Chemistry 2000, 275:24255–24263

50. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, Esposito K, Jönsson LS, Kolb H, Lansink M, Marcos A, Margioris A, Matusheski N, Nordmann

H, O'Brien J, Pugliese G, Rizkalla S, Schalkwijk C, Tuomilehto J, Wärnberg J, Watzl B, Winklhofer-Roob BM: Dietary factors and low-grade inflammation in relation to overweight and obesity. British Journal of Nutrition 2011, 106:S5–S78

51. Kawakami M, Murase T, Ogawa H, Ishibashi S, Mori N, Takaku F, Shibata S: Human recombinant TNF suppresses lipoprotein lipase activity and stimulates lipolysis. Journal of Biochemistry 1987, 101:331–338

52. Cawthorn WP, Sethi JK: TNF-α and adipocyte biology. FEBS Letters 2008, 582:117–131

53. Ruan H, Hacohen N, Golub TR, Parijs LV, Lodish HF: Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: Nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes 2002, 51:1319–1336

54. Hotamisligil GS, Murray DL, Choy LN, Speigelman BM: Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:4854–4858

55. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr.: Obesity is associated with macrophage accumulation in adipose tissue. Journal of Clinical Investigation 2003, 112:1796–1808

56. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993, 259:87–91

57. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. Journal of Clinical Investigation 1995, 95:2409–2415

58. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS: Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 1997, 389:610–614

59. Salles J, Tardif N, Landrier J-F, Mothe-Satney I, Guillet C, Boue-Vaysse C, Combaret L, Giraudet C, Patrac V, Bertrand-Michel J, Denis P, Chardigny J-M, Boirie Y, Walrand S: TNFα gene knockout differentially affects lipid deposition in liver and skeletal muscle of high-fat-diet mice. The Journal of Nutritional Biochemistry 2012, 23:1685–1693

60. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science 1996, 271:665–668

61. Stephens JM, Lee J, Pilch PF: Tumor Necrosis Factor-alpha -induced Insulin Resistance in 3T3-L1 Adipocytes Is Accompanied by a Loss of Insulin Receptor Substrate-1 and GLUT4 Expression without a Loss of Insulin Receptor-mediated Signal

Transduction. Journal of Biological Chemistry 1997, 272:971–976

62. Feinstein R, Kanety H, Papa MZ, Lunenfeld B, Karasik A: Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates. The Journal of Biological Chemistry 1993, 268:26055–26058

63. Turpeinen AM, Mutanen M, Aro A, Salminen I, Basu S, Palmquist DL, Griinari JM: Bioconversion of vaccenic acid to conjugated linoleic acid in humans. The American Journal of Clinincal Nutrition 2002, 76:504–510

64. Shingfield KJ, Bernard L, Leroux C, Chilliard Y: Role of trans fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. animal 2010, 4:1140–1166

65. Khanal RC, Dhiman TR, Boman RL: Changes in fatty acid composition of milk from lactating dairy cows during transition to and from pasture. Livestock Science 2008, 114:164–175

66. Bargo F, Delahoy JE, Schroeder GF, Baumgard LH, Muller LD: Supplementing total mixed rations with pasture increase the content of conjugated linoleic acid in milk. Animal Feed Science and Technology 2006, 131:226–240

67. Griinari JM, Corl BA, Lacy SH, Chouinard PY, Nurmela KVV, Bauman DE: Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by delta-9 desaturase. The Journal of Nutrition 2000, 130:2285–2292

68. Corl BA, Baumgard LH, Griinari JM, Delmonte P, Morehouse KM, Yurawecz MP, Bauman DE: Trans-7, cis-9 CLA is synthesized endogenously by delta-9 desaturase in dairy cows. Lipids 2002, 37:681–688

69. Palladino RA, O'Donovan M, Murphy JJ, McEvoy M, Callan J, Boland TM, Kenny DA: Fatty acid intake and milk fatty acid composition of Holstein dairy cows under different grazing strategies: Herbage mass and daily herbage allowance. Journal of Dairy Science 2009, 92:5212–5223

70. Griinari JM, Bauman DE: Production of Low Fat Milk by Diet Induced Milk Fat Depression. Advances in Dairy Technology 2001, 13:197–212

71. Randle PJ, Garland PB, Hales CN, Newsholme EA: The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. The Lancet 1963, 1:785–789

72. Lee KN, Pariza MW, Ntambi JM: Conjugated Linoleic Acid Decreases Hepatic Stearoyl-CoA Desaturase mRNA Expression. Biochemical and Biophysical Research Communications 1998, 248:817–821

73. Chouinard PY, Corneau L, Barbano DM, Metzger LE, Bauman DE: Conjugated linoleic acid alters milk fatty acid composition and inhibits milk fat synthesis in dairy cows. Journal of Nutrition 1998, 129:1579–1585

74. Prandini A, Sigolo S, Piva G: Conjugated linoleic acid (CLA) and fatty acid composition of milk, curd and Grana Padano cheese in conventional and organic farming systems. Journal of Dairy Research 2009, 76:278

75. Gorissen L, Raes K, Weckx S, Dannenberger D, Leroy F, Vuyst L, Smet S: Production of conjugated linoleic acid and conjugated linolenic acid isomers by Bifidobacterium species. Applied Microbiology and Biotechnology 2010, 87:2257–2266

76. de Carvalho EBT, de Melo ILP, Mancini-Filho J: Chemical and physiological aspects of isomers of conjugated fatty acids. Ciência e Tecnologia de Alimentos 2010, 30:295–307

77. Demir AS, Talpur FN: Chemoenzymatic Conversion of Linoleic Acid into Conjugated Linoleic Acid. Journal of Agricultural and Food Chemistry 2010, 58:1646–1652

78. Park Y, Albright KJ, Liu W, Storkson J, Cook ME, Pariza MW: Effect of conjugated linoleic acid on body composition in mice. Lipids 1997, 32:853–858

79. Tsuboyama-Kasaoka N, Miyazaki H, Kasaoka S, Ezaki O: Increasing the amount of fat in a conjugated linoleic acid-supplemented diet reduces lipodystrophy in mice. The Journal of Nutrition 2003, 133:1793–1799

80. Tsuboyama-Kasaoka N, Takahashi M, Tanemura K, Kim H-J, Tange T, Okuyama H, Kasai M, Ikemoto S, Ezaki O: Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. Diabetes 2000, 49:1534–1542

81. Shen W, Martinez K, Chuang CC, McIntosh M: The Phospholipase C Inhibitor U73122 Attenuates trans-10, cis-12 Conjugated Linoleic Acid-Mediated Inflammatory Signaling and Insulin Resistance in Human Adipocytes. Journal of Nutrition 2013, 143:584–590

82. Martinez K, Shyamasundar S, Kennedy A, Chuang CC, Marsh A, Kincaid J, Reid T, McIntosh M: Diacylglycerol kinase inhibitor R59022 attenuates conjugated linoleic acid-mediated inflammation in human adipocytes. The Journal of Lipid Research 2013, 54:662–670

83. Kennedy A, Martinez K, Chung S, LaPoint K, Hopkins R, Schmidt SF, Andersen K, Mandrup S, McIntosh M: Inflammation and insulin resistance induced by trans-10, cis-12 conjugated linoleic acid depend on intracellular calcium levels in primary cultures of human adipocytes. The Journal of Lipid Research 2010, 51:1906–1917

84. LaRosa PC, Riethoven JJM, Chen H, Xia Y, Zhou Y, Chen M, Miner J, Fromm ME: Trans-10, cis-12 conjugated linoleic acid activates the integrated stress response pathway in adipocytes. Physiological Genomics 2007, 31:544–553

85. Chung S, Brown JM, Provo JN, Hopkins R, McIntosh M: Conjugated Linoleic Acid Promotes Human Adipocyte Insulin Resistance through NFkB-dependent Cytokine

Production. Journal of Biological Chemistry 2005, 280:38445–38456

86. Choi JS, Koh I-U, Jung MH, Song J: Effects of three different conjugated linoleic acid preparations on insulin signalling, fat oxidation and mitochondrial function in rats fed a high-fat diet. British Journal of Nutrition 2007, 98:264

87. Brown JM, Maria B, Chung S, Fabiyi O, Morrison RF, Mandrup S, McIntosh MK: Conjugated linoleic acid induces human adipocyte delipidation: Autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. Journal of Biological Chemistry 2004, 279:26735–26747

88. Simón E, Macarulla MT, Fernández-Quintela A, Rodríguez VM, Portillo MP: Body fat-lowering effect of conjugated linoleic acid is not due to increased lipolysis. Journal of Physiology & Biochemistry 2005, 61:363–370

89. Xu X, Storkson J, Kim S, Sugimoto K, Park Y, Pariza MW: Short-term intake of conjugated linoleic acid inhibits lipoprotein lipase and glucose metabolism but does not enhance lipolysis in mouse adipose tissue. The Journal of Nutrition 2003, 133:663–667

90. Blankson H, Stakkestad JA, Fagertun H, Thom E, Wadstein J, Gudmundsen O: Conjugated linoleic acid reduces body fat mass in overweight and obese humans. The Journal of Nutrition 2000, 130:2943–2948

91. Risérus U, L B, Vessby B: Conjugated linoleic acid reduced abdominal adipose tissue in obese middle-aged men with signs of the metabolic syndrome: a randomised controlled trial. International Journal of Obesity 2001, 25:1129–1135

92. Risérus U, Vessby B, Arner P, Zethelius B: Supplementation with trans10, cis12-conjugated linoleic acid induces hyperproinsulinaemia in obese men: close association with impaired insulin sensitivity. Diabetologia 2004, 47:

93. Whigham LD, Watras AC, Schoeller DA: Efficacy of conjugated linoleic acid for reducing fat mass: a meta-analysis in humans. The American Journal of Clinical Nutrition 2007, 85:1203–12011

94. Schoeller DA, Watras AC, Whigham LD: A meta-analysis of the effects of conjugated linoleic acid on fat-free mass in humans. Applied Physiology, Nutrition, and Metabolism 2009, 34:975–978

95. Risérus U, Basu S, Jovinge S, Fredrikson GN, Ärnlöv J, Vessby B: Supplementation With Conjugated Linoleic Acid Causes Isomer-Dependent Oxidative Stress and Elevated C-Reactive Protein. Circulation 2002, 106:1925–1929

96. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA: Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. The Journal of Nutrition 2001, 131:1548–1554

97. Tholstrup T, Raff M, Straarup EM, Lund P, Basu S, Bruun JM: An oil mixture with trans-10, cis-12 conjugated linoleic acid increases markers of inflammation and in vivo lipid peroxidation compared with cis-9, trans-11 conjugated linoleic acid in postmenopausal women. The Journal of Nutrition: Nutrition and Disease 2008, 138:1445–1451

98. Tricon S, Burdge GC, Kew S, Banerjee T, Russell JJ, Jones EL, Grimble RF, Williams CM, Yaqoob P, Calder PC: Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. The American Journal of Clinical Nutrition 2004, 80:614–620

99. Thrush AB, Chabowski A, Heigenhauser GJ, McBride BW, Or-Rashid M, Dyck DJ: Conjugated linoleic acid increases skeletal muscle ceramide content and decreases insulin sensitivity in overweight, non-diabetic humans. Applied Physiology, Nutrition, and Metabolism 2007, 32:372–382

100. Risérus U, Brismar K, Arner P, Vessby B: Treatment with dietary trans-10,cis-12 conjugated linoleic acid causes isomerspecific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 2002, 25:1516–1521

101. Plaizier JC, Krause DO, Gozho GN, McBride BW: Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. The Veterinary Journal 2008, 176:21–31

102. Mulligan FJ, Doherty ML: Production diseases of the transition cow. The Veterinary Journal 2008, 176:3–9

103. Krause KM, Oetzel GR: Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Animal Feed Science and Technology 2006, 126:215–236

104. Owens FN, Secrist DS, Hill JW, Gill DR: Acidosis in cattle: A review. Journal of Animal Science 1998, 76:275–286

105. Abel ED, Peroni O, Kim JK, Kim Y-B, Boss O, Hadro E, Minnemann T, Shulman GI, Kahn BB: Adipose-selective targeting of the *GLUT4* gene impairs insulin action in muscle and liver. Nature 2001, 409:729–733

106. Portney LG, Watkins MP: Foundations of Clinical Research: Applications to Practice. 2008,

107. Srinivasan K, Ramarao P: Animal models in type 2 diabetes research: an overview. Indian Journal of Medical Research 2007, 125:451–472

108. Fumeron F, Lamri A, Abi Khalil C, Jaziri R, Porchay-Balderelli I, Lantieri O, Vol S, Balkau B, Marre M, the Data from the Epidemiological Study on the Insulin Resistance Syndrome (DESIR) Study Group: Dairy Consumption and the Incidence of Hyperglycemia and the Metabolic Syndrome: Results from a French prospective study, Data from the Epidemiological Study on the Insulin Resistance Syndrome (DESIR).

Diabetes Care 2011, 34:813–817

109. Struijk EA, Heraclides A, Witte DR, Soedamah-Muthu SS, Geleijnse JM, Toft U, Lau CJ: Dairy product intake in relation to glucose regulation indices and risk of type 2 diabetes. Nutrition, Metabolism and Cardiovascular Diseases 2012,

110. Crichton GE, Howe PRC, Buckley JD, Coates AM, Murphy KJ: Dairy consumption and cardiometabolic health: outcomes of a 12-month crossover trial. Nutrition & Metabolism 2012, 9:1–11

111. Tong X, Dong J-Y, Wu Z-W, Li W, Qin L-Q: Dairy consumption and risk of type 2 diabetes mellitus: a meta-analysis of cohort studies. European Journal of Clinical Nutrition 2011, 65:1027–1031

112. Rideout TC, Marinangeli C, Martin H, Browne RW, Rempel CB: Consumption of low-fat dairy foods for 6 months improves insulin resistance without adversely affecting lipids or bodyweight in healthy adults: a randomized free-living cross-over study. Nutrition Journal 2013, 12:1–9

113. Raff M, Tholstrup T, Basu S, Nonboe P, Sørensen MT, Straarup EM: A Diet Rich in Conjugated Linoleic Acid and Butter Increases Lipid Peroxidation but Does Not Affect Atherosclerotic, Inflammatory, or Diabetic Risk Markers in Healthy Young Men. The Journal of Nutrition: Nutrition and Disease 2008, 138:509–514

114. Matsumoto M, Inoue R, Tsuruta T, Hara H, Yajima T: Long-term oral administration of cows' milk improves insulin sensitivity in rats fed a high-sucrose diet. British Journal of Nutrition 2009, 102:1324

115. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT, Ye J: Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice. Diabetes 2009, 58:1509–1517

116. Ohira H, Fujioka Y, Katagiri C, Mamoto R, Aoyama-Ishikawa M, Amako K, Izumi Y, Nishiumi S, Yoshida M, Usami M, Ikeda M: Butyrate attenuates inflammation and lipolysis generated by the interaction of adipocytes and macrophages. Journal of Atherosclerosis and Thrombosis 2013, 20:425–442

117. Säemann M, Böhmig GA, Österreicher CH, Burtscher H, Parolini O, Diakos C, Stöckl J, Hörl WH, Zlabinger GJ: Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. The FASEB Journal 2000, 14:2380–2392

118. Lavau M, Fried SK, Susini C, Freychet P: Mechanism of insulin resistance in adipocytes of rats fed a high-fat diet. Journal of Lipid Research 1979, 20:8–16

119. Winzell MS, Ahrén B: The high-fat diet–fed mouse: A model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. Diabetes 2004, 53:S215–S219

120. Chisholm DJ, Clark PW, Daley EA, Jenkins AB, Kraegen EW, Storlein LH: Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. Diabetes 1991, 40:1397–1403

121. Kraegen EW, James DE, Storlein LH, Burleigh KM, Chisholm DJ: In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. Diabetologia 1986, 29:192–198

122. Park S-Y, Cho Y-R, Kim H-J, Higashimori T, Danton C, Lee M-K, Dey A, Rothermel B, Kim Y-B, Kalinowski A, Russell KS, Kim JK: Unraveling the temporal pattern of diet-induced insulin resistance in individual organs and cardiac dysfunction in C57BL:6 mice. Diabetes 2005, 54:3530–3540

123. Tam CS, Viardot A, Clément K, Tordjman J, Tonks K, Greenfield JR, Campbell LV, Samocha-Bonet D, Heilbronn LK: Short-term overfeeding may induce peripheral insulin resistance without altering subcutaneous adipose tissue macrophages in humans. Diabetes 2010, 59:2164–2170

124. Brøns C, Jensen CB, Storgaard H, Hiscock NJ, White A, Appel JS, Jacobsen S, Nilsson E, Larsen CM, Astrup A, Quistorff B, Vaag A: Impact of short-term high-fat feeding on glucose and insulin metabolism in young healthy men. The Journal of Physiology 2009, 587:2387–2397

125. Hariri N, Thibault L: High-fat diet-induced obesity in animal models. Nutrition Research Reviews 2010, 23:270–299

126. Blaak E: Gender differences in fat metabolism. Current Opinion in Clinical Nutrition and Metabolic Care 2001, 4:499–502

127. Wu C-H, Yao W-J, Lu F-H, Yang Y-C, Wu J-S, Chang C-J: Sex differences of body fat distribution and cardiovascular dysmetabolic factors in old age. Age and Ageing 2001, 30:331–336

128. Mittendorfer B: Sexual Dimorphism in Human Lipid Metabolism. Journal of Nutrition 2005, 135:681–686

129. Taraschenko OD, Maisonneuve IM, Glick SD: Sex differences in high fat-induced obesity in rats: Effects of 18-methoxycoronaridine. Physiology & Behavior 2011, 103:308–314

130. Marino M, Masella R, Bulzomi P, Campesi I, Malorni W, Franconi F: Nutrition and human health from a sex–gender perspective. Molecular Aspects of Medicine 2011, 32:1–70

131. Crowther NJ, Ferris WF: The impact of insulin resistance, gender, genes, glucocorticoids and ethnicity on body fat distribution. Journal of Endocrinology, Metabolism and Diabetes of South Africa 2010, 15:115–120

132. Levin BE, Dunn-Meynell AA, Balkan B, Keesey RE: Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 1997, 273:R725–R730

133. Collins S, Martin TL, Surwit RS, Robidoux J: Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiology & Behavior 2004, 81:243–248

134. Boulangé CL, Claus SP, Chou CJ, Collino S, Montoliu I, Kochhar S, Holmes E, Rezzi S, Nicholson JK, Dumas ME, Martin F-PJ: Early Metabolic Adaptation in C57BL/6 Mice Resistant to High Fat Diet Induced Weight Gain Involves an Activation of Mitochondrial Oxidative Pathways. Journal of Proteome Research 2013, 12:1956–1968

135. Levin BE, Dunn-Meynell AA: Differential effects of exercise on body weight gain and adiposity in obesity-prone and -resistant rats. International Journal of Obesity 2006, 30:722–727

136. Dourmashkin JT, Chang GQ, Hill JO, Gayles EC, Fried SK, Leibowitz SF: Model for predicting and phenotyping at normal weight the long-term propensity for obesity in Sprague–Dawley rats. Physiology & Behavior 2006, 87:666–678

Appendix A

An identical replicate of the feeding trial in this study was performed but the time was extended. Twenty two weeks consuming a high fat diet did not produce any significant difference in the final body weight or growth curves between any of the 5 treatment groups (Figure xix). In fact, the group consuming the low-fat diet (10% kcal from fat) was the second largest by body weight after 22 weeks. Additionally, there was no difference in whole body glucose homeostasis between groups (data not shown).

However, there is precedent for these results in the literature evaluating the sex differences in fat metabolism in rodents. A recent study showed in a direct comparison of male and female Sprague-Dawley rats consuming a high fat diet that female rats are resistant to high fat diet-induced obesity as compared to males[129]. Additionally, female rats did not respond to the anti-obesity treatment in the study that reduced adiposity in male rats[129]. This suggests differential fat metabolism between genders in their response to high fat diets.

There may be other genetic factors that may be important to consider if a study endeavors to focus on the metabolic mechanisms involved in mediating the effects of a high fat diet rather than evaluate the effects of a high fat diet on a population level. For instance, it has been observed previously that up to 50% of Sprague-Dawley rats are resistant to diet-induced obesity regardless of gender[132], and small genetic differences within the same inbred rodent strains can have significant impacts on metabolism[133,134]. Sprague-Dawley

rats respond differently to exercise-induced weight loss depending on whether they were resistant to the effects of high fat feeding[135]. Dourmashkin et al. (2006) have developed a model for the prediction of which Sprague-Dawley rats are likely to develop obesity to account for this issue; early weight gain on a high fat diet is correlated with an obesity prone phenotype, including increased lipoprotein lipase activity in adipose tissue, but reduced activity in skeletal muscle, promoting fat storage over oxidation[136]. In the current study, such considerations may not have been relevant, since the goal was to evaluate the effect of SARA butter on a population, rather than the specific metabolic actions of $CLA_{t10,c12}$, however it would be important to sort out responders and non-responders to a treatment in order to isolate the mechanisms at play. Indeed a comparison of the different effects of a treatment between responders and non-responders would likely offer valuable insight into the relevant mechanisms for effective treatment

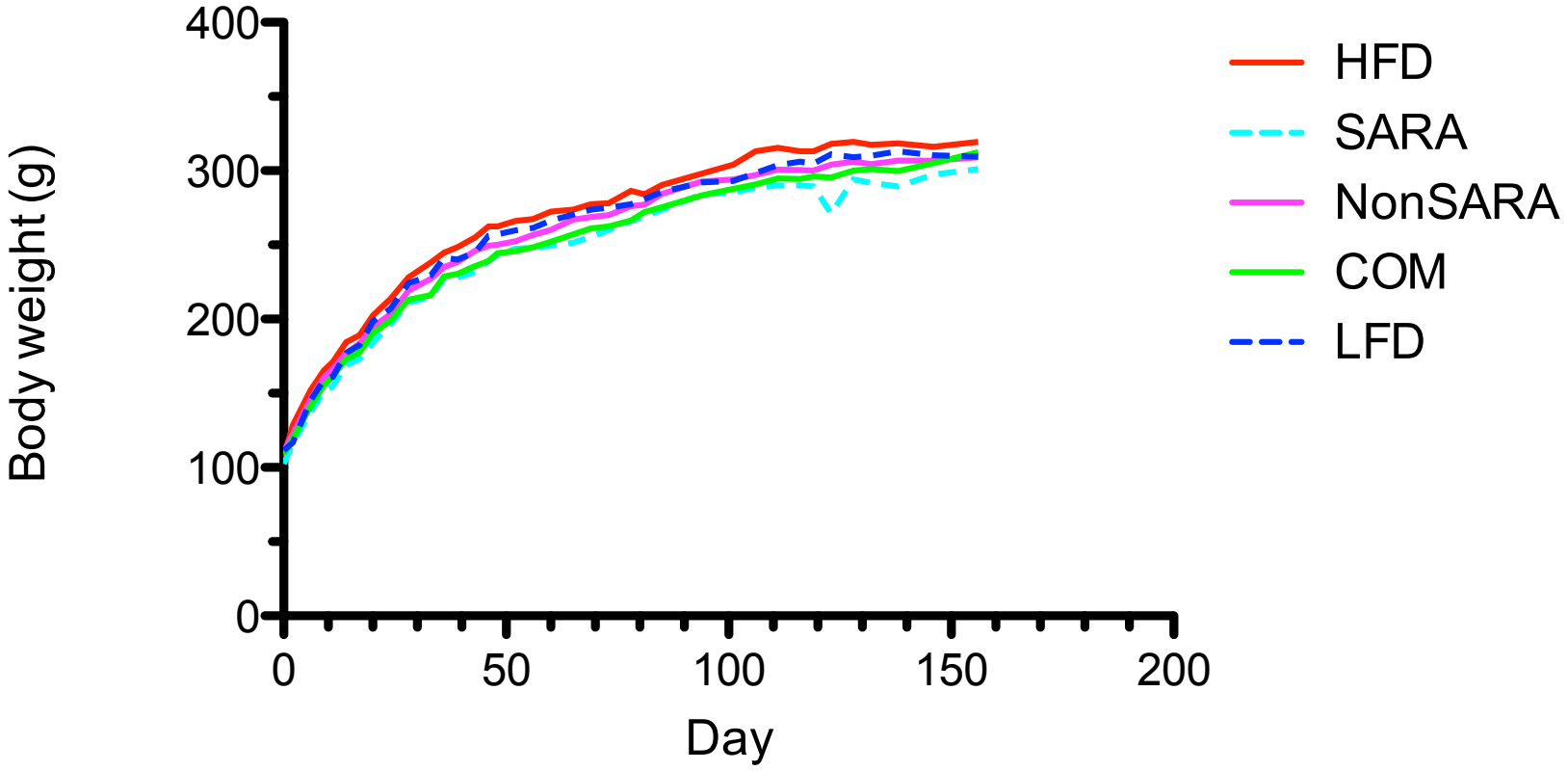

Figure xix: Growth curve of female Sprague-Dawley rats fed either a low or high fat diet for 22 weeks

Appendix B

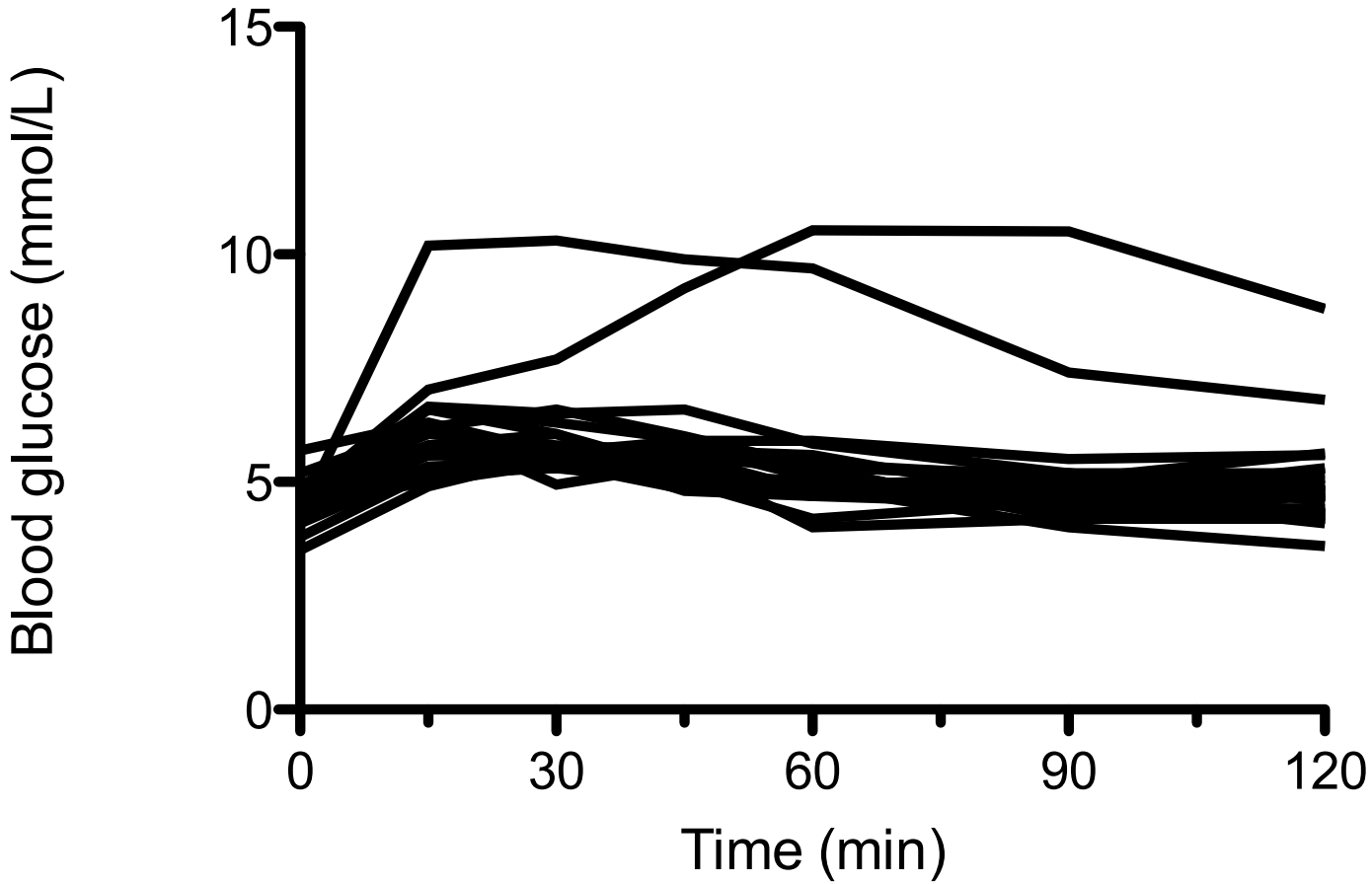

Figure xx: Individual AUC for the pyruvate tolerance test of twenty five female Sprague-Dawley rats